



**HAL**  
open science

## Résistance à la colistine chez les bacilles Gram négatif

Aurélie Jayol

► **To cite this version:**

Aurélie Jayol. Résistance à la colistine chez les bacilles Gram négatif. Microbiologie et Parasitologie. Université de Bordeaux; Université de Fribourg (Suisse), 2018. Français. NNT : 2018BORD0179 . tel-02063717

**HAL Id: tel-02063717**

**<https://theses.hal.science/tel-02063717>**

Submitted on 11 Mar 2019

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

THÈSE EN COTUTELLE PRÉSENTÉE  
POUR OBTENIR LE GRADE DE

**DOCTEUR DE**

**L'UNIVERSITÉ DE BORDEAUX, FRANCE**  
ÉCOLE DOCTORALE SCIENCES DE LA VIE ET DE LA SANTE  
SPÉCIALITÉ : MICROBIOLOGIE - IMMUNOLOGIE

**ET DE L'UNIVERSITÉ DE FRIBOURG, SUISSE**  
SPÉCIALITÉ : MEDICAL SCIENCES

Par Aurélie JAYOL

**RESISTANCE A LA COLISTINE  
CHEZ LES BACILLES A GRAM NEGATIF**

Sous la direction du Professeure Véronique DUBOIS  
et du Professeur Patrice NORDMANN

Soutenue le 18 octobre 2018

Membres du jury :

Pr. PLESIAT Patrick  
Pr. PERRETEN Vincent  
Pr. DUBOIS Véronique  
Pr. RUEGG Curzio  
Pr. NORDMANN Patrice  
Dr. POIREL Laurent

CHU de Besançon  
Institut vétérinaire de Bern  
CHU de Bordeaux  
Université de Fribourg  
Université de Fribourg  
Université de Fribourg

Rapporteur  
Rapporteur  
Examineur  
Président du jury  
Invité  
Invité

# REMERCIEMENTS

## *A mon jury de thèse*

Monsieur le Professeur Patrice NORDMANN et Madame la Professeure Véronique DUBOIS, mes deux co-directeurs de thèse, pour m'avoir accueillie dans leurs unités de recherche et m'avoir donnée la chance de travailler sur un sujet que j'affectionne tout particulièrement : la résistance aux antibiotiques. Merci pour votre encadrement, vos conseils, et pour m'avoir enseigné la rigueur scientifique.

Monsieur le Docteur Laurent POIREL, mon tuteur, pour son enseignement, ses encouragements et sa patience face à mon impatience.

Messieurs les Professeurs Patrick PLESIAT et Vincent PERRETEN pour m'avoir fait l'honneur d'analyser ce travail et d'en être les rapporteurs.

Monsieur le Professeur Curzio RUEGG pour avoir accepté d'être le président de ce jury.

## *A mes deux équipes de recherche*

Les membres de l'équipe de l'Unité de Microbiologie Moléculaire et Médicale de l'Université de Fribourg. Un merci tout particulier à Nicolas mon blopounet de codocotorant.

Les membres de l'équipe de l'Unité MFP UMR5234 de l'Université de Bordeaux pour leurs idées, leurs conseils, leur soutien apportés avec beaucoup d'enthousiasme, de gentillesse et de disponibilité. Cathy, ton petit « poisson rouge » te remercie pour ton aide technique et toutes les manips à 4 mains. Françoise, merci pour ta gentillesse, j'ai adoré enseigner à tes côtés. Camille, c'était un plaisir de travailler avec toi pendant ton année de master 2.

*A mes collègues de l'hôpital de Bordeaux*

Mes remerciements s'adressent également à l'ensemble du personnel du laboratoire de bactériologie de l'hôpital de Bordeaux, et tout particulièrement à mes anciens co-assistants, Julien et Pauline, devenus des amis, mais aussi à mes autres collègues biologistes, sans qui mes deux années d'assistantats n'auraient pas été les mêmes.

*A ma famille, vous êtes les piliers de mon existence*

A mes parents sans qui rien n'aurait été possible. Merci pour votre soutien pendant toutes ces années d'études.

A mon frère Arnaud. Nous avons grandi ensemble dans les rires et parfois dans les pleurs. Que de souvenirs passés et à construire.

A Guillaume, pour ton soutien de tous les jours. Merci pour ta compréhension envers mon travail parfois un peu envahissant et merci pour toutes ces fois où tu m'as emmené au laboratoire quel que soit le jour ou l'heure. Tu me combles de bonheur.

*A notre amour et à notre avenir...*



## **Titre : Résistance à la colistine chez les bacilles à Gram négatif**

### **Résumé :**

Les entérobactéries productrices de carbapénèmases peuvent être responsables d'impasses thérapeutiques puisque ces souches sont multirésistantes aux antibiotiques. La colistine, un antibiotique de la famille des polymyxines, fait partie des molécules de derniers recours potentiellement utilisables pour le traitement des patients infectés par ces souches. Son utilisation est ainsi en augmentation constante mais des résistances émergent.

Ce travail a contribué à l'amélioration du diagnostic de la résistance à la colistine par le développement de deux nouveaux outils diagnostiques : un test rapide, le Rapid Polymyxin NP test et un milieu de culture sélectif, la gélose SuperPolymyxin. Il a permis d'identifier de nouvelles mutations chromosomiques au sein des gènes *pmrA*, *pmrB*, *phoP*, *phoQ*, *mgrB* et *crrB* responsables de l'acquisition de résistances et d'hétérorésistance à la colistine chez *K. pneumoniae* et *K. oxytoca*. Il a révélé que les mutations chromosomiques et les résistances plasmidiques étaient additionnelles et pouvaient entraîner l'acquisition d'un haut niveau de résistance à la colistine chez *E. coli*. Il a permis d'identifier une épidémie de souches de *K. pneumoniae* productrices de la carbapénémase OXA-48 et résistantes à la colistine en France en 2014. Il a démontré que *Hafnia* était un genre d'entérobactéries présentant une résistance naturelle de bas niveau à la colistine. Enfin, il a permis de proposer une option thérapeutique, le ceftazidime/avibactam en association ou non avec l'aztréonam, pour traiter les patients infectés par les souches de *K. pneumoniae* productrices de carbapénèmases et résistantes à la colistine.

Ce travail a ainsi contribué à améliorer les connaissances sur la résistance à la colistine chez les BGN au niveau du diagnostic, des mécanismes de résistance acquis, des résistances naturelles, et de l'épidémiologie et a permis de proposer des combinaisons d'antibiotiques actives *in vitro* sur les souches de *K. pneumoniae* productrices de carbapénèmases et résistantes à la colistine.

**Mots clés : Colistine, Polymyxines, test rapide, milieu de screening, mécanismes de résistance, épidémiologie, entérobactéries, bacilles Gram négatif**

**Title :** Resistance to colistin in Gram negative rods

**Abstract :**

Carbapenemase-producing Enterobacteriaceae may be responsible for therapeutic failures since such strains are usually multidrug-resistant. Colistin, an antibiotic of the polymyxin family, is one of the last-resort molecules potentially active for treating patients infected with these strains. Its use is thus constantly increasing but resistances occur.

Our work contributed to improve the diagnosis of colistin resistance by developing two new diagnostic tools: a rapid test, the Rapid Polymyxin NP test and a selective culture medium, the SuperPolymyxin agar. In term of resistance mechanisms, we identified novel chromosomal mutations within the *pmrA*, *pmrB*, *phoP*, *phoQ*, *mgrB* and *crrB* genes responsible for the acquired colistin resistance and heteroresistance in *K. pneumoniae* and *K. oxytoca*. We revealed that chromosomal mutations and plasmid resistance were additional and could lead to the acquisition of a high level of colistin resistance in *E. coli*. We investigated an outbreak caused by colistin- and carbapenem- resistant OXA-48-producing *K. pneumoniae* strains in France in 2014. We also identified the low-level intrinsic resistance to colistin of *Hafnia* spp. Finally, we evaluated the usefulness of a novel therapeutic option, namely ceftazidime / avibactam, in combination or not with aztreonam, for potential treatment of infections caused by colistin-resistant and carbapenemase-producing *K. pneumoniae*.

This work has significantly contributed to improve the knowledge of colistin resistance in Gram negatives in diagnostic, in characterization of acquired or intrinsic resistance mechanisms, and in epidemiology.

**Keywords :** Colistin, polymyxin, rapid test, screening medium, mechanism of resistance, epidemiology, Enterobacteriaceae, Gram negative rods

Unités de recherche :

**Laboratoire de Microbiologie Fondamentale et Pathogénicité CNRS UMR-5234, Université de Bordeaux Segalen, 146 rue Léo Saignat Batiment 3A, 33076 BORDEAUX, FRANCE**

**Unité de Microbiologie Médicale et Moléculaire, Laboratoire européen INSERM, Département de médecine, Université de Fribourg, route Albert Gockel 3, 1700 FRIBOURG, SUISSE**

# TABLE DES MATIERES

<b>ETUDE BIBLIOGRAPHIQUE .....</b>	<b>6</b>
<b>Introduction .....</b>	<b>7</b>
<b>A. Les polymyxines.....</b>	<b>7</b>
1. Généralités sur les polymyxines .....	7
2. Structure des polymyxines .....	8
3. Mécanismes d'action des polymyxines .....	9
4. Spectre d'activité des polymyxines .....	11
5. Indications du colistiméthate.....	11
<b>B. Résistances naturelles à la colistine .....</b>	<b>12</b>
1. <i>Bacillus polymyxa</i> subspecies colistinus .....	12
2. <i>Proteus, Providencia, Morganella</i> et <i>Serratia marcescens</i> .....	12
3. <i>Enterobacter</i> spp. hétérorésistants.....	12
<b>C. Résistances acquises à la colistine.....</b>	<b>13</b>
1. Résistances chromosomiques .....	13
1.1. Modifications du lipopolysaccharide .....	13
1.1.1. Ajout de groupements cationiques à la surface du LPS .....	13
a. Gènes codant pour les enzymes qui modifient le LPS .....	16
b. Systèmes à deux composants .....	17
c. Gène régulateur du système à deux composants PhoPQ.....	21
d. Gènes connecteurs des systèmes à deux composants .....	21
1.1.2. Modification de l'acétylation du LPS (chez <i>K. pneumoniae</i> ) .....	23
1.1.3. Augmentation de production du LPS (chez <i>K. pneumoniae</i> ) .....	23
1.1.4. Absence de production de LPS (chez <i>A. baumannii</i> ).....	23
1.2. Modifications de la capsule polysaccharidique (CPS).....	24
1.2.1. Hyperproduction de la CPS (chez <i>K. pneumoniae</i> ).....	24
1.2.2. Libération de la CPS : phénomène de trapping (chez <i>K. pneumoniae</i> et <i>P. aeruginosa</i> ) .....	24
1.3. Implication des porines .....	24
1.4. Implication des pompes d'efflux.....	25
2. Résistances plasmidiques.....	25
2.1. Gène de résistance plasmidique <i>mcr-1</i> et variants.....	25
2.1.1. Gène de résistance plasmidique <i>mcr-1</i> .....	25
2.1.2. Variants du gène de résistance plasmidique <i>mcr-1</i> .....	26
2.2. Gène de résistance plasmidique <i>mcr-2</i> .....	26
2.3. Gène de résistance plasmidique <i>mcr-3</i> et variants.....	27
2.3.1. Gène de résistance plasmidique <i>mcr-3</i> .....	27
2.3.2. Variants du gène de résistance plasmidique <i>mcr-3</i> .....	27
2.4. Gène de résistance plasmidique <i>mcr-4</i> et variants.....	27
2.4.1. Gène de résistance plasmidique <i>mcr-4</i> .....	27
2.4.2. Variants du gène de résistance plasmidique <i>mcr-4</i> .....	28
2.5. Gène de résistance plasmidique <i>mcr-5</i> .....	28
3. Epidémiologie de la résistance.....	28
3.1. Facteurs de risque associés à la résistance à la colistine .....	28
3.2. Pressions de sélection pour l'acquisition de résistances plasmidiques à la colistine .....	29
<b>D. Détermination de la sensibilité à la colistine .....</b>	<b>29</b>
1. Méthodes de dilutions .....	30
1.1. Dilutions en milieu liquide : méthode de référence .....	30
1.2. Dilutions en milieu solide .....	31
2. Méthode des disques .....	31

3.	Colispot .....	32
4.	Bandelettes par gradient de diffusion.....	32
4.1.	Bandelettes Etest® (bioMérieux).....	32
4.2.	Bandelettes MIC Test Strip® (Liofilchem) .....	33
5.	Panels commerciaux pour la détermination des CMI en milieu liquide .....	33
5.1.	Système Sensititre® (ThermoFisher Scientific).....	33
5.2.	Système UMIC® (Biocentric).....	34
5.3.	Système MICRONAUT-S® (Merlin Diagnostika GmbH).....	34
5.4.	Système MICRONAUT MIC strip® (Merlin Diagnostika GmbH) .....	34
5.5.	Système Sensitest® ou ComASP® (Liofilchem) .....	34
6.	Systèmes automatisés.....	35
6.1.	Système Vitek® 2 (bioMérieux) .....	35
6.2.	Système BD Phoenix® (BD Diagnostics).....	35
6.3.	Système MicroScan® (Beckman Coulter Diagnostics) .....	36
7.	Diamètres et concentrations critiques .....	36
8.	Contrôles de qualité .....	37
<b>OBJECTIFS.....</b>		<b>38</b>
<b>MATERIEL ET METHODES.....</b>		<b>39</b>
<b>A. Techniques bactériologiques .....</b>		<b>40</b>
1.	Souches bactériennes .....	40
1.1.	Souches cliniques résistantes à la colistine .....	40
1.2.	Autres souches.....	40
2.	Plasmides .....	40
3.	Milieux de culture .....	41
4.	Identification de l'espèce .....	41
5.	Antibiogrammes.....	41
6.	Détermination des Concentrations Minimales Inhibitrices (CMI) .....	41
<b>B. Techniques de biologie moléculaire et de génétique .....</b>		<b>42</b>
1.	Extraction des acides nucléiques .....	42
1.1.	Extraction d'ADN génomique.....	42
1.2.	Extraction d'ADN plasmidique .....	42
1.3.	Extraction d'ARN .....	42
2.	Réactions d'amplification génique .....	42
2.1.	Réactions de <i>Polymerase Chain Reaction</i> (PCR).....	42
2.2.	Réactions de séquençage.....	43
2.3.	Réactions de <i>Quantitative Real Time-Polymerase Chain Reaction</i> (qRT-PCR) .....	43
3.	Complémentation des souches possédant des gènes mutés.....	44
4.	Etude de la clonalité.....	45
4.1.	<i>Multi Locus Sequence Typing</i> (MLST) .....	45
4.2.	Rep-PCR (Diversilab®) .....	45
4.3.	Electrophorèse en champ pulsé après restriction enzymatique (PFGE) .....	46
<b>C. Analyse in silico.....</b>		<b>46</b>
1.	Analyse des séquences nucléotidiques et protéiques .....	46
2.	Prédiction des séquences promotrices des gènes .....	47
3.	Recherche de données sur la structure et la fonction des protéines .....	47
4.	Prédiction de la structure secondaire des protéines .....	47
5.	Prédiction de l'impact des altérations géniques sur la fonction protéique .....	47

<b>RESULTATS EXPERIMENTAUX .....</b>	<b>48</b>
<b>A. Développement de tests diagnostiques pour la détection de la résistance aux polymyxines</b>	<b>49</b>
1. Développement d'un test rapide .....	49
1.1. Détection rapide des BGN résistants à la colistine à partir de colonies bactériennes .....	49
1.2. Détection rapide des BGN résistants à la colistine à partir d'hémocultures .....	63
2. Développement d'une gélose sélective pour le screening des BGN résistants à la colistine .....	70
<b>B. Evaluation des tests phénotypiques et des tests automatisés pour la détection de la résistance à la colistine .....</b>	<b>78</b>
1. Evaluation de l'automate BD Phoenix® .....	78
2. Evaluation des systèmes UMIC®, MicroScan® et Sensititre® .....	85
<b>C. Epidémiologie de la résistance à la colistine chez les BGN.....</b>	<b>94</b>
1. Détermination du taux de résistance à la colistine chez les entérobactéries productrices de carbapénèmases en France .....	94
2. Détermination de la prévalence du portage fécal de BGN résistants à la colistine dans l'Ouest de la France .....	104
<b>D. Mécanismes chromosomiques de résistance à la colistine.....</b>	<b>107</b>
1. Diversité des mécanismes chromosomiques de résistance chez <i>K. pneumoniae</i> .....	107
1.1. Identification d'une mutation dans la protéine PmrB responsable de l'acquisition d'une résistance à la colistine .....	107
1.2. Identification d'altérations dans la protéine PhoP responsables de l'acquisition d'une hétérorésistance à la colistine .....	116
1.3. Identification d'altérations dans la protéine MgrB responsables de l'acquisition de résistances à la colistine .....	124
a. Les altérations dans la protéine MgrB sont un mécanisme fréquent d'acquisition de résistances à la colistine .....	125
b. L'insertion du gène <i>blaCTX-M</i> au sein du gène <i>mgrB</i> est responsable de l'acquisition d'une résistance croisée aux céphalosporines de troisième génération et à la colistine .....	132
1.4. Identification de mutations dans la protéine CrrB responsables de l'acquisition de résistances à la colistine .....	136
2. Mécanismes chromosomiques de résistance chez <i>K. oxytoca</i> .....	141
<b>E. Impact de l'association des résistances chromosomique et plasmidique .....</b>	<b>145</b>
<b>F. <i>Hafnia</i>, un genre d'entérobactérie naturellement résistant à la colistine .....</b>	<b>149</b>
<b>G. Options thérapeutiques pour le traitement des <i>K. pneumoniae</i> produisant une carbapénémase et résistantes à la colistine .....</b>	<b>156</b>
<b>DISCUSSION ET PERSPECTIVES .....</b>	<b>162</b>
<b>REFERENCES BIBLIOGRAPHIQUES.....</b>	<b>170</b>
<b>ANNEXES.....</b>	<b>182</b>

# ABREVIATIONS

<b>AMM</b>	Autorisation de Mise sur le Marché
<b>ANSM</b>	Agence Nationale de Sécurité du Médicament
<b>BGN</b>	Bacilles à Gram négatif
<b>BLSE</b>	Bêta-Lactamase à Spectre Etendu
<b>CASFM</b>	Comité de l'Antibiogramme de la Société Française de Microbiologie
<b>CLSI</b>	<i>Clinical and Laboratory Standards Institute</i>
<b>CMI</b>	Concentration Minimale Inhibitrice
<b>CMS</b>	Colistiméthate de Sodium
<b>CNR</b>	Centre National de Référence
<b>CPS</b>	Capsule polysaccharidique
<b>CS</b>	Colistine
<b>DO</b>	Densité optique
<b>EUCAST</b>	<i>European Committee on Antimicrobial Susceptibility Testing</i>
<b>Kb</b>	Kilobases
<b>LAra4N</b>	4-amino-4-deoxy-L-arabinose
<b>LB</b>	Luria-Bertani
<b>LPS</b>	Lipopolysaccharide
<b>McF</b>	Mac Farland
<b>MDR</b>	Multidrug résistant
<b>MH</b>	Mueller-Hinton
<b>MLST</b>	<i>Multi Locus Sequence Typing</i>
<b>IF</b>	Facteur d'impact
<b>Pb</b>	Paire de bases
<b>PCR</b>	<i>Polymerase Chain Reaction</i>
<b>PEtN</b>	Phosphoéthanolamine
<b>PFGE</b>	<i>Pulsed-Field Gel Electrophoresis</i>
<b>R</b>	Résistant
<b>S</b>	Sensible
<b>ST</b>	Séquence-type
<b>UFC</b>	Unité formant colonies
<b>UI</b>	Unité internationale

## LISTE DES FIGURES

**Figure 1.** Structures de la colistine, du colistiméthate et de la polymyxine B.

**Figure 2.** Mécanismes d'action des polymyxines.

**Figure 3.** Représentations schématiques de la régulation des gènes impliqués dans la résistance aux polymyxines chez *Escherichia coli* (A) et *Klebsiella pneumoniae* (B).

**Figure 4.** Représentations schématiques de la régulation des gènes impliqués dans la résistance aux polymyxines chez *Acinetobacter baumannii* (A) et *Pseudomonas aeruginosa* (B).

**Figure 5.** Activation directe et indirecte de l'opéron *pmrHFIJKLM* par le système à deux composants PhoPQ chez *K. pneumoniae*.

## LISTE DES TABLEAUX

**Tableau 1.** Diamètres et concentrations critiques pour la colistine selon le CLSI et l'EUCAST en 2018.

**Tableau 2.** Protéines de résistance plasmidiques à la colistine.

**Tableau 3.** Variants de la protéine MCR-1 disponibles dans la base de données GenBank.

**Tableau 4.** Variants de la protéine MCR-3 disponibles dans la base de données GenBank.

**Tableau 5.** Variants de la protéine MCR-4 disponibles dans la base de données GenBank.

**Tableau 6.** Conditions de réalisation des PCR.

**Tableau 7.** Composition des solutions utilisées pour l'électrophorèse en champ pulsé.

## LISTE DES ANNEXES

**Annexe A.** Amorces utilisées pour la recherche des mécanismes de résistance à la colistine chez les bacilles à Gram négatif.

**Annexe B.** Mutations chromosomiques responsables de l'acquisition de résistances à la colistine chez les bacilles à Gram négatif.

**Annexe C (Revue).** Polymyxins: Antibacterial Activity, Susceptibility Testing, and Resistance Mechanisms Encoded by Plasmids or Chromosomes.

# ***ETUDE BIBLIOGRAPHIQUE***



## **Introduction**

La lutte contre les bacilles à Gram négatif (BGN) multi-résistants (MDR) aux antibiotiques représente un enjeu majeur de santé publique. Autrefois sensibles à la quasi-totalité des antibiotiques, certaines souches de BGN sont devenues résistantes à de nombreux antibiotiques, voire à tous ceux utilisables cliniquement. L'acquisition de plasmides porteurs de gènes de résistance (gènes de carbapénèmases notamment) ainsi que l'accumulation de mutations chromosomiques ont entraîné l'émergence de souches résistantes à toutes les  $\beta$ -lactamines mais aussi aux autres classes d'antibiotiques (aminosides, quinolones...). Cette multirésistance a contraint les cliniciens à réintroduire une classe d'antibiotique quasiment abandonnée depuis des décennies en raison de sa toxicité, les polymyxines (colistine et polymyxine B). Cependant, la résistance aux polymyxines est en constante augmentation. L'émergence de la résistance aux polymyxines chez les souches multirésistantes aux antibiotiques est particulièrement inquiétante puisqu'elle compromet le dernier rempart sur la route vers la toto-résistance aux antibiotiques, ce d'autant que les perspectives de nouvelles molécules thérapeutiques actives sur les BGN MDR sont assez limitées.

## **A. Les polymyxines**

### **1. Généralités sur les polymyxines**

La polymyxine E, aussi appelée colistine (CS), et la polymyxine B sont les deux polymyxines utilisées en clinique (1). La colistine est naturellement synthétisée par *Bacillus polymyxa* subspecies colistinus et a été découverte à la fin des années 1940 au Japon. Elle était à l'époque l'une des premières molécules connues ayant une activité sur les BGN.

Les polymyxines ont été largement utilisées jusqu'au milieu des années 1970 puis ont ensuite été abandonnées en raison de leurs néphrotoxicité et neurotoxicité (2), de leur faible diffusion tissulaire et suite au développement de nouveaux antibiotiques (carbapénèmes, fluoroquinolones, aminosides...). Pendant trente ans, l'utilisation des polymyxines chez l'Homme a été limitée au traitement des infections pulmonaires à BGN MDR chez les patients

atteints de mucoviscidose. Dans le milieu vétérinaire, elle était par contre très utilisée pour le traitement ou la prophylaxie des diarrhées du porc ou du veau notamment.

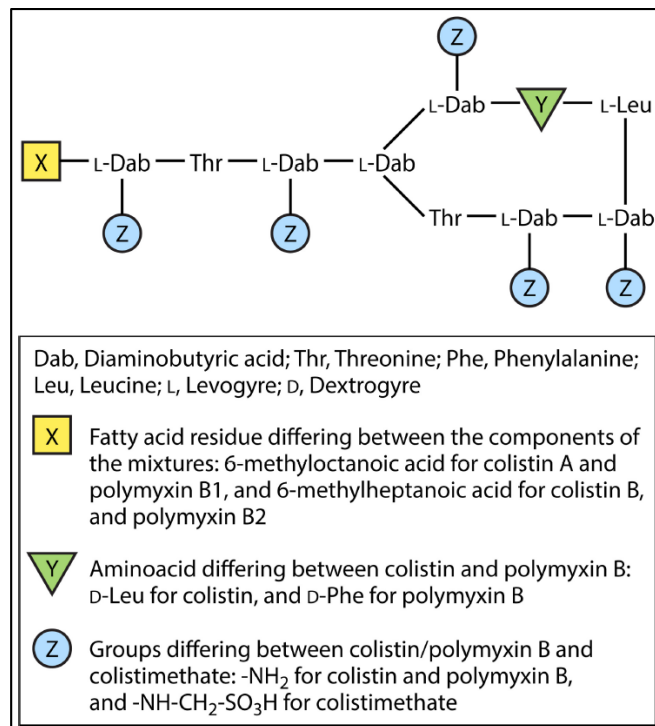
Cependant, l'émergence récente de bactéries résistantes à toutes les classes d'antibiotiques et l'absence de commercialisation de nouvelles classes d'antibiotiques ont entraîné une utilisation renouvelée des polymyxines dans le traitement des infections à BGN MDR chez l'Homme, et notamment ceux producteurs de carbapénèmases (3–5).

## **2. Structure des polymyxines**

La structure des polymyxines est similaire aux peptides antimicrobiens cationiques naturels (défensines, gramicidines) qui représentent la première ligne de défense contre la colonisation bactérienne chez les cellules eucaryotes (6).

La colistine est un polypeptide cationique composé d'un cycle constitué de sept acides aminés, d'une chaîne tripeptidique et d'une queue constituée d'un acide gras hydrophobe (Figure 1) (5, 7). La colistine est un mélange de colistine A (polymyxine E1) et colistine B (polymyxine E2) qui diffèrent par un acide gras terminal. Le cycle heptapeptidique est le même que celui de la polymyxine B à l'exception d'un acide aminé, qui est la phénylalanine pour la polymyxine B et la leucine pour la colistine (Figure 1) (8).

Le colistiméthate de sodium (CMS) est la prodrogue inactive de la colistine (Figure 1) (1). Il est hydrolysé *in vivo* en colistine ainsi qu'en de multiples dérivés inactifs (5).

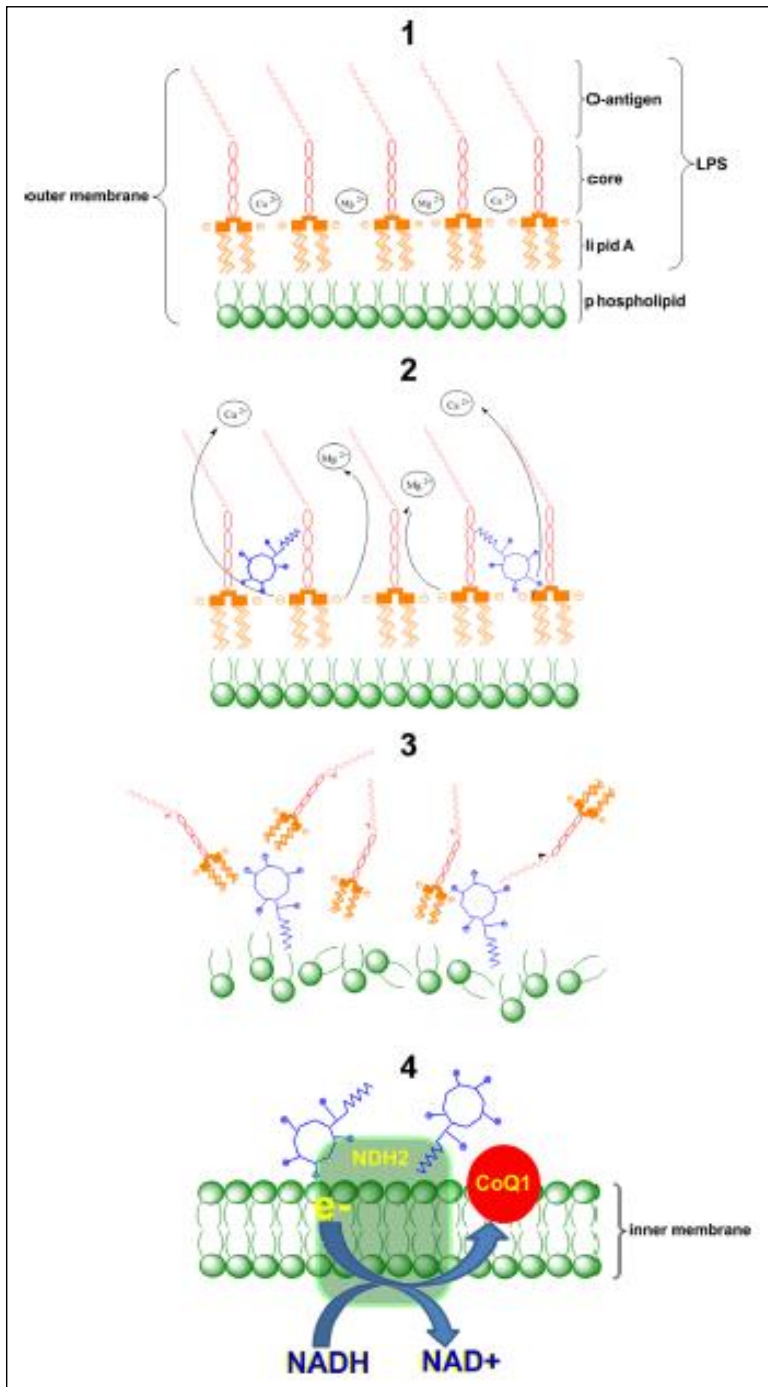


**Figure 1.** Structures de la colistine, du colistiméthate et de la polymyxine B. D’après Poirel et al, Clinical Microbiology Reviews 2014 (9).

### 3. Mécanismes d’action des polymyxines

La paroi des BGN est constituée d’une membrane interne (ou membrane cytoplasmique), d’un espace périplasmique et d’une membrane externe. Les polymyxines ont comme cible la membrane externe de la paroi des BGN dont le composant essentiel est le lipopolysaccharide (LPS). Une interaction électrostatique entre les résidus acide  $\alpha,\gamma$ -diaminobutyrique (Dab) chargés positivement des polymyxines et les groupements phosphates chargés négativement du lipide A du LPS (Figure 2-1) entraîne un déplacement des ions divalents ( $Mg^{2+}$  et  $Ca^{2+}$ ) (Figure 2-2) et ainsi une déstabilisation du LPS (Figure 2-3). L’extrémité hydrophobe des polymyxines interagit alors avec le LPS permettant ainsi leur insertion dans la membrane externe. Ceci entraîne une augmentation de la perméabilité membranaire et une fuite du contenu intracellulaire conduisant à la lyse bactérienne et à la mort cellulaire (4). Une fois à l’intérieur de la membrane interne, les polymyxines inhibent l’activité d’une enzyme respiratoire, la NADH-quinone oxydoréductase (NDH-2), ce qui constitue un deuxième mécanisme conduisant à la mort cellulaire (Figure 2-4) (10). Grâce à ces deux mécanismes d’action, les polymyxines ont une activité bactéricide dépendante de leur concentration (5, 11).

A la différence de la plupart des antibiotiques, les polymyxines possèdent une activité anti-endotoxinique (4). La liaison des polymyxines au lipide A bactérien empêche la libération du LPS (qui constitue l'endotoxine des BGN), ce qui évite ainsi la survenue de chocs endotoxiniques lors de la lyse bactérienne.



1. Structure de la membrane externe (la cible des polymyxines est le LPS).
2. Déplacement des cations divalents à la surface de la membrane externe par les polymyxines chargées positivement.
3. Déstabilisation de la membrane externe à cause des interactions électrostatiques entre les polymyxines (chargées +) et les groupements phosphates du LPS (chargés -).
4. Pénétration des polymyxines dans la membrane interne responsable d'une fuite du contenu intracellulaire et d'une inhibition de l'enzyme respiratoire NDH-2.

**Figure 2.** Mécanismes d'action des polymyxines.  
D'après Deris et al, Bioconjugate Chemistry 2014 (10).

#### **4. Spectre d'activité des polymyxines**

La colistine est active vis-à-vis de la plupart des BGN aérobies : *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Haemophilus influenzae*, et la majorité des espèces d'entérobactéries (*Escherichia coli*, *Klebsiella spp*, *Citrobacter spp*, *Salmonella spp*, *Shigella spp* et *Yersinia pseudotuberculosis*). Elle a aussi une activité vis-à-vis de certaines espèces de mycobactéries dont *Mycobacterium tuberculosis* (4).

Cependant, certaines espèces de BGN sont inconstamment sensibles (résistances acquises > 10 %) comme *Enterobacter spp.*, *Stenotrophomonas maltophilia* ou *Achromobacter xylosoxidans*. D'autres espèces sont naturellement résistantes à la colistine dont *Proteus spp.*, *Morganella morganii*, *Providencia spp.*, *Serratia marcescens*, *Burkholderia cepacia*, *Chromobacterium spp.*, *Edwardsiella spp.*, *Brucella*, *Legionella*, *Campylobacter spp.*, *Vibrio cholerae*. Les polymyxines ne sont actives ni contre les cocci à Gram négatif (*Neisseria spp.*), ni contre les bactéries à Gram positif, ni contre les bactéries anaérobies strictes (4).

#### **5. Indications du colistiméthate**

En France, l'Autorisation de Mise sur le Marché (AMM) du colistiméthate (CMS) sodique (Colimycin<sup>®</sup>), qui est la prodrogue de la colistine, a été modifiée en février 2013 par l'Agence Nationale de Sécurité du Médicaments (ANSM). Cette molécule peut désormais être utilisée chez l'adulte et chez l'enfant dans le traitement des infections microbiologiquement documentées dues à des BGN définis comme sensibles, lorsqu'aucun autre antibiotique n'est actif *in vitro*, notamment chez les patients atteints de mucoviscidose et chez les patients hospitalisés en service de réanimation. Le CMS doit être utilisé en dernier recours lorsqu'aucun autre traitement n'est possible et toujours en association. Il peut être administré par voie intraveineuse, en aérosols pour le traitement des infections respiratoires ou par voie intrathécale ou intraventriculaire en cas de méningite (4).

En Suisse, seul le CMS sous forme de poudre pour solution pour inhalation (Colistilab<sup>®</sup> ou ColiFin<sup>®</sup>) est commercialisé. Il est indiqué pour le traitement intermittent par aérosol des infections des voies respiratoires à *Pseudomonas aeruginosa* chez les patients atteints de mucoviscidose.

## **B. Résistances naturelles à la colistine**

### **1. *Bacillus polymyxa* subspecies colistinus**

*Bacillus polymyxa*, l'espèce productrice des polymyxines, est naturellement résistante à la colistine grâce à la production d'une enzyme qui hydrolyse la colistine, la colistinase (12). A ce jour, aucun mécanisme de résistance enzymatique n'a été décrit chez d'autres espèces bactériennes.

### **2. *Proteus*, *Providencia*, *Morganella* et *Serratia marcescens***

La résistance naturelle de *Proteus mirabilis* et *Serratia marcescens* est due à l'ajout de groupements cationiques (groupements phosphoéthanolamine et/ou 4-amino-4-deoxy-L-arabinose) sur le lipide A, ce qui augmente la charge positive du lipopolysaccharide (LPS) bactérien diminuant ainsi l'affinité des polymyxines qui sont elles-même cationiques (13–15). Cet ajout de groupements est lié à l'activation constitutive de l'expression de l'opéron *pmrHFIJKLM* et du gène *eptB* (13–15).

### **3. *Enterobacter* spp. hétérorésistants**

Les souches de *E. cloacae* peuvent être répartis en 13 clusters (C-I à XII) par séquençage du gène *hsp60*. Guérin et al ont montré que les souches appartenant aux clusters I, II, IV, VII, IX, X, XI et XII présentaient généralement un phénotype d'hétérorésistance à la colistine (présence de plusieurs sous-populations présentant des sensibilités différentes à la colistine) (16). Pour déterminer le mécanisme de résistance à la colistine, ils ont inactivé les gènes impliqués dans la modification du lipopolysaccharide (*pmr* et *pho*) et ont montré que les souches redevenaient ainsi sensibles à la colistine. Ceci suggère donc que cette hétérorésistance naturelle est due à l'expression de l'opéron *pmrHFIJKLM* qui est sous régulation du système à deux composants PhoP/PhoQ (PhoPQ). Une deuxième étude a confirmé l'implication du système PhoPQ dans le phénotype d'hétérorésistance à la colistine et a prouvé l'implication des sous-populations résistantes à la colistine dans les échecs de traitement par de la colistine chez la souris (17).

Cependant, une troisième étude a révélé des résultats discordants comparés aux deux premières études en indiquant que c'était l'hyperexpression du régulateur transcriptionnel *soxRS* qui induit l'hyperexpression du système d'efflux *acrAB-tolC* qui était responsable de l'hétérorésistance à la colistine

chez *Enterobacter cloacae* et *Enterobacter asburiae* (18). Le mécanisme d'hétérorésistance à la colistine chez *Enterobacter* spp. reste donc encore à préciser.

## **C. Résistances acquises à la colistine**

La résistance acquise aux polymyxines a été identifiée dans plusieurs genres de BGN, comme *Klebsiella*, *Escherichia*, *Enterobacter*, *Salmonella*, *Acinetobacter* et *Pseudomonas*.

Les mécanismes de résistance à la colistine sont encore mal connus mais plusieurs mécanismes moléculaires ont été identifiés. L'acquisition de résistances aux polymyxines est dans la majorité des cas due à l'ajout de groupements cationiques à la surface du lipopolysaccharide de façon similaire à ce qui a été observé chez les genres *Proteus* ou *Serratia*. Cet ajout de groupements peut être dû à des mutations chromosomiques mais aussi à l'acquisition de gènes portés par des plasmides.

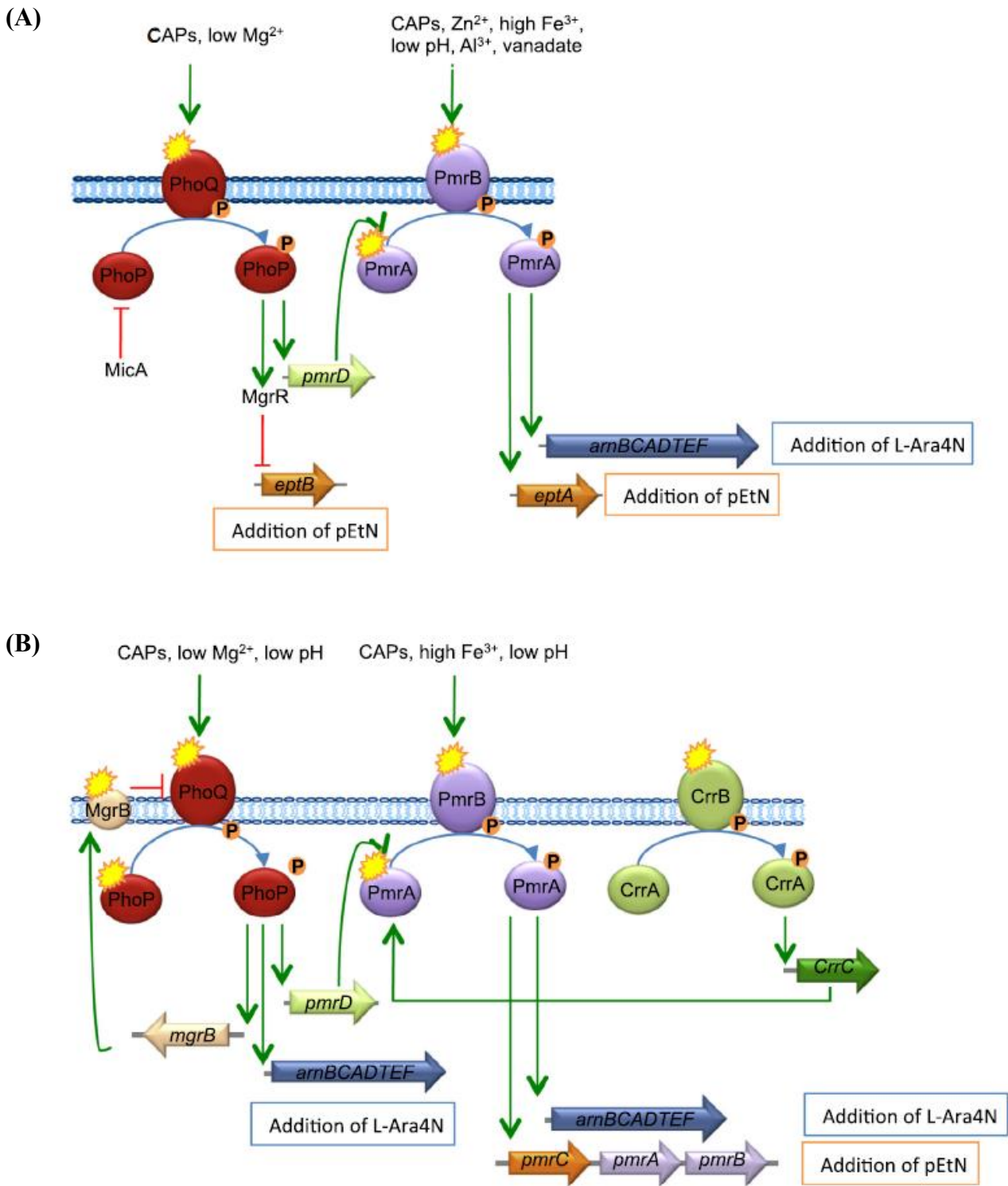
### **1. Résistances chromosomiques**

#### **1.1. Modifications du lipopolysaccharide**

##### **1.1.1. Ajout de groupements cationiques à la surface du LPS**

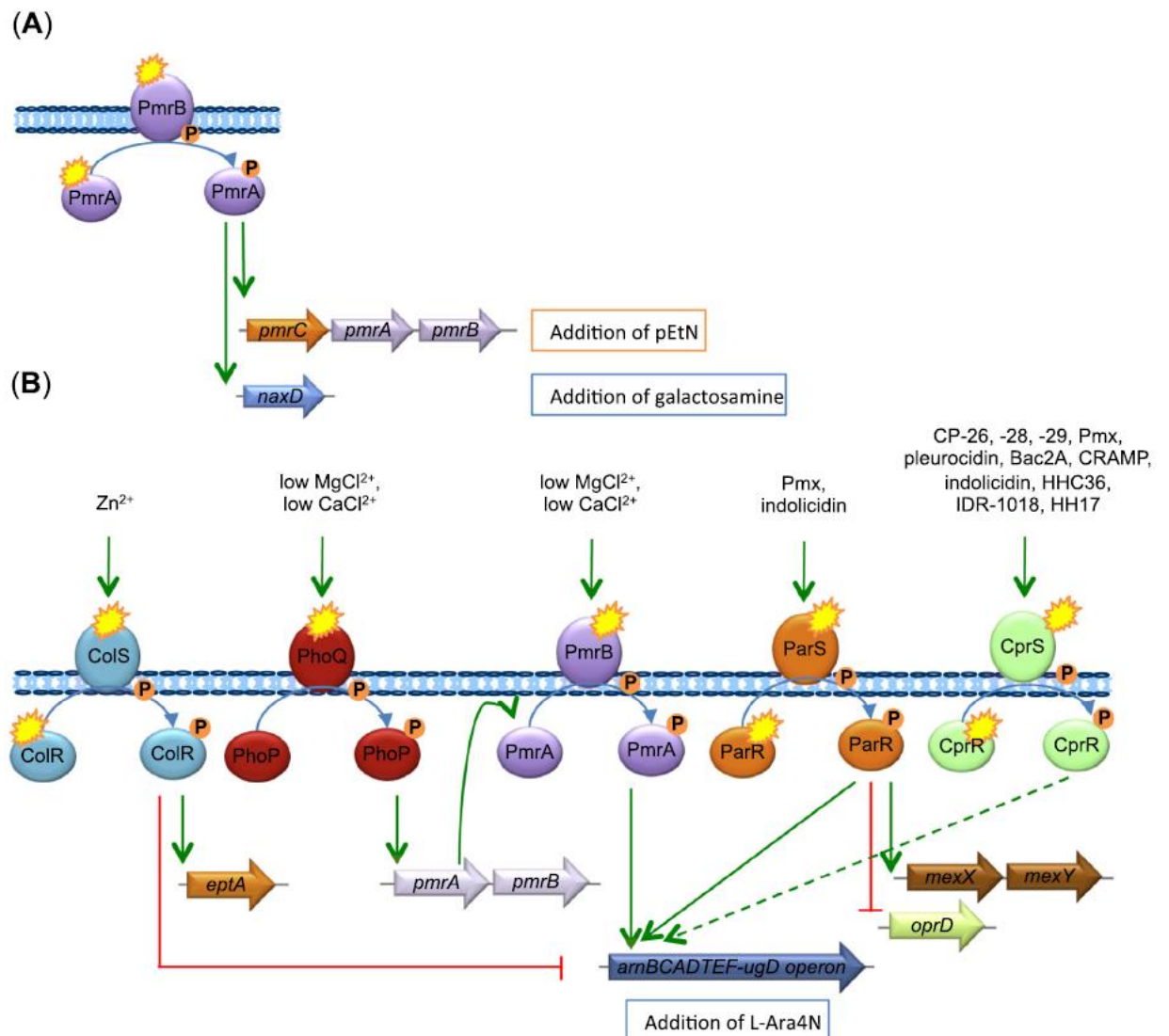
Plusieurs gènes et opérons sont impliqués dans la modification qualitative du LPS (Figures 3 et 4) :

- des gènes codant pour des enzymes qui sont directement impliquées dans la modification du LPS (synthèse et/ou ajout de groupements cationiques sur le LPS) : le gène *pmrC*, le gène *naxD*, le gène *pmrE* et l'opéron *pmrHFIJKLM*.
- des gènes régulant l'expression des gènes impliqués dans la modification du LPS : les systèmes à deux composants PmrA/PmrB (PmrAB), PhoP/PhoQ (PhoPQ), CrrA/CrrB (CrrAB), ParR/ParS (ParRS), CprR/CprS (CprRS), et ColR/ColS (ColRS).
- un gène régulateur du système à deux composants PhoPQ : le gène *mgrB*.
- des gènes connecteurs des systèmes à deux composants : le gène *pmrD* qui connecte les systèmes PmrAB et PhoPQ, et le gène *crrC* qui connecte les systèmes CrrAB et PmrAB.



**Figure 3.** Représentations schématiques de la régulation des gènes impliqués dans la résistance aux polymyxines chez *Escherichia coli* (A) et *Klebsiella pneumoniae* (B). D'après Jeannot et al, International Journal of Antimicrobial Agents 2017 (19).





**Figure 4.** Représentations schématiques de la régulation des gènes impliqués dans la résistance aux polymyxines chez *Acinetobacter baumannii* (A) et *Pseudomonas aeruginosa* (B). D'après Jeannot et al, International Journal of Antimicrobial Agents 2017 (19).

### a. Gènes codant pour les enzymes qui modifient le LPS

L'ajout des groupements cationiques phosphoéthanolamine (pEtN), L-amino-4-arabinose (LAra4N) et galactosamine sur le LPS est médié par différents gènes selon les espèces (Figures 3 et 4):

- les gènes *eptB*, *eptA* et *pmrHFIJKLM* chez *E. coli* (ajout de pEtN et de LAra4N)
- les gènes *pmrC* et l'opéron *pmrHFIJKLM* chez *K. pneumoniae* (ajout de pEtN et de LAra4N)
- les gènes *pmrC* et *naxD* chez *A. baumannii* (ajout de pEtN et de galactosamine)
- le gène *eptA* et l'opéron *pmrHFIJKLM* chez *P. aeruginosa* (ajout de LAra4N)

L'ajout de ces groupements cationiques diminue l'affinité de la colistine pour le LPS bactérien et ainsi conduit à l'acquisition de résistances.

#### ➤ Gène *pmrC* (chez *K. pneumoniae* et *A. baumannii*)

Chez *K. pneumoniae* et *A. baumannii*, le gène *pmrC* est le premier gène de l'opéron *pmrCAB* qui code pour trois protéines : la protéine PmrC, la protéine régulatrice PmrA, et une protéine kinase senseur membranaire PmrB. La protéine PmrC est une aminotransférase qui ajoute un groupement PEtN sur le LPS (20).

#### ➤ Gènes *eptA* (chez *E. coli* et *P. aeruginosa*) et *eptB* (chez *E. coli*)

Chez *E. coli* et *P. aeruginosa*, les gènes *eptA* et *eptB* codent pour des aminotransférases qui ajoutent un groupement PEtN sur le LPS.

#### ➤ Opéron *pmrHFIJKLM* (chez toutes les espèces sauf *A. baumannii*)

L'opéron *pmrHFIJKLM* (aussi appelé opéron *arnBCADTEF*) code pour un total de sept protéines qui synthétisent et ajoutent un groupement LAra4N sur le LPS (21).

#### ➤ Gène *naxD* (chez *A. baumannii* uniquement)

Chez *A. baumannii*, le gène *naxD* code pour une déacétylase qui synthétise et ajoute un groupement galactosamine sur le LPS (22).

## **b. Systèmes à deux composants**

La synthèse de pEtN et de LAra4N est régulée par les systèmes à deux composants.

### ➤ **Système à deux composants PmrAB (chez toutes les espèces)**

#### Rôle physiologique du système à deux composants PmrAB

Les systèmes à deux composants PmrAB et PhoPQ sont normalement activés lorsque la bactérie est phagocytée par les macrophages, c'est-à-dire quand elle est intracellulaire (20). La synthèse de LAra4N est en effet nécessaire à la survie de la bactérie dans les macrophages de l'hôte (23, 24).

#### Régulation par le système à deux composants PmrAB

Pour s'adapter à l'environnement (forte concentration en  $\text{Fe}^{3+}/\text{Al}^{3+}/\text{H}^+$  chez *E. coli* ou *K. pneumoniae*, ou faible concentration en  $\text{MgCl}_2/\text{CaCl}_2$  chez *P. aeruginosa*), la protéine senseur PmrB est activée par autophosphorylation et active alors à son tour la protéine régulatrice PmrA par phosphorylation (Figures 3 et 4) (20, 24–26).

#### *Régulation par le système à deux composants PmrAB chez E. coli et K. pneumoniae*

La protéine PmrA phosphorylée active à son tour le gène *eptA* chez *E. coli*, et l'opéron *pmrCAB* chez *K. pneumoniae*, ce qui conduit à l'ajout de pEtN (Figure 3).

Chez *E. coli* et *K. pneumoniae*, la protéine PmrA active aussi l'opéron *pmrHFIJKLM*, ce qui conduit à l'ajout de groupement LAra4N sur le LPS.

#### *Régulation par le système à deux composants PmrAB chez P. aeruginosa*

Chez *P. aeruginosa*, l'activation de PmrA entraîne l'activation de l'opéron *pmrHFIJKLM*, ce qui conduit à l'ajout de groupements LAra4N sur le LPS (Figure 4) (22).

#### *Régulation par le système à deux composants PmrAB chez A. baumannii*

Chez *A. baumannii*, l'activation de PmrA entraîne l'activation de l'opéron *pmrCAB* et du gène *naxD*, ce qui conduit à l'ajout, respectivement, de groupements pEtN et galactosamine, sur le LPS (Figure 4) (22).

## Conséquences des mutations au sein des gènes *pmrA* et *pmrB*

Des mutations au sein des gènes *pmrA* et *pmrB* sont impliquées dans l'acquisition de résistances à la colistine chez *E. coli*, *K. pneumoniae*, *Salmonella enterica*, *P. aeruginosa* et *A. baumannii* (Annexe B). Ces mutations entraînent une activation constitutive du régulon PmrAB (27) et donc la production de groupements cationiques et la modification du LPS.

### ➤ Système à deux composants PhoPQ (chez toutes les espèces sauf *A. baumannii*)

#### Structure de l'opéron *phoPQ*

L'opéron PhoPQ code pour deux protéines : une protéine régulatrice PhoP et une protéine kinase senseur membranaire PhoQ.

#### Rôle physiologique du système à deux composants PhoPQ

Le système à deux composants PhoPQ permet l'expression de gènes qui codent pour des transporteurs de  $Mg^{2+}$ , des enzymes qui modifient le LPS pour permettre la résistance aux peptides antimicrobiens cationiques, des enzymes qui réduisent le stress cellulaire dû à un pH acide ou encore des facteurs de virulence (28, 29). Le système PhoPQ permet ainsi la survie bactérienne dans des conditions de faible concentration de magnésium ou de pH acide ou en présence de peptides antimicrobiens.

#### Régulation par le système à deux composants PhoPQ

Pour s'adapter à l'environnement (faible concentration en  $MgCl^{2+}/CaCl_2$ ), la protéine senseur PhoQ est activée par autophosphorylation et active alors à son tour la protéine régulatrice PhoP par phosphorylation (Figures 3 et 4) (28, 29).

#### *Régulation par le système à deux composants PhoPQ chez E. coli et K. pneumoniae*

La protéine PhoP activée active le gène *eptB* chez *E. coli* et l'opéron *pmrHFIJKLM* chez *K. pneumoniae*, ce qui conduit à la synthèse de pEtN et de LAra4N respectivement (Figure 3).

Chez *E. coli* et *K. pneumoniae*, PhoP peut aussi activer indirectement PmrA par l'intermédiaire de la protéine PmrD. L'activation de la protéine PmrA conduit à la synthèse de pEtN et de LAra4N comme décrit précédemment.

#### *Régulation par le système à deux composants PhoPQ chez P. aeruginosa*

Chez *P. aeruginosa*, la protéine PhoP active directement la protéine PmrA, ce qui conduit à la synthèse de LAra4N comme décrit précédemment (Figure 4).

### Conséquences des mutations au sein des gènes *phoP* et *phoQ*

Des mutations au sein des gènes *phoP* et *phoQ* sont impliquées dans l'acquisition de résistances à la colistine chez *E. coli*, *K. pneumoniae*, et *P. aeruginosa* (Annexe B). Ces mutations entraînent une activation constitutive du régulon PhoPQ et par conséquent la production des groupements cationiques et la modification du LPS.

#### ➤ **Système à deux composants CrrAB (chez *K. pneumoniae* uniquement)**

### Rôle physiologique du système à deux composants CrrAB

L'opéron *crrAB* n'est pas présent dans le génome de toutes les souches de *K. pneumoniae* (30). Ce système n'est donc pas indispensable à la bactérie.

### Conséquences des mutations au sein du gène *crrB*

Des mutations au sein du gène *crrB* sont impliquées dans l'acquisition de résistances de haut niveau à la colistine chez *K. pneumoniae* (Annexe B) (30, 31). Ces mutations entraînent une activation constitutive du régulon PmrAB par l'intermédiaire d'un connecteur CrrC (31) et donc la production des groupements cationiques pEtN et LAra4N et la modification du LPS (Figure 3).

Une étude récente a révélé que des mutations dans la protéine CrrB entraînent l'augmentation de l'expression d'un gène codant pour une probable pompe d'efflux de type Resistance-Nodulation-Division (RND) (32). En effet, la délétion du gène codant pour cette pompe d'efflux chez les souches avec CrrB mutée entraîne une diminution des CMIs de la colistine. La résistance à la colistine liée aux mutations du gène *crrB* serait donc également médiée par l'expression d'une pompe d'efflux (32).

#### ➤ **Système à deux composants ParRS (chez *P. aeruginosa* uniquement)**

### Structure de l'opéron *parRS*

L'opéron *parRS* code pour deux protéines : une protéine régulatrice ParR et une protéine kinase senseur membranaire ParS.

### Régulation par le système à deux composants ParRS

Pour s'adapter à l'environnement (présence d'indolicidine et concentrations subinhibitrices de colistine ou de polymyxine B), la protéine senseur ParS est activée par autophosphorylation et active alors à son tour la protéine régulatrice ParR par phosphorylation (33, 34). Cette dernière active l'opéron *pmrHFIJKLM*, ce qui conduit à la synthèse de LAra4N.

### Conséquences des mutations au sein des gènes *parR* et *parS*

Des mutations au sein des gènes *parR* et *parS* sont responsables de l'acquisition de résistances à la colistine (Annexe B), mais aussi de l'augmentation de la production du système d'efflux MexXY et d'une dérégulation de la porine OprD (Figure 4). Ces altérations conduisent donc à une résistance aux polymyxines mais aussi aux aminoglycosides, aux fluoroquinolones et à certaines  $\beta$ -lactamines (35).

#### ➤ **Système à deux composants CprRS (chez *P. aeruginosa* uniquement)**

### Régulation par le système à deux composants CprRS

Pour s'adapter à l'environnement (présence d'indolicidine et concentrations subinhibitrices de colistine ou de polymyxine B), la protéine senseur CprS est activée par autophosphorylation et active alors à son tour la protéine régulatrice CprR par phosphorylation (34, 36, 37). Cette dernière active l'opéron *pmrHFIJKLM*, ce qui conduit à la synthèse de LAra4N.

### Conséquences de l'altération des gènes *cprR* et *cprS*

L'inactivation du gène *phoQ* couplée à des altérations des gènes *cprR* et *cprS* est impliquée dans l'acquisition de résistances à la colistine chez *P. aeruginosa* (Annexe B) (36).

#### ➤ **Système à deux composants ColRS (chez *P. aeruginosa* uniquement)**

### Régulation par le système à deux composants ColRS

Pour s'adapter à l'environnement (présence d'ions zinc), la protéine senseur membranaire ColS est activée par autophosphorylation et active alors à son tour la protéine régulatrice ColR par phosphorylation (36). La protéine ColR active la transcription du gène *eptA* et réprime la transcription de l'opéron *pmrHFIJKLM* (Figure 4), ce qui conduit à la synthèse de pEtN comme décrit précédemment.

### Conséquences de l'altération des gènes *colR* et *colS*

L'inactivation du gène *phoQ* couplée à des altérations des gènes *colR* et *colS* est impliquée dans l'acquisition de résistances à la colistine chez *P. aeruginosa* (Annexe B) (36).

### c. Gène régulateur du système à deux composants PhoPQ

#### ➤ Gène *mgrB* (chez *K. pneumoniae* uniquement)

Le système à deux composants PhoPQ active la transcription du gène *mgrB*

Chez *K. pneumoniae*, la stimulation de PhoQ par une faible concentration extracellulaire d'ions magnésium ou par des peptides antimicrobiens cationiques entraîne la phosphorylation de PhoP, qui active alors la transcription du gène *mgrB* (38).

La protéine MgrB exerce un rétrocontrôle négatif sur le système à deux composants PhoPQ

Après son activation, la protéine MgrB réprime PhoQ, ce qui diminue alors la phosphorylation de PhoP. MgrB exerce ainsi un rétrocontrôle négatif sur le système à deux composants PhoPQ (Figure 3) (38).

Conséquences des mutations au sein du gène *mgrB*

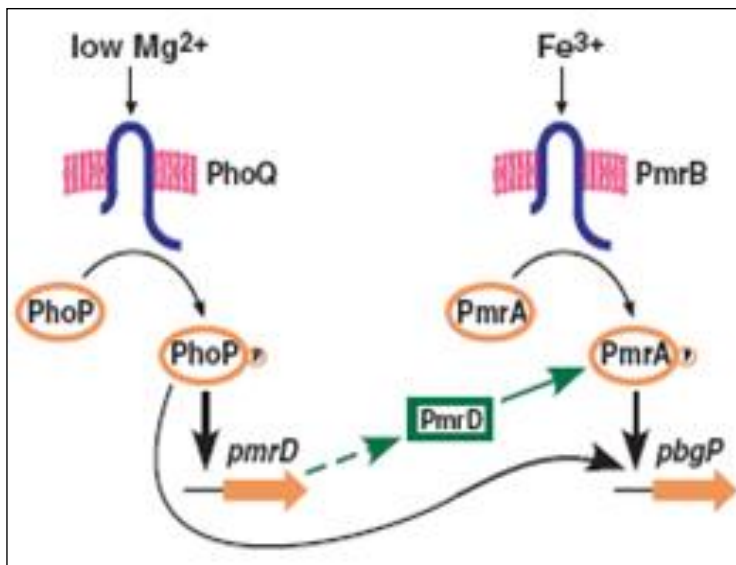
L'inactivation du gène *mgrB* (insertion ou délétion de séquences nucléotidiques, mutations) est un mécanisme d'acquisition de résistances à la colistine chez *K. pneumoniae* et *Klebsiella oxytoca* (Annexe B) (39, 40). L'inactivation du gène *mgrB*, régulateur négatif du système à deux composants PhoPQ, entraîne une surexpression des gènes *phoP* et *phoQ*. L'augmentation de transcription de l'opéron *phoPQ* induit alors l'activation de l'opéron *pmrHFIJKLM* conduisant ainsi à la production de LAra4N responsable de l'acquisition de résistances à la colistine.

### d. Gènes connecteurs des systèmes à deux composants

#### ➤ Gène *pmrD* (chez *E. coli*, *K. pneumoniae* et *S. enterica*)

Le système à deux composants PhoPQ active le système à deux composants PmrAB par l'intermédiaire du gène *pmrD*

Situé en aval de l'opéron *pmrHFIJKLM*, le gène *pmrD* joue un rôle essentiel chez *E. coli*, *K. pneumoniae* et *S. enterica* car il permet la connexion entre les systèmes à deux composants PhoPQ et PmrAB (41) (Figure 5). En présence d'un taux faible d'ions magnésium dans le milieu extracellulaire, la protéine PhoP phosphorylée active la transcription du gène *pmrD*. La protéine PmrD se lie à la forme phosphorylée de la protéine régulatrice PmrA, ce qui la protège de la déphosphorylation par la protéine PmrB et donc maintient son activation (41). La protéine PmrA active alors la transcription de l'opéron *pmrHFIJKLM* comme décrit précédemment.



**Figure 5.** Activation directe et indirecte de l'opéron *pmrHFIJKLM* par le système à deux composants PhoPQ chez *K. pneumoniae*.

D'après Mitrophanov et al, PLOS Genetics 2008 (41).

La protéine PhoP active ainsi l'expression de l'opéron *pmrHFIJKLM* de deux manières différentes chez *E. coli*, *K. pneumoniae* et *S. enterica* (Figure 5) (41) :

- directement par liaison de la protéine PhoP activée au promoteur de l'opéron *pmrHFIJKLM*
- indirectement par l'intermédiaire de la protéine PmrD qui maintient l'activation de la protéine PmrA qui active à son tour la transcription de l'opéron *pmrHFIJKLM*

La protéine PmrA n'exerce pas de rétrocontrôle négatif sur le gène *pmrD* chez *K. pneumoniae*

A l'inverse de ce qui se passe chez *S. enterica*, la transcription du gène *pmrD* n'est pas réprimée par la protéine PmrA chez *K. pneumoniae* (absence de rétrocontrôle négatif) en raison de l'absence de site de liaison de PmrA sur la région promotrice du gène *pmrD* (41).

Conséquences des modifications d'expression du gène *pmrD*

Chez *S. enterica*, l'amplification du gène *pmrD* qui entraîne sa surexpression est responsable de cas d'hétérorésistance à la colistine (42).



➤ **Gène *crrC* (chez *K. pneumoniae* uniquement)**

Le système à deux composants CrrAB active le système à deux composants PmrAB par l'intermédiaire du gène *crrC*

Le gène *crrC* permet la connexion entre les systèmes à deux composants CrrAB et PmrAB (42) (Figure 3).

**1.1.2. Modification de l'acétylation du LPS (chez *K. pneumoniae*)**

➤ **Gène *lpxM* (chez *K. pneumoniae*)**

Le gène *lpxM* est responsable de l'addition de chaînes acétyl sur le lipide A des entérobactéries. Des mutations dans ce gène sont responsables de l'acquisition de résistances à la colistine chez *K. pneumoniae* (Annexe B) (43). Il est probable que cette résistance à la colistine soit due à la modification de l'acétylation du LPS.

**1.1.3. Augmentation de production du LPS (chez *K. pneumoniae*)**

➤ **Gène *yciM* (chez *K. pneumoniae*)**

Le gène *yciM* est un régulateur de la synthèse du LPS. Des mutations dans ce gène sont responsables de l'acquisition de résistances à la colistine chez *K. pneumoniae* (Annexe B) (43). Il est probable que cette résistance à la colistine soit due à l'augmentation de la production du LPS.

**1.1.4. Absence de production de LPS (chez *A. baumannii*)**

Chez *A. baumannii*, il a été montré que la résistance à la colistine pouvait être la conséquence d'une perte complète de la production de lipopolysaccharide (modification quantitative du LPS) suite à des mutations au sein des gènes *lpxA*, *lpxC*, *lpxD* qui codent pour les trois premières enzymes impliquées dans la synthèse du LPS (Annexe B) (44). Un des mécanismes décrit est l'insertion d'une séquence d'insertion IS*Abal1* dans *lpxA* et *lpxC* (45). Cependant, ces mutations engendreraient une perte de fitness (46). Ceci pourrait expliquer pourquoi ces souches sont rarement rencontrées dans des souches cliniques.

Par ailleurs, des mutations au sein du gène *lpsB*, qui code pour une glycosyltransférase impliquée dans la synthèse du LPS, sont impliquées dans l'acquisition de résistances à la colistine chez *A. baumannii* (47–49).

## **1.2. Modifications de la capsule polysaccharidique (CPS)**

### **1.2.1. Hyperproduction de la CPS (chez *K. pneumoniae*)**

Chez *K. pneumoniae*, la CPS agit comme une barrière protectrice. L'hyperproduction de CPS confère une résistance aux polymyxines car elle limite les interactions des polymyxines avec leurs sites de fixation à la surface du LPS (50). Des mutants ne produisant pas de CPS sont ainsi plus sensibles aux peptides antimicrobiens.

### **1.2.2. Libération de la CPS : phénomène de trapping (chez *K. pneumoniae* et *P. aeruginosa*)**

Chez *K. pneumoniae* et *P. aeruginosa*, la libération de la CPS anionique par la bactérie entraîne la capture des peptides antimicrobiens cationiques (phénomène de trapping), réduisant ainsi la quantité de peptides antimicrobiens qui atteint la surface bactérienne et empêchant ainsi leur interaction électrostatique avec la surface bactérienne (51). La CPS est reliée à la surface bactérienne par une interaction ionique avec le LPS et cette interaction est stabilisée par des cations divalents (52). Il est donc probable que la libération de CPS en présence de polymyxines s'explique par la perturbation des ponts ioniques cation-dépendants entre les molécules de LPS.

## **1.3. Implication des porines**

Chez *S. enterica*, des protéines périplasmiques (YdeI et YgiW) pourraient interagir avec les porines OmpD et OmpF, respectivement, pour augmenter la résistance bactérienne aux polymyxines et aux peptides antimicrobiens cationiques (53).

L'investigation des protéines OMPs chez des souches de *K. pneumoniae* et *E. asburiae* résistantes à la colistine révèle la perte d'une protéine de membrane externe (perte d'un domaine LysM d'une taille de 16 kDa) qui possède une fonction de liaison au peptidoglycane et révèle une surexpression du système régulateur *phoP-pmrD-arn* (54).

L'implication des porines dans l'acquisition de résistances à la colistine reste à préciser.

## 1.4. Implication des pompes d'efflux

La résistance à la colistine causée par un système antiport de transport d'ions potassium a été décrite chez *Yersinia pseudotuberculosis* (55). L'hypothèse proposée est que la pompe d'efflux préviendrait l'action des peptides cationiques en acidifiant le cytoplasme et en rejetant l'antibiotique hors de la bactérie.

Plusieurs études suggérant un rôle des pompes d'efflux dans l'acquisition de résistances à la colistine chez *K. pneumoniae* ont récemment été publiées (32, 56–59). Une étude a notamment révélé que l'ajout d'inhibiteurs de pompes d'efflux diminue la survie des bactéries en présence de polymyxines (58). Des études complémentaires sont nécessaires afin de préciser l'implication des pompes d'efflux dans l'acquisition de résistances à la colistine.

## 2. Résistances plasmidiques

Actuellement, cinq protéines (MCR-1 à MCR-5) conférant des résistances plasmidiques à la colistine ont été décrites chez les entérobactéries (Tableau 2).

**Tableau 2.** Protéines de résistance plasmidiques à la colistine.

<b>Protéine</b>	<b>Taille de la protéine</b>	<b>Pays de découverte</b>	<b>Espèce progénitrice supposée</b>	<b>Numéro Genbank</b>	<b>Référence</b>
MCR-1	541 aa	Chine	<i>Moraxella</i> spp.	KP347127	(60)
MCR-2	538 aa	Belgique	<i>Moraxella pluranimalium</i>	LT598652	(61)
MCR-3	541 aa	Chine	<i>Aeromonas</i> spp.	KY924928	(62)
MCR-4	541 aa	Espagne, Belgique	<i>Shewanella frigidimarina</i>	MF543359	(63)
MCR-5	547 aa	Allemagne	<i>Cupriavidus gilardii</i>	KY807921	(64)

### 2.1. Gène de résistance plasmidique *mcr-1* et variants

#### 2.1.1. Gène de résistance plasmidique *mcr-1*

Le gène de résistance plasmidique *mcr-1* (1 626 pb) a été découvert en Chine en 2015 chez des souches de *E. coli* et de *K. pneumoniae* isolées chez le porc et l'homme (60). Ce gène a ensuite été décrit dans le monde entier au sein de souches d'entérobactéries d'espèces variées (*Enterobacter*, *Salmonella*, *Shigella*...) provenant de l'environnement, de l'animal, ou de l'homme (9). Le gène *mcr-1* est mobilisé par un transposon composite appelé Tn6330.2 constitué du gène *mcr-1* entouré de deux séquences d'insertion IS*Apl1* (famille IS30) (43). Ce transposon composite cible préférentiellement des séquences riches en AT et

la transposition génère une duplication de 2 pb. Le gène *mcr-1* peut être porté par des types de plasmides très variés (IncX4, IncI2, IncHI2, IncF, IncY, et IncP). Le progéniteur de MCR-1 semble être une espèce bactérienne du genre *Moraxella* car l'élément génétique mobile IS1595 a pour origine génétique *Moraxella* et l'analyse phylogénétique de MCR-1 a révélé une forte homologie de la protéine avec les phosphoéthanolamines transférases de *Moraxella* spp.

### 2.1.2. Variants du gène de résistance plasmidique *mcr-1*

Plusieurs variants de la protéine MCR-1 ont été décrits chez les entérobactéries (Tableau 3).

**Tableau 3.** Variants de la protéine MCR-1 disponibles dans la base de données GenBank.

<b>Variant</b>	<b>Substitution protéique</b>	<b>Numéro Genbank</b>	<b>Référence</b>
MCR-1.2	Q3L	KX236309	(65)
MCR-1.3	I38V	KU934208	(66)
MCR-1.4	D440N	KY041856	(67)
MCR-1.5	H452Y	KY283125	(67)
MCR-1.6	R536H	KY352406	(68)
MCR-1.7	A215T	KY488488	(67)
MCR-1.8	Q3R	KY683842	(67)
MCR-1.9	V407A	KY964067	-
MCR-1.11	Ins V en 7-8	KY853650	-
MCR-1.12	Q3H	LC337668	-

Ins, insertion

### 2.2. Gène de résistance plasmidique *mcr-2*

Le gène de résistance plasmidique *mcr-2* (1 617 pb) a été découvert dans des souches de *E. coli* isolées chez le porc et le boeuf en Belgique (61). Il est localisé sur un élément génétique mobile de la famille IS1595 (séquences d'insertion IS1595 de chaque côté du gène *mcr-2* et présence d'une transposase de type ISXO<sub>2</sub>-like). Cet élément mobile est porté par un plasmide de type IncX4 (61). La protéine MCR-2 (538 aa) partage 80,7 % d'identité en acides aminés avec MCR-1. Le progéniteur du gène codant pour MCR-2 est très probablement *Moraxella pluranimalium* car l'élément génétique mobile IS1595 a été identifié chez *M. pluranimalium* et l'analyse phylogénétique de MCR-2 a révélé une forte homologie (99 % d'identité protéique) avec une phosphoéthanolamine transférase de *M. pluranimalium* (69).

## **2.3. Gène de résistance plasmidique *mcr-3* et variants**

### **2.3.1. Gène de résistance plasmidique *mcr-3***

Le gène de résistance plasmidique *mcr-3* (1 626 pb) a été découvert dans des souches de *E. coli* isolées chez le porc en Chine (62). Il est porté par un plasmide de type IncHI2 portant un transposon TnAs2 (62). La protéine MCR-3 (541 aa) partage 32,5 % et 31,7 % d'identité en acides aminés avec MCR-1 et MCR-2, respectivement. Le progéniteur du gène codant pour MCR-3 serait une espèce bactérienne du genre *Aeromonas* car le transposon TnAs2 n'a été identifié que chez *Aeromonas salmonicida* et l'analyse phylogénétique de MCR-3 a révélé une forte homologie (94,1 à 94,8 % d'identité en acides aminés) avec les phosphoéthanolamines transférases de *Aeromonas* spp. (62).

### **2.3.2. Variants du gène de résistance plasmidique *mcr-3***

Plusieurs variants de la protéine MCR-3 ont été décrits chez les entérobactéries (Tableau 4).

**Tableau 4.** Variants de la protéine MCR-3 disponibles dans la base de données GenBank.

<b>Variant</b>	<b>Substitution protéique</b>	<b>Numéro Genbank</b>	<b>Référence</b>
MCR-3.2	T488I	WP_094315354	-
MCR-3.4	G373V	OYJ46098	-
MCR-3.5	M23V, A457E, T488I	MF463699	(70)
MCR-3.10	V122G	MG214533	-
MCR-3.11	V373G, T468Q	AUO29399	-

## **2.4. Gène de résistance plasmidique *mcr-4* et variants**

### **2.4.1. Gène de résistance plasmidique *mcr-4***

Le gène de résistance plasmidique *mcr-4* (1626 pb) a été découvert dans une souche de *S. enterica* serovar Typhimurium isolée chez le porc en Italie et dans des souches de *E. coli* isolées chez le porc en Espagne et en Belgique (63). Il est porté par un plasmide de type ColE10 qui peut être mobilisé à l'aide d'un plasmide conjugatif « helper » (63). La protéine MCR-4 (541 aa) partage 34 %, 35 % et 49 % d'identités en acides aminés avec MCR-1, MCR-2 et MCR-3, respectivement. Le progéniteur du gène codant pour MCR-4 serait *Shewanella frigidimarina* car la séquence d'insertion ISKpn6 située en amont du gène *mcr-4* montre 99 % d'identité nucléotidique avec une séquence d'insertion identifiée chez *S. frigidimarina* et l'analyse phylogénétique de MCR-4 a révélé une forte homologie (82 à 99 % d'identité protéique) avec les phosphoéthanolamines transférases de *Shewanella* spp.

## 2.4.2. Variants du gène de résistance plasmidique *mcr-4*

Plusieurs variants de la protéine MCR-4 ont été décrits chez les entérobactéries (Tableau 5).

**Tableau 5.** Variants de la protéine MCR-4 disponibles dans la base de données GenBank.

<b>Variant</b>	<b>Substitution protéique</b>	<b>Numéro Genbank</b>	<b>Référence</b>
MCR-4	-	ASR73329	(63)
MCR-4.2	Q331R	AVP73879	(71)
MCR-4.3	V236F	PYZ20165	(72)
MCR-4.4	H205N, Q331R	WP_109545055	(73)
MCR-4.5	P110L, Q331R	WP_109545054	(73)

## 2.5. Gène de résistance plasmidique *mcr-5*

Le gène de résistance plasmidique *mcr-5* (1644 pb) a été découvert dans une souche de *S. enterica* serovar Paratyphi B isolée chez de la volaille et dans l'alimentation en Allemagne (64). Il fait probablement parti d'un opéron qui code pour la protéine MCR-5 mais aussi pour une protéine ChrB impliquée dans la résistance au chromate et pour deux fragments de protéines de transport de la superfamille majeure de facilitateurs (MFS). L'opéron contenant le gène *mcr-5* est localisé sur un transposon de la famille Tn3 porté par un plasmide de type ColE (64). La protéine MCR-5 (547 aa) partage 36,1 %, 35,3 %, 34,7 % et 33,7 % d'identités en acides aminés avec MCR-1, MCR-2, MCR-3 et MCR-4, respectivement (64). Le progéniteur du gène codant pour MCR-5 est indéterminé mais la protéine MCR-5 et le transposon de type Tn3 ont tous les deux été identifiés au sein de *Cupriavidus gilardii* suggérant que cette espèce bactérienne pourrait être l'espèce progénitrice (64). Le gène *mcr-5* a récemment été identifié dans une souche de *Aeromonas hydrophila* dans des selles de cochons en Chine (74). Ce gène était porté par un plasmide capable de répllication chez *Pseudomonas* faisant craindre une dissémination de ce gène chez ce genre bactérien.

## 3. Epidémiologie de la résistance

### 3.1. Facteurs de risque associés à la résistance à la colistine

Une pression de sélection liée à l'utilisation de colistine semble être un facteur de risque indépendant pour l'acquisition de la résistance à la colistine (75, 76). La décontamination du tractus gastrointestinal par de la colistine entraîne l'acquisition de souches résistantes à la colistine et la sélection de souches naturellement résistantes à la colistine (77–79). L'utilisation inappropriée de la colistine, et notamment un dosage suboptimal ou une monothérapie prolongée, favoriserait l'émergence de sous populations résistantes à la colistine, correspondant au phénomène dit d'hétérorésistance (11). De même, l'administration à des

intervalles de temps importants (toutes les 12 ou 24 heures) semblerait favoriser la sélection de souches résistantes (80).

### **3.2. Pressions de sélection pour l'acquisition de résistances plasmidiques à la colistine**

L'acquisition de résistances plasmidiques à la colistine a été décrite au sein de souches d'entérobactéries isolées chez l'homme en l'absence de pression de sélection par des polymyxines (81, 82). Plusieurs arguments sont en faveur d'une origine animale des gènes de résistances plasmidiques identifiés chez l'homme : l'isolement fréquent d'entérobactéries productrices de *mcr-1* chez l'animal, l'origine animale de *Moraxella* spp. qui est le progéniteur probable de *mcr-1* et *mcr-2*, et la co-localisation sur le même plasmide du gène *mcr-1* et de gènes de résistances à des antibiotiques d'usage vétérinaire. La résistance à la colistine d'abord sélectionnée chez l'animal aurait ensuite diffusée chez l'homme.

Les peptides antimicrobiens cationiques (cathélicidines et défensines) qui font partie du système immunitaire intrinsèque de l'homme ne semblent pas être une pression de sélection pour l'acquisition de résistances à la colistine. En effet, les mutations chromosomiques et le gène de résistance plasmidique *mcr-1* responsable de l'acquisition de résistances à la colistine ne sont pas responsables de résistances croisées aux peptides antimicrobiens cationiques LL-37,  $\alpha$ -défensine 5 (HD5) et  $\beta$ -défensine 3 (HDB3) (83).

### **D. Détermination de la sensibilité à la colistine**

La détermination de la sensibilité à la colistine est difficile et les délais d'obtention des résultats sont longs (24-48h). Plusieurs facteurs sont à l'origine des difficultés pour tester la sensibilité à la colistine :

- le haut poids moléculaire de la colistine qui entraîne une faible diffusion de la colistine dans l'agarose responsable de résultats de fausses sensibilités obtenus avec la méthode des disques et les bandelettes de gradient de diffusion
- les propriétés cationiques de la colistine qui entraînent sa fixation sur les matières plastiques chargées négativement
- les résultats non reproductibles ou non interprétables obtenus avec la méthode de dilutions en milieu liquide qui entraînent des difficultés pour déterminer les CMI des souches hétérorésistantes
- l'absence de méthode de référence fiable et de réalisation facile.

Par ailleurs, les études comparatives d'évaluation des tests et automates disponibles ont souvent des limites :

- absence de vérification de la non duplication des souches testées
- faible nombre de souches résistantes à la colistine testées
- manque de diversité des espèces bactériennes testées
- manque de diversité des mécanismes de résistances testés (chromosomiques et plasmidiques)
- absence de souches avec un faible niveau de résistance à la colistine (4 mg/L, sachant qu'une souche est résistante si la CMI de la colistine est  $\geq 4$  mg/L)
- la méthode de référence utilisée dans les anciennes études n'est pas toujours celle recommandée actuellement (dans beaucoup d'anciennes études, les dilutions en milieu liquide étaient réalisées avec ajout de Tween, ce qui n'est plus recommandé maintenant)

## **1. Méthodes de dilutions**

L'objectif des méthodes de dilution est de déterminer la concentration minimale inhibitrice (CMI) qui correspond à la plus petite concentration de colistine qui inhibe la croissance bactérienne visible après une incubation de 16 à 24 heures à 37°C. Les méthodes de dilutions sont des techniques testant une suspension bactérienne dans plusieurs concentrations de colistine dans un milieu liquide (dilutions en milieu liquide) ou en gélose (dilutions en milieu solide).

### **1.1. Dilutions en milieu liquide : méthode de référence**

La méthode de dilutions en milieu liquide (BMD) est actuellement la seule méthode recommandée par les comités américain (Clinical Laboratory Standards Institute (CLSI)) et européen (European Committee on Antimicrobial Susceptibility Testing (EUCAST)) ([www.eucast.org](http://www.eucast.org)) pour tester la sensibilité à la colistine.

D'après ces recommandations, la méthode de dilutions en milieu liquide doit être réalisée dans du milieu Mueller Hinton ajusté en cations (MHB-CA), sans addition de surfactant (polysorbate ou Tween 80 utilisé auparavant pour empêcher la fixation de la colistine sur les plaques en polystyrène) et dans des plaques en polystyrène non traitées. La gamme de concentration testée s'étend de 0,12 à 128 mg/L et la concentration finale bactérienne est de  $5.10^5$  UFC/mL dans chaque puit (84).

Cependant, la méthode de dilutions en milieu liquide est fastidieuse, nécessite une préparation manuelle des solutions stocks d'antibiotique pouvant conduire à des erreurs et n'est ainsi pas utilisable par



la majorité des laboratoires de microbiologie de routine. Par ailleurs, des CMI non reproductibles et non interprétables sont décrites en raison de la présence de puits manqués (c'est-à-dire, des puits sans croissance bactérienne alors qu'il y a croissance à des concentrations plus élevées d'antibiotiques) pour certaines souches de *Enterobacter* (85), de *P. aeruginosa* (86) et de *A. baumannii* (87, 88). Ce phénomène est appelé hétérorésistance (85).

Malgré ces inconvénients, la méthode de dilutions en milieu liquide reste la méthode de référence pour déterminer les CMI en raison de sa reproductibilité, de sa fiabilité, et de la possibilité d'automatisation.

## **1.2. Dilutions en milieu solide**

La méthode de dilutions en milieu solide étudie la croissance d'une suspension bactérienne sur des géloses Mueller Hinton (MH) contenant différentes concentrations de colistine (en général de 0,12 mg/L à 512 mg/L) (89) et l'inoculum bactérien testé est de  $10^4$  UFC de bactéries.

La méthode de dilutions en milieu solide évite théoriquement l'adsorption de la colistine sur le polystyrène des boîtes. De nombreuses études ont montré une grande corrélation entre les méthodes de dilutions en milieu solide et en milieu liquide (89–91), à l'exception des résultats obtenus pour les souches de *P. aeruginosa* et de *S. maltophilia* isolées des patients atteints de mucoviscidose (92, 93). L'avantage de cette méthode est qu'elle permet de tester plusieurs souches sur une même gélose et qu'il est possible d'automatiser l'inoculation des suspensions bactériennes. Cependant, la préparation manuelle des géloses est fastidieuse, et les géloses doivent être utilisées dans la semaine suivant leur préparation.

Beaucoup d'études ont employé la méthode de dilutions en milieu solide comme méthode de référence, cependant le comité joint CLSI-EUCAST a récemment décidé que cette méthode n'était pas recommandée pour la détermination de la sensibilité à la colistine ([www.eucast.org](http://www.eucast.org)).

## **2. Méthode des disques**

La méthode des disques est une méthode de diffusion d'une quantité donnée de colistine à partir d'un disque imprégné d'antibiotique dans une gélose MH ensemencée avec une suspension bactérienne. Le diamètre d'inhibition est mesuré après une incubation de 18 à 24 heures à 37°C.

La méthode des disques est une méthode facile et peu coûteuse qui ne nécessite pas d'équipement spécifique. Ces avantages expliquent pourquoi cette méthode est très utilisée pour tester la sensibilité aux antibiotiques des bactéries. Cependant la faible diffusion de la colistine dans la gélose est responsable des très mauvaises performances obtenues avec cette méthode. Des taux élevés de résultats de fausses sensibilités sont en effet décrits (jusqu'à 35%) comparés aux résultats obtenus par la méthode de dilutions

(77, 91, 93–95). C’est pourquoi les valeurs seuils des diamètres d’inhibition ont été retirées par l’EUCAST, l’utilisation des disques pour tester la sensibilité à la colistine est désormais proscrite et la détermination des CMI est nécessaire avant son utilisation clinique (96).

### **3. Colisport**

Le « Colisport » est une méthode pour détecter la résistance à la colistine chez *E. coli* qui a récemment été proposée par Jouy et al (97). Il s’agit d’un test qui consiste en l’application d’une goutte de 10 µl d’une solution de colistine à 8 mg/L à la surface d’une gélose MH ensemencée avec une suspension bactérienne à 10<sup>8</sup> UFC/mL. Le diamètre d’inhibition est mesuré après une incubation de 16 à 18 heures à 35°±2°C.

Les performances de ce test ont été évaluées avec une collection de 42 souches sensibles et de 89 souches résistantes à la colistine (incluant des souches *mcr-1* positives). Une zone d’inhibition de 8 à 10 mm a été obtenue pour toutes les souches sensibles à la colistine, tandis que les souches résistantes à la colistine ne présentaient pas de zone d’inhibition. D’autres études sont nécessaires pour préciser les performances de ce test, notamment sur d’autres espèces bactériennes que *E. coli*.

### **4. Bandelettes par gradient de diffusion**

Les bandelettes par gradient de diffusion (bandelettes Etest<sup>®</sup>, MIC tests Strip<sup>®</sup>) sont des systèmes contenant un gradient de concentrations de colistine. Ces systèmes sont déposés sur une gélose MH après ensemencement par une suspension bactérienne. Le gradient de concentrations de colistine contenu dans la bandelette diffuse dans la gélose et les CMI sont lues après une incubation de 16 à 24 h à 37°C.

#### **4.1. Bandelettes Etest<sup>®</sup> (bioMérieux)**

Les bandelettes Etest<sup>®</sup> (bioMérieux) sont des systèmes contenant un gradient de colistine de 0,016 à 256 mg/L. De nombreuses études ont décrit des taux élevés de résultats de fausses sensibilités (jusqu’à 32 %) comparés aux résultats obtenus par la méthode de dilutions en milieu liquide (85, 89, 93, 94). Les bandelettes Etest<sup>®</sup> ne détectent pas la résistance à la colistine chez certaines souches même lorsqu’elles présentent des CMI élevées à la colistine par la méthode de dilutions en milieu liquide (85, 94). De plus, il y a des discordances entre les CMI mesurées par Etest<sup>®</sup> et les CMI mesurées par dilution (85, 98). Les

bandelettes Etest<sup>®</sup> sous-estiment les CMI pour les souches résistantes (MIC  $\geq$  4 mg/L) et surestiment les CMI pour les souches sensibles (MIC < 4 mg/L) (85).

#### **4.2. Bandelettes MIC Test Strip<sup>®</sup> (Liofilchem)**

Les bandelettes MIC Test Strip<sup>®</sup> (Liofilchem) sont des systèmes contenant un gradient de colistine de 0,064 à 1 024 mg/L. Une étude a révélé que ces bandelettes sous-estimaient les CMI en particulier pour les souches présentant des CMI proches des valeurs seuils (2-8 mg/L) conduisant à des résultats de fausses sensibilités (99).

Plusieurs études ont donc révélé de mauvaises performances des bandelettes par gradient de diffusion pour la détermination des CMI et pour la catégorisation des souches (sensibles ou résistantes). Ces mauvais résultats sont probablement liés à la faible diffusion de la colistine dans les géloses. En raison des forts taux de résultats de fausses sensibilités décrits, ces systèmes ne sont plus recommandés par l'EUCAST pour tester la sensibilité à la colistine ([www.eucast.org](http://www.eucast.org)).

### **5. Panels commerciaux pour la détermination des CMI en milieu liquide**

Plusieurs panels commerciaux (les systèmes Sensititre<sup>®</sup>, UMIC<sup>®</sup>, MICRONAUT<sup>®</sup> et Sensitest<sup>®</sup>) permettent de déterminer les CMI en milieu liquide. En raison de leurs développements très récents, les performances de ces systèmes sont mal connues.

#### **5.1. Système Sensititre<sup>®</sup> (ThermoFisher Scientific)**

Le système Sensititre<sup>®</sup> (ThermoFisher Scientific) consiste en des plaques de 96 puits testant des concentrations de colistine de gammes de concentrations variables (100). L'inoculation et la lecture peuvent être automatisées. La croissance est mesurée après une incubation de 18 à 24h.

Il existe plusieurs panels Sensititre<sup>®</sup> permettant de tester la colistine et trois d'entre eux ont été évalués jusqu'à présent (89, 99, 101). Le premier est le panel TREK GNXF testant des concentrations de colistine de 0,25 à 4 mg/L qui a montré une concordance de catégorisation des souches de 96 % avec la méthode de référence et l'absence de résultats de fausses sensibilités (89). Le deuxième est le panel GNX3F testant également des concentrations de colistine de 0,25 à 4 mg/L qui a montré une parfaite détection des souches productrices du gène de résistance plasmidique à la colistine *mcr-1* (101). Le dernier est le panel

SEMPA1 (panel réalisé sur demande testant des concentrations de colistine de 0,25 à 64 mg/L) qui a montré une bonne catégorisation des souches ( $\geq 95$  %) avec des performances un peu moins bonnes pour *Acinetobacter* spp. (bonne catégorisation dans 91 % des cas) (99).

### **5.2. Système UMIC<sup>®</sup> (Biocentric)**

Le système UMIC<sup>®</sup> (Biocentric) correspond à une barrette de dilutions en milieu liquide permettant de tester une seule souche pour des concentrations de colistine de 0,06 à 64 mg/L.

Une étude a révélé une bonne catégorisation des souches dans 92 % des cas mais a également souligné des variations de CMI's importantes (notamment pour *Pseudomonas* spp. et *Acinetobacter* spp.) par rapport à la méthode de référence (99).

### **5.3. Système MICRONAUT-S<sup>®</sup> (Merlin Diagnostika GmbH)**

Le système MICRONAUT-S<sup>®</sup> (MERLIN Diagnostika GmbH) consiste en des plaques de 96 puits permettant de tester 8 souches pour des concentrations de colistine de 0,12 à 128 mg/L.

Une étude a révélé les performances moyennes de ce système avec une bonne catégorisation des souches dans 89 % des cas selon les espèces (99).

### **5.4. Système MICRONAUT MIC strip<sup>®</sup> (Merlin Diagnostika GmbH)**

Le système MICRONAUT MIC strip<sup>®</sup> (MERLIN Diagnostika GmbH) correspond à une barrette de dilutions en milieu liquide permettant de tester une seule souche pour des concentrations de colistine de 0,12 à 128 mg/L.

Les performances de ce système sont similaires au système non unitaire (MICRONAUT-S) de la même marque avec une bonne catégorisation des souches dans 91 % des cas (99).

### **5.5. Système Sensitest<sup>®</sup> ou ComASP<sup>®</sup> (Liofilchem)**

Le système SensiTest<sup>®</sup>, nouvellement appelé Compact Antimicrobial Susceptibility panel, ComASP<sup>®</sup> (Liofilchem) consiste en des plaques de 32 puits permettant de tester 4 souches pour des concentrations de colistine de 0,25 à 16 mg/L.

Une étude a révélé des performances moyennes de ce système avec une bonne catégorisation des souches dans 82 à 94 % des cas selon les espèces (performances moins bonnes pour *Acinetobacter* spp.) (99). Une autre étude a montré de bonnes performances du système Sensitest avec une bonne catégorisation

des souches dans 98,9 % des cas (98,8 % pour les souches résistantes à la colistine produisant le gène *mcr-1*) (102).

## **6. Systèmes automatisés**

Plusieurs systèmes automatisés permettent de tester la sensibilité à la colistine. Deux instruments, le Vitek<sup>®</sup> 2, et le BD Phoenix<sup>®</sup> peuvent générer des résultats rapides (3,5-16 h) grâce à des systèmes de détection optique qui détectent des changements subtils de croissance bactérienne, tandis que d'autres, le MicroScan<sup>®</sup> et le Sensititre<sup>®</sup>, sont des systèmes dont l'incubation est plus longue (16 à 24 h).

### **6.1. Système Vitek<sup>®</sup> 2 (bioMérieux)**

Le système Vitek<sup>®</sup> 2 (bioMérieux) utilise des panels testant des concentrations de colistine de 0,5 à 16 mg/L (100). La turbidimétrie liée à la croissance bactérienne est lue toutes les 15 min. Les résultats sont rendus entre 4 et 10 h après le début de l'incubation.

Comparé aux méthodes de dilutions, le système Vitek<sup>®</sup> 2 a une faible sensibilité pour détecter les souches résistantes à la colistine en raison de taux de résultats de fausses sensibilités très importants (36 %) (101) et d'une mauvaise détection des souches hétérorésistantes (91). Une étude a également révélé que ce système ne détectait que 43 % des souches produisant le gène plasmidique *mcr-1* (101). A l'inverse, une autre étude a révélé des taux de résultats de fausses résistances extrêmement élevées (38 %) chez *Acinetobacter baumannii* (103).

### **6.2. Système BD Phoenix<sup>®</sup> (BD Diagnostics)**

Le système BD Phoenix<sup>®</sup> (BD Diagnostics) utilise des panels testant des concentrations de colistine de 0,5 à 4 mg/L (100). Ces panels peuvent êtreensemencés de façon manuelle ou automatisée et les résultats sont rendus entre 6 et 16 h après le début de l'incubation.

Une étude a révélé une catégorisation correcte des souches de *A. baumannii* dans seulement 89 % des cas avec des taux de résultats de fausses résistances extrêmement élevés (41 %) (103).

### **6.3. Système MicroScan® (Beckman Coulter Diagnostics)**

Le système MicroScan® (Beckman Coulter Diagnostics) utilise des panels testant des concentrations de colistine de 2 et 4 mg/L. Les panels sont inoculés manuellement et incubés dans l'instrument pendant 16 à 20 heures (100).

Comparé aux méthodes de dilutions, le panel Microscan® NC42 catégorise correctement 87 % des souches de *Acinetobacter* spp. (104) et le panel MicroScan® NC66 catégorise correctement 87 % des souches de *K. pneumoniae* (105) pour la détection de la résistance aux polymyxines. Une étude récente a montré que le panel NM44 détectait parfaitement les souches d'entérobactéries productrices du gène *mcr-1* (101).

## **7. Diamètres et concentrations critiques**

Il n'y a actuellement pas de consensus entre les organisations américaines (CLSI) (106) et européennes (EUCAST) (96) concernant les valeurs seuils pour déterminer la sensibilité à la colistine. Les diamètres critiques et les concentrations critiques pour la colistine sont présentés dans le Tableau 1. Cependant, une étude pharmacocinétique a suggéré que ces valeurs seuils seraient trop hautes car des concentrations plasmatiques de 2 mg/L sont rarement atteintes (107). Par ailleurs, une étude récente a montré qu'une valeur seuil fixée à 1 mg/L permettrait une meilleure identification des souches productrices du gène plasmidique *mcr-1* (101).

**Tableau 1.** Diamètres et concentrations critiques pour la colistine selon le CLSI et l'EUCAST en 2018.

	Charge du disque	Diamètres critiques (mm) <sup>a</sup>		Concentrations critiques (mg/L) <sup>a</sup>		
		S	R	S	I	R
<b>CLSI</b>						
<i>Enterobacteriaceae</i>	-	-	-	-	-	-
<i>Acinetobacter</i> spp.	-	-	-	≤ 2	-	≥ 4
<i>Pseudomonas</i> spp.	10 µg	≥ 11	≤ 10	≤ 2	4	≥ 8
<b>EUCAST</b>						
<i>Enterobacteriaceae</i>	-	-	-	≤ 2	-	> 2
<i>Acinetobacter</i> spp.	-	-	-	≤ 2	-	> 2
<i>Pseudomonas</i> spp.	-	-	-	≤ 2	-	> 2

<sup>a</sup>-, non déterminé

## 8. Contrôles de qualité

Des contrôles de qualité sont nécessaires lors de la détermination des CMI de la colistine afin de vérifier la conformité de la procédure utilisée. Les souches pouvant être utilisées à cet effet sont les souches *E. coli* ATCC 25922 (valeurs de la CMI acceptables entre 0,25 et 2 mg/L), *P. aeruginosa* ATCC 27853 (valeurs de la CMI acceptables entre 0,5 et 4 mg/L) et *E. coli* NCTC 13846 (valeurs de la CMI acceptables entre 2 et 8 mg/L) (106).

## ***OBJECTIFS***

Malgré une utilisation clinique de la colistine depuis des décennies et en raison d'un intérêt limité pour cette molécule, il n'existe pas de méthode optimale pour tester la sensibilité ou la résistance à la colistine. Les difficultés pour tester la sensibilité à la colistine sont liées à plusieurs facteurs : la faible diffusion de la colistine dans les géloses, les propriétés cationiques de la colistine entraînant sa fixation sur les matières polystyrènes, l'existence de souches hétérorésistantes difficiles à détecter et enfin une méthode de référence, la méthode de dilutions en milieu liquide, difficilement utilisable en routine car trop longue et fastidieuse. Le premier objectif de notre travail était donc de mettre au point des tests diagnostiques (test rapide et milieu de culture sélectif) pour détecter de façon fiable et facile les souches résistantes à la colistine.

Le deuxième objectif de cette étude était ensuite d'évaluer les tests phénotypiques et les automates disponibles afin de préciser leurs performances en les comparant à la technique de référence de dilutions en milieu liquide.

Ensuite, l'épidémiologie de la résistance à la colistine étant très mal connue chez l'Homme en raison du manque d'outils pour la détection de cette résistance, le troisième objectif était de réaliser des études grâce aux outils développés afin de préciser l'épidémiologie de la résistance à la colistine puis de caractériser les mécanismes de résistance à la colistine au sein des souches collectées (identification de mécanismes déjà connus ou identification de nouveaux mécanismes de résistance).

Enfin, étant donné le fait que l'émergence et la diffusion de la résistance à la colistine chez les souches productrices de carbapénèmases peut conduire à des impasses thérapeutiques, un autre objectif de notre travail était de tester de nouvelles molécules et/ou de nouvelles associations d'antibiotiques sur ces souches multirésistantes, ceci afin d'identifier de nouvelles options thérapeutiques.



# ***MATERIEL ET METHODES***

## **A. Techniques bactériologiques**

### **1. Souches bactériennes**

#### **1.1. Souches cliniques résistantes à la colistine**

360 souches de BGN (*K. pneumoniae*, *Klebsiella oxytoca*, *E. coli*, *Hafnia spp*, *S. enterica*, *P. aeruginosa*, *A. baumannii*) ont été étudiées. Ces souches ont été isolées de prélèvements cliniques d'origines humaine ou animale et provenaient de différentes régions du monde (Afrique du Sud, Colombie, France, Suisse, Turquie).

#### **1.2. Autres souches**

D'autres souches bactériennes de référence ont été utilisées comme souches contrôles ou pour les expériences de clonage et de conjugaison :

- *E. coli* ATCC 25922 et *P. aeruginosa* ATCC 27853 : utilisés comme contrôles pour la détermination des CMI de la colistine.
- *K. pneumoniae* ATCC 53153 : utilisée comme souche de référence.
- *E. coli* Top10 (Invitrogen, Illkirch, France) : utilisé pour les expériences de clonage.
- *E. coli* C600 et *E. coli* K12 : utilisés pour les expériences de conjugaison.

### **2. Plasmides**

Plusieurs plasmides de clonage ont été utilisés :

- le plasmide pCR-BluntII-TOPO (Invitrogen) est un plasmide de 3,5 kb à nombre élevé de copies qui permet le clonage direct de produits de PCR présentant des bouts francs. Les cellules ayant reçu un plasmide dépourvu d'insert sont normalement éliminées du fait de l'expression du gène toxique *ccdB*. Ce plasmide confère une résistance à la kanamycine et à la zéocine.
- le plasmide pBR322 est un plasmide de clonage de 4,4 kb à faible nombre de copies qui confère une résistance à l'ampicilline et à la tétracycline.
- le plasmide pF2 est un plasmide de clonage de 3,3 kb à nombre élevé de copies dérivé de pBluescript qui confère une résistance au triclosan (gène *mFabI*) (108).

### **3. Milieux de culture**

Le milieu Mueller-Hinton (MH) agar a été utilisé pour la réalisation des antibiogrammes et le milieu MH ajusté en cations (CA-MHB) a été utilisé pour la détermination des CMI<sub>s</sub> en milieu liquide.

Le milieu Luria Bertani (LB) additionné d'antibiotiques a été utilisé pour la sélection des clones lors des conjugaisons et des clonages.

Le milieu cœur-cerveau (Brain Heart Infusion, BHI) a été utilisé pour la préparation des cellules compétentes et l'étude de la croissance bactérienne.

Le milieu chromogène UriSelect®4 a été utilisé pour vérifier l'absence de contamination des souches.

### **4. Identification de l'espèce**

Les identifications bactériennes des souches ont été réalisées par une approche phénotypique à l'aide de galeries API 20E (bioMérieux, Marcy l'Etoile, France), ou par spectrométrie de masse MALDI-TOF (bioMérieux). Ces deux techniques n'étant pas capables de discriminer les différentes espèces de *Hafnia*, la détermination de l'espèce bactérienne des souches de *Hafnia* a été réalisée par séquençage partiel de l'ARN16S à l'aide des amorces 8F (5-AGAGTTTGATCCTGGCTCAG-3) et 1489R (5-TACCTTGTTACGACTTCA-3) et analyse des séquences sur le site internet de l'université de Lyon (<https://umr5558-bibiserv.univ-lyon1.fr/lebibi/lebibi.cgi>) (109).

### **5. Antibiogrammes**

Les antibiogrammes ont été réalisés par la méthode de diffusion sur milieu gélosé MH avec un inoculum de 0,5 McF, comme recommandé par l'EUCAST ([www.eucast.org](http://www.eucast.org)). Les milieux ont été incubés 16 à 24 heures à 37°C et les résultats ont été interprétés selon les recommandations de l'EUCAST.

### **6. Détermination des Concentrations Minimales Inhibitrices (CMI<sub>s</sub>)**

Les CMI<sub>s</sub> ont été déterminées par la méthode de dilutions en milieu liquide dans un milieu CA-MHB selon les recommandations de l'EUCAST et du CLSI. La colistine et la polymyxine B ont été testées selon une gamme de concentrations de 0,12 à 128 mg/L, sans addition de Tween 80 et avec un inoculum bactérien final de 5x10<sup>5</sup> UFC/mL. Les milieux ont été incubés 16 à 24 heures à 37°C et les résultats ont été interprétés selon les recommandations de l'EUCAST.

## **B. Techniques de biologie moléculaire et de génétique**

### **1. Extraction des acides nucléiques**

#### **1.1. Extraction d'ADN génomique**

L'ADN total (ADN génomique et plasmidique) a été extrait à l'aide du kit QIAamp DNA MiniKit (Qiagen, Courtaboeuf, France). Le principe de ce kit repose sur une lyse cellulaire en présence de lysozyme et de protéinase K. L'ADN est ensuite purifié (phénol-chloroforme), précipité (NaCl, isopropanol) et resuspendu dans de l'eau. L'ADN extrait peut être quantifié par spectrophotométrie à une longueur d'onde de 260 nm.

#### **1.2. Extraction d'ADN plasmidique**

L'ADN plasmidique a été extrait à l'aide du kit QIAprep Spin Miniprep (Qiagen). Le principe de ce kit repose sur une étape de lyse alcaline suivie d'une fixation de l'ADN plasmidique sur une résine échangeuse d'anions hautement sélective. Les ARN, protéines et autres impuretés sont éliminés par plusieurs lavages successifs de la résine. Une étape d'élution suivie d'une précipitation à l'isopropanol permet de récupérer l'ADN plasmidique concentré.

#### **1.3. Extraction d'ARN**

L'ARN a été extrait à l'aide du kit RNeasy Plus Mini Kit (Qiagen). Le matériel biologique est tout d'abord lysé et homogénéisé dans des conditions hautement dénaturantes en présence d'un tampon contenant du  $\beta$ -mercaptoéthanol et du guanidium isothiocyanate (GITC), qui inactive les RNases contenues dans l'extrait biologique et assure l'extraction des ARNs intacts. L'ARN est ensuite fixé sur une membrane et les contaminants sont éliminés par plusieurs lavages successifs. Une étape d'élution avec de l'eau RNase-free permet de récupérer l'ARN.

### **2. Réactions d'amplification génique**

#### **2.1. Réactions de *Polymerase Chain Reaction* (PCR)**

Les réactions de PCR ont été effectuées dans un volume réactionnel final de 50  $\mu$ l comprenant 0,2 mM de chaque dNTP, du tampon de réaction 1X, 10  $\mu$ M de chaque amorce, 2 unités de *Taq* DNA polymérase

(Roche ou Proméga) et 2 µg d'ADN. L'amplification des gènes susceptibles d'être impliqués dans la résistance à la colistine chez *K. pneumoniae* a été réalisée à l'aide des amorces listées dans l'annexe A.

Un thermocycleur 2720 Thermal Cycler (Applied Biosystems) a permis de réaliser les réactions de PCR selon le programme suivant (Tableau 6).

**Tableau 6.** Conditions de réalisation des PCR.

Étape	Température	Durée
Dénaturation initiale	94°	10 min
Dénaturation	94°	1 min
Hybridation des amorces	X <sup>oa</sup>	1 min
Elongation	72°	1 min / kb <sup>b</sup>
Terminaison de la synthèse des brins	72°	10 min

<sup>a</sup> Température d'hybridation des amorces selon la température de fusion (T<sub>m</sub>) des amorces

<sup>b</sup> Temps d'élongation selon la taille du fragment à amplifier

L'électrophorèse des produits de PCR a été réalisée en gel d'agarose 1 % dans du tampon Tris-Acétate-EDTA (TAE) avec 0,5 µg/mL de SybrGreen puis les amplicons ont été visualisés sous UV. Les produits de PCR ont ensuite été purifiés à l'aide de colonnes du kit QIAquick PCR Purification (Qiagen). Le principe de ce kit repose sur une technique de centrifugation-filtration. En présence d'une forte concentration saline, l'ADN est fixé à une matrice de silice et les nucléotides, les enzymes et le tampon de réaction sont éliminés par lavage. L'ADN est ensuite élué à l'aide d'une solution à faible concentration saline.

## **2.2. Réactions de séquençage**

Les réactions de séquences ont été réalisées par les entreprises Microsynth (Balgach, Suisse) ou Eurofins (Ebersberg, Allemagne) et le séquençage a été réalisé selon la méthode de Sanger.

## **2.3. Réactions de *Quantitative Real Time-Polymerase Chain Reaction* (qRT-PCR)**

La qRT-PCR permet de quantifier la quantité d'ARN initialement présent dans un extrait pour déterminer le niveau d'expression des gènes. Elle a été réalisée en une étape à l'aide du kit QuantiFast SYBR Green RT-PCR (Qiagen) et d'un automate, le Rotor-Gene (Qiagen).

Les réactions ont été effectuées dans un volume réactionnel final de 25 µL comprenant 25 µM de chaque amorce (amorces listées dans l'annexe 3), 1X de marqueur fluorescent SYBR Green, 0,25 µL de reverse transcriptase et 100 ng d'ARN. L'ARN est tout d'abord rétrotranscrit grâce à la transcriptase inverse puis l'ADN complémentaire (ADNc) ainsi obtenu permet la réalisation des PCR. La synthèse des fragments

d'ADN est détectée grâce à la fluorescence du SYBR green lorsqu'il se lie à l'ADN. L'augmentation du signal de fluorescence est mesurée en temps réel par le Rotor-Gene, permettant ainsi le suivi de l'augmentation du nombre de copies du produit de PCR. Le cycle seuil ( $C_t$ ) correspond au seuil à partir duquel l'amplification du produit de PCR est détectable. Plus il existe de copies d'un ADNc dans la réaction au temps 0, plus le  $C_t$  est petit car l'amplification est plus rapidement détectée. Pour chaque expérience, les données obtenues ont été normalisées par rapport à celles obtenues pour le gène *rpsL*, gène encodant une protéine ribosomale (référence endogène), puis la variation de la quantité d'ADN a été comparée aux souches sensibles à la colistine à l'aide de l'équation  $2e^{-\Delta\Delta C_T}$  (quantification relative). Les expériences ont été répétées trois fois.

### **3. Complémentation des souches possédant des gènes mutés**

#### Préparation des cellules électrocompétentes

Les bactéries ont été mises en culture à 37°C dans du milieu BHI jusqu'à une phase exponentielle de croissance (DO entre 0,7 et 0,8) puis centrifugées à trois reprises (20 min, 4000 g, 4°C) avec mise en suspension du culot dans des volumes de plus en plus faibles de glycérol 10 % froid pour les concentrer.

#### Clonage du gène d'intérêt dans le plasmide vecteur pCR-BluntII-TOPO

Le gène d'intérêt de la souche de référence *K. pneumoniae* ATCC 53153 (CMI colistine = 0,12 mg/L) a été amplifié par PCR à l'aide des amorces listées dans l'annexe A et d'une Phusion DNA polymérase (Finnzymes, Life Technologies, Illkirch, France) qui permet d'obtenir un produit de PCR à bouts francs. Un fragment d'ADN non codant a également été amplifié à l'aide des amorces *mdhF* et *mdhR*. Les produits de PCR purifiés ont ensuite été mis en présence de 0,5 unité de DNA polymérase puis ont été ligués dans le vecteur pCR-BluntII-TOPO.

#### Transformation dans *E. coli* Top 10

Les plasmides recombinants ont ensuite été transformés par électroporation dans *E. coli* Top10 à l'aide d'un électroporateur Gene Pulser II (Bio-Rad). Les souches transformantes ont été sélectionnés par incubation toute la nuit à 37°C sur milieu MH contenant 50 mg/L de kanamycine. L'insertion correcte de l'insert dans le plasmide a été vérifiée par séquençage des clones à l'aide des amorces M13F et M13R contenues dans le kit.

## Extraction du plasmide pTOPO contenant le gène et transformation dans les souches résistantes à la colistine

Les plasmides des transformants ont été extraits à l'aide du kit Miniprep (Qiagen) et transformés par électroporation dans les souches résistantes à la colistine. Les transformants ont été sélectionnés par incubation overnight à 37°C sur milieu MH contenant 50 mg/L de kanamycine, ou 100 mg/L de zéocine lorsque les souches cliniques étaient résistantes à la kanamycine. Les clones ont ensuite été vérifiés par séquençage à l'aide des amorces M13F et M13R.

## **4. Etude de la clonalité**

### **4.1. Multi Locus Sequence Typing (MLST)**

La MLST est une technique d'épidémiologie moléculaire basée sur la détermination du Séquence-Type (ST) des souches de *K. pneumoniae*. Elle a été réalisée selon la méthode décrite par Diancourt et al (110).

Des fragments internes de 7 gènes de ménage (*rpoB*, *gapA*, *mdh*, *pgi*, *phoE*, *infB*, *tonB*) ont été amplifiés puis séquencés. Le profil allélique a ensuite été déterminé en utilisant le logiciel du site internet de l'Institut Pasteur ([www.pasteur.fr/mlst](http://www.pasteur.fr/mlst)).

### **4.2. Rep-PCR (Diversilab®)**

Cette technique permet la caractérisation génotypique des souches bactériennes. Elle est basée sur la rep-PCR, dont le principe consiste à amplifier de nombreux fragments de tailles différentes (amplicons) correspondant à des séquences courtes répétées, conservées, non codantes et dispersées sur le génome.

L'ADN a été extrait à l'aide du kit MoBio Ultra Clean microbial DNA selon les recommandations du fournisseur (bioMérieux). La quantité d'ADN extrait a ensuite été déterminée par spectrophotométrie et si nécessaire diluée pour obtenir une concentration d'ADN à amplifier comprise entre 25 et 50 ng/mL. Les réactions d'amplification ont ensuite été effectuées à l'aide des amorces du kit Diversilab® pour *K. pneumoniae* et les fragments amplifiés ont été séparés par électrophorèse réalisée dans un système microfluidique (LabChip). Après électrophorèse, l'analyse et la comparaison des profils génotypiques ont été effectuées via le site web Diversilab®, les souches pouvant être considérées comme identiques si les profils partagent plus de 95 % d'identité.

### **4.3. Electrophorèse en champ pulsé après restriction enzymatique (PFGE)**

L'électrophorèse en champ pulsé permet de mettre en évidence le polymorphisme génétique de différentes souches bactériennes d'une même espèce et de déterminer si des souches sont clonales. Cette technique consiste à analyser sur gel d'agarose les fragments d'ADN obtenus après digestion enzymatique de l'ADN total des souches bactériennes.

Les bactéries ont été incubées dans du milieu BHI jusqu'à l'obtention d'une DO à 600 nm entre 0,8 et 1. Un millilitre de cellules a été centrifugé, lavé et resuspendu dans le tampon de suspension préalablement équilibré à 50°C. Les bactéries ont été incluses dans un bloc d'agarose à 2 % à basse température de fusion. Les blocs ont été incubés pendant 2 h à 37°C dans un tampon de lyse puis dans une solution de protéinase K pendant 16 h à 50°C. La protéinase K a ensuite été éliminée par plusieurs lavages dont un avec une solution de lavage contenant du phénylméthane sulfonyl fluoride (PMSF) à 1 mM (inhibiteur de protéase). La composition des tampons est présentée dans le tableau 7.

L'ADN total des cellules a ensuite été digéré avec l'enzyme de restriction *BamHI* pour *K. pneumoniae* et *SfiI* pour *Hafnia* spp. pendant 16 h à 37°C puis la digestion a été arrêtée par lavages. Les plugs ont été incorporés dans un gel d'agarose 0,8 % préparé avec du tampon TBE 0,5X. Les conditions utilisées pour l'électrophorèse sont les suivantes : 6 Volts par cm pendant 20 h avec des changements de direction du courant toutes les 2 à 32 s. Après électrophorèse, le gel a été coloré au BET pendant 30 minutes puis lavé dans un bain d'eau milliQ et les profils génétiques ont été visualisés sous UV.

**Tableau 7.** Composition des solutions utilisées pour l'électrophorèse en champ pulsé.

<b>Solution</b>	<b>Composition</b>
Tampon de suspension	10 mM Tris pH 7.2, 20 mM NaCl, 50 mM EDTA
Tampon de lyse	10 mM Tris pH 7.2, 50 mM NaCl, 0.2 % Na-laurylsarcosine deoxycholate, 0.5 % Na-laurylsarcosine, 50 µg/mL lysosyme
Tampon protéinase K	100 mM EDTA pH 8, 0.2 % Na-laurylsarcosine deoxycholate, 1 % Na-laurylsarcosine, 20 µg/mL protéinase K
Tampon de lavage	20 mM Tris pH 8, 50 mM EDTA

## **C. Analyse in silico**

### **1. Analyse des séquences nucléotidiques et protéiques**

L'analyse des séquences a été faite à l'aide des logiciels 4Peaks (Softonic) et DNA Strider 1.4f puis les séquences ont été comparées aux séquences disponibles sur le site internet du NCBI (*National Center for Biotechnology Information*) ([www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)).



## **2. Prédiction des séquences promotrices des gènes**

La prédiction des séquences promotrices des gènes a été réalisée à l'aide du programme BPROM (*Bacterial Promoter Prediction Program*) accessible à partir de la plateforme Softberry-BPROM (<http://www.softberry.com/>).

## **3. Recherche de données sur la structure et la fonction des protéines**

La structure des protéines a été recherchée dans la banque de données Protein Data Bank du Research Collaboratory for Structural Bioinformatics ([www.pdb.org/](http://www.pdb.org/)) et dans la banque de donnée Pfam de l'Institut Sanger (<http://pfam.sanger.ac.uk/>).

La fonction des domaines protéiques a été recherchée dans la banque de données Ensembl Bacteria (<http://bacteria.ensembl.org/index.html>).

## **4. Prédiction de la structure secondaire des protéines**

En l'absence de données sur la structure des protéines étudiées dans les banques de données, la prédiction de la structure secondaire des protéines a été réalisée à l'aide du programme EMBOSS 6.3.1 accessible à partir de la plateforme de l'Institut Pasteur de Paris (<http://mobylye.pasteur.fr/>).

## **5. Prédiction de l'impact des altérations géniques sur la fonction protéique**

La prédiction de l'impact des altérations géniques sur la fonction protéique a été réalisée à l'aide du programme PROVEAN (*Protein Variation Effect Analyzer*) de l'Institut J. Craig Venter (<http://provean.jcvi.org/index.php>).

# ***RESULTATS EXPERIMENTAUX***

## **A. Développement de tests diagnostiques pour la détection de la résistance aux polymyxines**

### **1. Développement d'un test rapide**

#### **1.1. Détection rapide des BGN résistants à la colistine à partir de colonies bactériennes**

##### **Contexte :**

La méthode des disques, les bandelettes Etest<sup>®</sup> et certains automates (Vitek<sup>®</sup> 2 par exemple) sont peu fiables pour détecter la résistance à la colistine. La méthode de dilutions en milieu liquide de référence est fastidieuse, longue et demande du personnel qualifié. Par ailleurs, pour toutes ces techniques, les résultats ne sont obtenus qu'après un délai de 16 à 20 h. Il n'est actuellement pas possible de détecter toutes les souches résistantes à la colistine par biologie moléculaire car certains mécanismes sont encore inconnus et cette recherche nécessite le séquençage de plusieurs gènes impliqués dans la synthèse ou la modification du LPS.

##### **Objectif de l'étude :**

Cette étude avait pour objectif de mettre au point un test biochimique pour détecter de façon fiable et rapide les souches résistantes à la colistine. Ce test devait avoir une bonne sensibilité pour la détection des souches résistantes à la colistine quel que soit le mécanisme de résistance et devait être rapide. Il devait être facile d'emploi pour permettre une utilisation en routine mais aussi peu coûteux pour la réalisation d'études épidémiologiques.

##### **Principaux résultats :**

Nous avons mis au point un test, le Rapid Polymyxin NP test, basé sur la détection du métabolisme du glucose lié à la croissance bactérienne en présence d'une concentration définie de colistine. La formation de métabolites acides consécutive au métabolisme du glucose est mise en évidence par un changement de couleur (orange à jaune) d'un indicateur de pH (rouge de phénol).

Ainsi :

- si une bactérie est résistante à la colistine, elle croît dans le milieu Polymyxin NP qui contient de la colistine, elle métabolise le glucose du milieu et libère des métabolites acides qui font virer le milieu de l'orange au jaune grâce à l'indicateur de pH (rouge de phénol).

- à l'inverse, si une bactérie est sensible à la colistine, sa croissance est inhibée par la colistine et elle ne métabolise plus le glucose, ainsi le milieu reste orange.

Lors de la mise au point du test, la composition du milieu de culture Polymyxin NP a été déterminée à l'aide de tests sur 20 souches d'entérobactéries sensibles et résistantes à la colistine. Plusieurs paramètres ont été ajustés :

- le milieu de culture : le milieu MHB-CA a été choisi car c'est le milieu de référence pour la détermination des CMI en milieu liquide. L'ajustement en cations nous semblait indispensable car les cations jouent un rôle important dans la régulation des systèmes à deux composants PmrAB et PhoPQ. Nous avons aussi testé le milieu Luria Bertani mais des résultats faussement positifs ont été obtenus suite à l'acidification de l'environnement par ce milieu.
- le choix de l'indicateur de pH : le rouge de phénol a été choisi. La réalisation du test avec le bleu de bromothymol donnait de moins bons résultats.
- le choix du pH de la solution : un pH de 6,7 a été choisi. Un pH trop faible entraînait une coloration initiale trop orangée et rendait ainsi la lecture finale difficile, tandis qu'un pH trop haut entraînait un retard de rendu de résultats.
- La concentration de glucose : 1 %.

Une fois ce milieu mis au point, les autres paramètres du test ont été ajustés :

- la concentration finale de colistine et de polymyxine B : des concentrations de 1 à 5 mg/L ont été testées et c'est la concentration de 3,75 mg/L qui était la plus optimale
- l'inoculum bactérien : un inoculum bactérien de 3 McF a été choisi. Un inoculum trop important (densité optique  $\geq 4$  McF) entraînait une acidification du milieu même avec des souches sensibles et ainsi des résultats faussement positifs, tandis qu'un inoculum trop faible (densité optique  $\leq 2$  McF) entraînait un virage de couleur plus lent et ainsi un délai d'obtention des résultats plus importants.
- l'atmosphère d'incubation (avec ou sans CO<sub>2</sub>) : un délai d'obtention des résultats un peu plus court a été observé pour certaines souches de *Enterobacter* spp. résistantes à la colistine lors de l'incubation sous CO<sub>2</sub>.
- l'éventuel intérêt d'une agitation : une agitation du milieu n'entraînait pas un délai d'obtention des résultats raccourci.
- Les milieux de cultures utilisés pour la culture des bactéries avant la réalisation du test : les colonies bactériennes dont la croissance a été réalisée sur les géloses Drigalski, Mac Conkey ou au pourpre de bromocrésol ne peuvent pas être utilisées pour la réalisation du test en raison de l'obtention de résultats faussement positifs. Les milieux adéquats pour la culture des bactéries avant la réalisation du test sont les géloses LB, MH, les géloses au sang, les géloses chocolat, la gélose chromogénique UriSelect®4 et la gélose sélective Eosine Bleu de Méthylène (EMB).

Pour évaluer les performances du Rapid Polymyxin NP test dans sa version finalisée, nous avons testé 200 souches d'entérobactéries d'espèces variées, incluant 165 souches résistantes (résistances naturelles ou acquises, chromosomiques ou plasmidiques) et 35 souches sensibles à la colistine. Une seule souche de *E. coli* résistante à la colistine (CMI = 8 mg/L) avec un mécanisme de résistance inconnu a donné un résultat faussement négatif. Trois souches de *K. pneumoniae* sensibles à la colistine ont donné un résultat faussement positif mais ces souches présentaient des CMIs de la colistine égales à 1 ou 2 mg/L, c'est-à-dire juste en dessous de la valeur seuil de l'EUCAST (sensible si  $\leq 2$  mg/L). La corrélation entre les résultats du test et la catégorisation des souches selon les valeurs des CMIs obtenues par dilutions en milieu liquide était excellente, avec une sensibilité de 99,3 % pour détecter les souches résistantes, y compris les souches productrices du gène plasmidique *mcr-1*, et une bonne spécificité (95,4 %).

### **Conclusion :**

Nous avons mis au point un test biochimique, le Rapid Polymyxin NP test, qui permet la détection rapide (en moins de 2 h) des souches d'entérobactéries résistantes à la colistine à partir de cultures bactériennes. Ce test est une avancée importante pour la détermination de la sensibilité à la colistine car aucune méthode avant celle-ci n'était capable de détecter la résistance à la colistine dans un délai aussi court (minimum 18 h avec les autres techniques). De plus, ses performances pour détecter les souches résistantes à la colistine sont excellentes (sensibilité égale à 99,3 %) et bien supérieures à de nombreuses techniques (taux de résultats de fausses sensibilités jusqu'à 32 % avec la méthode des disques et les bandelettes Etest<sup>®</sup>). Par ailleurs, ce test est très facile à réaliser et ne nécessite pas de qualification particulière. Il est peu coûteux et peut ainsi être utilisé dans n'importe quel laboratoire de routine.

**Ce travail a fait l'objet d'une demande de dépôt de brevet auprès de l'Office européen des brevets en mars 2015 et a été publié dans *Emerging Infectious Diseases* en juin 2016 (Facteur d'impact (IF) = 6.994).**

# Rapid Detection of Polymyxin Resistance in *Enterobacteriaceae*

Patrice Nordmann, Aurélie Jayol, Laurent Poirel

For identification of polymyxin resistance in *Enterobacteriaceae*, we developed a rapid test that detects glucose metabolism associated with bacterial growth in the presence of a defined concentration of colistin or polymyxin B. Formation of acid metabolites is evidenced by a color change (orange to yellow) of a pH indicator (red phenol). To evaluate the test, we used bacterial colonies of 135 isolates expressing various mechanisms of colistin resistance (intrinsic, chromosomally encoded, and plasmid-mediated MCR-1) and 65 colistin-susceptible isolates. Sensitivity and specificity were 99.3% and 95.4%, respectively, compared with the standard broth microdilution method. This new test is inexpensive, easy to perform, sensitive, specific, and can be completed in <2 hours. It could be useful in countries facing endemic spread of carbapenemase producers and for which polymyxins are last-resort drugs.

**A**mong the most clinically significant multidrug-resistant bacteria are carbapenemase-producing *Enterobacteriaceae*. Because these bacteria usually remain susceptible to polymyxins, an old class of antimicrobial drugs almost abandoned in the 1970s because of their potential toxicity, interest in polymyxins (colistin and polymyxin B) has been renewed worldwide (1,2). However, the increasing use of colistin explains why acquired colistin resistance may now be added to the carbapenem resistance trait in *Enterobacteriaceae* (3).

The standard reference technique for determining susceptibility to polymyxins is broth microdilution, which requires fastidious attention and a long time (24 h) to perform (4). Other techniques for determining susceptibility to polymyxins (disk diffusion and Etest) have been proposed and also provide results in 18–24 h. Because of poor diffusion of polymyxin molecules in agar, rates of false susceptibility are high (up to 32%) (4,5).

Acquired resistance to colistin in *Enterobacteriaceae* results mostly from modification of lipopolysaccharide (6). Addition of phosphoethanolamine, 4-amino-L-arabinose cationic groups, or both to lipopolysaccharide decreases polymyxin binding to the bacterial outer membrane. Addition of these groups may be associated with chromosome-encoded mechanisms (mutations in PmrAB or PhoPQ

two-component systems or alterations of the *mgrB* gene) (6). A recent report revealed that addition of phosphoethanolamine may also be plasmid mediated through the *mcr-I* gene, which confers the first known plasmid-mediated resistance to colistin in isolates from humans and animals (7). More recently, the *mcr-I* gene was identified in several plasmid backbones, mostly in *Escherichia coli* (8–10). There is therefore a need for a test that enables rapid detection of polymyxin resistance in *Enterobacteriaceae* and that may contribute to its containment.

We developed a test (the rapid polymyxin NP [Nordmann/Poirel] test) that detects bacterial growth in the presence of a defined concentration of a polymyxin. Bacterial growth detection (or absence) is based on carbohydrate metabolism (11). Acid formation associated with carbohydrate metabolism in *Enterobacteriaceae* can be observed through the color change of a pH indicator. This test is rapid (<2 h) and easy to perform.

## Materials and Methods

### Isolate Collection

To evaluate the performance of the rapid polymyxin NP test, we used 200 isolates collected from clinical samples worldwide. This collection included 135 *Enterobacteriaceae* isolates resistant to polymyxin: 5 isolates of intrinsically polymyxin-resistant species (*Morganella morganii*, *Proteus mirabilis*, *Proteus vulgaris*, *Providencia stuartii*, and *Serratia marcescens*) and 130 isolates of various enterobacterial species (*Klebsiella* spp., *E. coli*, *Enterobacter* spp., and *Hafnia alvei*) with acquired resistance to polymyxins (online Technical Appendix, <http://wwwnc.cdc.gov/EID/article/22/6/15-1840-Techapp1.pdf>), including a previously reported heteroresistant *Klebsiella pneumoniae* isolate for which MIC for colistin was high (12). The other 65 enterobacterial isolates belonged to various species and were susceptible to polymyxins (online Technical Appendix).

### MIC Determination

To determine MICs for polymyxins, we used the broth microdilution method in cation-adjusted Mueller-Hinton broth (MHB-CA, reference 69444; Bio-Rad, Marnes-La-Coquette, France) as recommended by Clinical Laboratory

Author affiliation: University of Fribourg, Fribourg, Switzerland

DOI: <http://dx.doi.org/10.3201/eid2206.151840>

Standard Institute (CLSI) guidelines (13–15). We considered this method to be the standard for comparison with the rapid polymyxin NP results. Polymyxin B and colistin sulfate (Sigma-Aldrich, St. Louis, MO, USA) were tested over a range of dilutions (0.12–128 µg/mL). All experiments were repeated in triplicate in separate experiments. As recommended by CLSI, microdilution was performed without addition of Tween 80 (15), and *E. coli* ATCC 25922 was used as a control strain.

Because no breakpoint is available for polymyxins for *Enterobacteriaceae* according to CLSI guidelines (14), we used the breakpoints of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) for reference (16). Enterobacterial isolates with colistin or polymyxin B MICs  $\leq 2$  µg/mL were categorized as susceptible; those with MICs  $> 2$  µg/mL were categorized as resistant.

### PCR Amplification and Sequencing

We recovered the chromosomal DNA of the isolates by using a commercially available kit (QIAquick; QIAGEN, Courtaboeuf, France) according to the manufacturer's instructions. We sequenced the *pmrA*, *pmrB*, *phoP*, *phoQ*, and *mgrB* genes possibly involved in colistin resistance in *K. pneumoniae* and *K. oxytoca*, as described previously (12,17–19). We performed PCR amplification for detection of the plasmid-mediated *mcr-1* gene as described (7).

### Isolate Genotyping by Pulsed-Field Gel Electrophoresis

We assessed the genetic relatedness of the colistin-resistant isolates with identical molecular mechanisms of colistin resistance. We used pulsed-field gel electrophoresis with *XbaI*-digested genomic DNA as described previously (20).

### Rapid Polymyxin NP Test

#### Reagents and Solutions

The rapid polymyxin NP test uses 2 reagents and solutions: stock solutions of polymyxins and rapid polymyxin NP solution. Each is described below.

For stock solutions of polymyxins, colistin sulfate and polymyxin B powders (Sigma Aldrich) were diluted into MHB-CA medium in glass tubes to obtain a concentration of 0.2 mg/mL. These powders can be stored at 4°C before use, and the diluted polymyxin solutions can be kept at –20°C for 1 year. Of note, polymyxin-containing batches from commercial origin can be used, but the colistimethate sulfate powder, a therapeutic prodrug of colistin, cannot be used.

To prepare 250 mL of the rapid polymyxin NP solution, we mixed the culture medium and the pH indicator in a glass bottle as follows: 6.25 g of MHB-CA powder, 0.0125 g of phenol red (Sigma Aldrich), and 225 mL of distilled water. The pH of the solution was adjusted to 6.7

by adding drops of 1 mol/L HCL. This solution was then autoclaved at 121°C for 15 min. After cooling the solution to room temperature, we added 25 mL of 10% anhydrous D(+)-glucose (Roth, Karlsruhe, Germany) sterilized by filtration. The final concentrations in the rapid polymyxin NP solution were consequently 2.5% of MHB-CA powder, 0.005% of phenol red indicator, and 1% of D(+)-glucose. This rapid polymyxin NP solution can be kept at 4°C for 1 week or at –20°C for 1 year. This solution must be prewarmed at 37°C before use to prevent growth delay and therefore a delayed color change.

Just before performing the experiment, we added colistin to the rapid polymyxin NP solution and mixed it into sterile glass tubes to obtain a rapid polymyxin NP solution containing a final colistin concentration of 5 µg/mL. For example, we added 25 mL of colistin stock solution at 0.2 mg/mL to 1 mL of rapid polymyxin NP test solution for the testing of 1 isolate and respective negative and positive controls.

#### Bacterial Inoculum Preparation

We prepared a standardized enterobacterial inoculum by using freshly obtained (overnight) bacterial colonies grown on Luria-Bertani or Mueller-Hinton plates. We resuspended the bacterial colonies into 10 mL of sterile NaCL (0.85%) to obtain a 3.0–3.5 McFarland optical density ( $\approx 10^9$  CFU/mL), which corresponds to an  $\approx 10$ -mL full loop of bacterial colonies diluted in 10 mL of NaCL. A bacterial suspension was prepared for each isolate to be tested and for the colistin-susceptible and -resistant isolates used as controls (isolates FR-01 and FR-136; respectively; online Technical Appendix). As recommended by the EUCAST guidelines for susceptibility testing, we used the bacterial suspensions within 15 min of preparation and for no longer than 60 min after preparation (16).

#### Tray Inoculation

We performed testing in a 96-well polystyrene microtest plate (round base, with lid, sterile, reference 82.1582.001; Sarstedt, Nümbrecht, Germany). For each isolate, bacterial suspension was inoculated in parallel into 2 wells, with and without colistin. The following steps of the rapid polymyxin NP test were then performed (Figure):

Step 1: 150 µL of colistin-free solution was transferred to wells A1–A4.

Step 2: 150 µL of the rapid polymyxin NP solution containing colistin was transferred to wells B1–B4.

Step 3: 50 µL of NaCl 0.85% was added to wells A1 and B1.

Step 4: 50 µL of the colistin-susceptible isolate suspension used as negative control was added to wells A2 and B2.



Step 5: 50 mL of the colistin-resistant isolate suspension used as positive control was added to wells A3 and B3.

Step 6: 50 mL of the tested isolate suspension was added to wells A4 and B4.

We mixed the bacterial suspension with the reactive medium by pipetting up and down. The final concentration of bacteria was  $\approx 10^8$  CFU/mL in each well, and the final concentration of colistin sulfate was 3.75  $\mu$ g/mL.

#### Tray Incubation

We incubated the inoculated tray for up to 4 h at  $35 \pm 2^\circ\text{C}$  in ambient air, without being sealed and without agitation. We did not seal the tray because oxygen is required for carbohydrate metabolism.

#### Tray Reading

We visually inspected the tray (checked for no spontaneous color change) after 10 min and then every hour for 4 h. We considered the test result positive (polymyxin resistance) if the polymyxin-resistant isolate grew in presence of colistin and negative (polymyxin susceptibility) if the polymyxin-susceptible isolate did not grow in presence of colistin. We considered the test result interpretable if the following 4 conditions were met: 1) both wells with 0.85% NaCl without bacterial suspension (wells A1 and B1) remained orange (absence of medium contamination); 2) the wells with bacterial suspension and colistin-free (wells A2–A4) turned from orange to yellow, confirming the metabolism of glucose by the isolates; 3) the wells with the colistin-susceptible reference bacterial suspension (negative control)

gave negative results (wells A2 and B2); and 4) the wells with the colistin-resistant reference bacterial suspension (positive control) gave positive results (wells A3 and B3). The test result was positive when the well containing colistin (well B3) and the isolate to be tested turned from orange to yellow, giving exactly the same color as the well without colistin (well A3), indicating glucose metabolism and growth in presence of colistin (i.e., colistin resistance) (Figure). The test result was negative when the well containing colistin (well B2) with the isolate to be tested remained orange (unchanged color) (Figure) or was more clear than the wells with 0.85% NaCl but not exactly the same color as the well without colistin (not shown). Results were interpreted by 2 technicians who did not know which isolates were colistin resistant and colistin susceptible.

#### Other Experimental Conditions Tested

##### Polymyxin B Instead of Colistin

We evaluated the possibility of adapting the test to susceptibility testing of polymyxin B in countries where polymyxin B is prescribed. To do so, we performed the rapid polymyxin NP test with 20 colistin/polymyxin B-susceptible isolates and 20 colistin/polymyxin B-resistant isolates with polymyxin B at the same concentrations of colistin sulfate.

##### Incubation Conditions

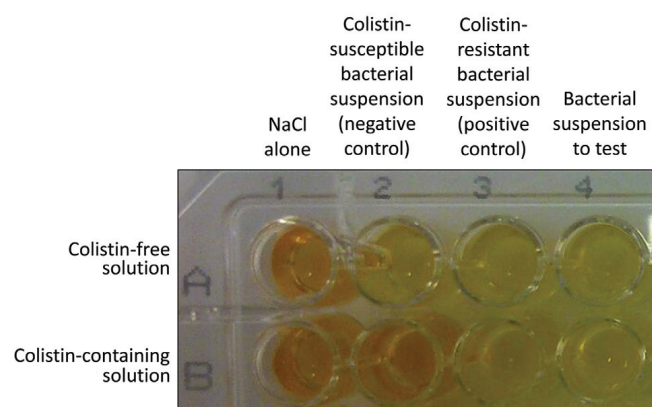
To determine effects of the incubation atmosphere on time to result, we incubated the tray with 20 colistin-susceptible isolates and 20 colistin-resistant isolates, in parallel, under 2 conditions: ambient air and atmosphere with 5%  $\text{CO}_2$ . We also incubated the tray for 20 colistin-susceptible isolates and 20 colistin-resistant isolates in parallel with and without agitation.

##### Culture Media

To determine the potential effects of culture medium on the test results, we performed the test with 20 colistin-susceptible isolates and 20 colistin-resistant isolates cultured overnight on different agar plates. The following culture media were tested: 1) nonselective culture medium such as Columbia agar + 5% sheep blood (bioMérieux, La-Balme-Les-Grottes, France); 2) chocolate agar + PolyVitex (bioMérieux); 3) nonselective chromogenic medium UriSelect 4 (Bio-Rad); 4) Eosin methylene blue agar (Sigma Aldrich); 5) Drigalski agar (Bio-Rad); 6) MacConkey agar (VWR BDH Prolabo, Leuven, Belgium); and 7) bromocresol purple (bioMérieux).

#### Results

Of the 200 enterobacterial isolates tested to evaluate the performance of the rapid polymyxin NP test (online Technical Appendix), 5 isolates belonged to bacterial species that are



**Figure.** Representative results of the rapid polymyxin NP [Nordmann/Poirel] test. Noninoculated wells are shown as controls (first column). The rapid polymyxin NP test was performed with a reference colistin-susceptible isolate (second column) and with a reference colistin-resistant isolate (third column) in a reaction medium without (upper row) and with (lower row) colistin. The tested isolate grew in the presence (and absence) of colistin (wells B4 and A4, respectively) and was therefore reported to be colistin-resistant.



intrinsically resistant to colistin (*M. morgani*, *P. mirabilis*, *P. vulgaris*, *P. stuartii*, and *S. marcescens*) and 130 isolates of various species displayed an acquired mechanism of resistance to colistin. For 87 *Klebsiella* spp. isolates, resistance to colistin was associated with various chromosomal gene changes responsible for lipopolysaccharide modifications (online Technical Appendix): 10 isolates had mutations in the PmrAB two-component system (n = 3 in *pmrA* gene, n = 7 in *pmrB* gene); 2 isolates had structural modifications in the PhoPQ two-component system (n = 1 in *phoP* gene, n = 1 in *phoQ* gene); and 75 isolates had various alterations in *mgrB* gene, the negative regulator of the PhoPQ system (online Technical Appendix). Seven nonduplicate *E. coli* isolates harbored a plasmid-mediated *mcr-1* gene. Pulsed-field gel electrophoresis revealed that isolates with identical mechanisms of colistin resistance (chromosomal or plasmid-encoded) were not clonally related (data not shown). The mechanism(s) of colistin resistance remained unknown for the 43 remaining enterobacterial isolates (online Technical Appendix). With regard to performance of the rapid polymyxin NP test with colistin-susceptible strains, the 65 colistin-susceptible isolates tested (MICs of colistin 0.12–2 µg/mL) gave negative results, except for 3 isolates (isolates FR-180, 181, and 182) for which colistin MICs were 1–2 µg/mL (just below the EUCAST breakpoint) and which gave a positive (false-positive) result (online Technical Appendix).

As expected, isolates that were intrinsically resistant to colistin (n = 5), such as *Proteus* spp., *P. stuartii* and *S. marcescens*, gave a positive test result (online Technical Appendix). Colistin-resistant enterobacterial isolates (n = 130, MICs of colistin ranging from 4 to >128 µg/mL) also gave positive results, except for 1 colistin-resistant *E. coli* isolate (isolate FR-119) for which colistin MIC was 8 µg/mL and which gave a negative (false-negative) result (online Technical Appendix).

Correlation was high between colistin resistance and positive rapid polymyxin NP test results and, conversely, colistin susceptibility and negative test results (online Technical Appendix). Sensitivity (99.3%) and specificity (95.4%) of the test were also high, compared with the standard broth microdilution method.

By reading the color change of the wells every hour, we determined that final results were obtained 2 h after incubation when the tray was incubated at 35 ± 2°C under an ambient atmosphere. However, positive results (frank color change) were obtained as early as 1 h after incubation for *Klebsiella* spp. and *E. coli* isolates. Half of the *Enterobacter* spp. isolates gave positive results within 1 h of incubation and the other half within 2 h. In addition, all resistant isolates gave positive results after 1 h of incubation when trays were incubated at 35 ± 2°C under 5% CO<sub>2</sub>. Agitating the tray did not improve the speed with which results were obtained.

The rapid polymyxin NP test results were the same whether performed with polymyxin B or with colistin (data not shown). Testing of several agar media revealed that 30% of the colistin-susceptible tested isolates gave false-positive results when bacterial colonies were recovered from acidifying media such as Drigalski, MacConkey, or bromocresol purple agar. Media that were adequate for culturing bacteria before performing the rapid polymyxin NP test were Luria Bertani agar, Mueller-Hinton agar, Columbia agar + 5% sheep blood, chocolate agar, UriSelect 4 agar, and eosin methylene blue agar.

## Discussion

The rapid polymyxin NP test is easy-to-perform, rapid, sensitive, and specific. It detects polymyxin-resistant and -susceptible isolates from any enterobacterial species, regardless of the molecular mechanism of polymyxin resistance. This test offers the possibility of detecting polymyxin resistance from bacterial cultures from infected samples or from selective media before any antimicrobial drug susceptibility testing results are obtained. Results are obtained at least 16 h sooner with this test than with the reference broth microdilution method. This test is as reliable as the reference dilution technique but much less cumbersome and is not based on diffusion of large polymyxin molecules in agar (as are the Etest and the disk-diffusion techniques), which therefore prevents false susceptibility results (15). A commercial test for research-use only is available for determining MICs of polymyxins (TREK Diagnostic Systems, Inc., Cleveland, OH, USA) (15); however, this test is adapted to testing series of isolates rather than single isolates, and results are available in 16–20 h. Sensitivity and specificity of the rapid polymyxin NP test were high (99.3% and 95.4%, respectively), making it a potential useful clinical technique.

The rapid polymyxin NP test can be performed on cultured bacteria grown on media such as Luria Bertani, Mueller-Hinton, UriSelect-4, eosin-methylene blue, blood agar, and chocolate agar. Interference may be observed with colonies grown on acidifying culture media such as Drigalski, Mac Conkey, and bromocresol purple agars. The test has been optimized in its present form; testing under other conditions (e.g., changes in pH indicator, inoculum size, glucose and pH indicator concentrations; preparation of the solution with non-cation-adjusted culture medium; or use of other polystyrene-containing trays) gave less optimal results.

The rapid polymyxin NP test uses commercially available polymyxin B and colistin sulfate powders, which are unspecified mixtures of chemically related compounds that differ by single amino acid changes and fatty acyl moieties. The changes of the relative proportion of the polymyxin components of these mixtures is poorly defined and a potential source of variability (21).

We believe that the rapid polymyxin NP test may be useful for first-step screening of polymyxin resistance because use of molecular-based techniques for identification of all polymyxin-resistance mechanisms cannot be foreseen. Indeed, polymyxin resistance in *Enterobacteriaceae* may be associated with many nonrelated mechanisms, some of which are known (defects in outer-membrane proteins, structural modification of lipopolysaccharide, efflux over-expression; 16) and others still unknown.

This study was subject to at least 4 limitations. First, we did not assess the ability of the rapid polymyxin NP test to detect heteroresistant isolates with low MICs for polymyxin by broth microdilution; such isolates are problematic to detect. Second, the rapid polymyxin NP test involves an orange-to-yellow color change, which is readily apparent for resistant organisms, but interpretation may require more vigilance from laboratory technicians testing organisms with low-level resistance; thus, larger scale studies in different laboratories are needed to fully evaluate the reliability of rapid polymyxin NP test performance. Third, we could not determine the mechanism for colistin resistance in 43 isolates for which several mechanisms of colistin resistance may exist and may be expressed in various ways. Last, we did not evaluate the rapid polymyxin NP test in species of bacteria with metabolic pathways that differed from those of *Enterobacteriaceae*; further work is needed to adapt the rapid polymyxin NP test to detection of polymyxin-resistant *Pseudomonas aeruginosa* and *Acinetobacter baumannii*, which have different metabolic pathways.

The rapid polymyxin NP test can be used to determine susceptibility or resistance to polymyxins in countries facing endemic spread of carbapenemase producers and for which polymyxins are last-resort drugs (22). The test can also rapidly identify carriers of polymyxin-resistant isolates, leading to rapid implementation of adequate hygiene measures to control their spread. This test can also support the development of novel polymyxin-like molecules, facilitating patient enrollment in pivotal clinical trials.

### Acknowledgments

We thank the following colleagues, who provided polymyxin-resistant isolates: Adrian Brink, Jean-Winoc Decusser, Javier Fernandez-Dominguez, Jean-Yves Madec, Maria-Virginia Villegas, and Mesut Yilmaz.

This work was financed by the University of Fribourg, Switzerland. An international patent form, corresponding to this test, has been filed on behalf of the University of Fribourg.

Dr. Nordmann is professor of medicine, chair of the Microbiology Unit, Department of Medicine, Faculty of Science, University of Fribourg, Switzerland. His main field of research interest includes the genetics, biochemistry, and molecular epidemiology

of resistance in gram-negative bacteria, and the development of rapid diagnostic tests for detection of emerging antibiotic resistance traits.

### References

1. Nordmann P, Naas T, Poirel L. Global spread of carbapenemase-producing *Enterobacteriaceae*. *Emerg Infect Dis*. 2011;17:1791–8. <http://dx.doi.org/10.3201/eid1710.110655>
2. Falagas ME, Kasiakou SK. Colistin: the revival of polymyxins for the management of multidrug-resistant gram-negative bacterial infections. *Clin Infect Dis*. 2005;40:1333–41. <http://dx.doi.org/10.1086/429323>
3. Monaco M, Giani T, Raffone M, Arena F, Garcia-Fernandez A, Pollini S, et al. Colistin resistance superimposed to endemic carbapenem-resistant *Klebsiella pneumoniae*: a rapidly evolving problem in Italy, November 2013 to April 2014. *Eur Surveill*. 2014;19:pii:20939.
4. Hindler JA, Humphries RM. Colistin MIC variability by method for contemporary clinical isolates of multidrug-resistant Gram-negative bacilli. *J Clin Microbiol*. 2013;51:1678–84. <http://dx.doi.org/10.1128/JCM.03385-12>
5. Tan TY, Ng SY. Comparison of Etest, Vitek and agar dilution for susceptibility testing of colistin. *Clin Microbiol Infect*. 2007;13:541–4. <http://dx.doi.org/10.1111/j.1469-0691.2007.01708.x>
6. Olaitan AO, Morand S, Rolain JM. Mechanisms of polymyxin resistance: acquired and intrinsic resistance in bacteria. *Front Microbiol*. 2014;5:643. <http://dx.doi.org/10.3389/fmicb.2014.00643>
7. Liu YY, Wang Y, Walsh TR, Yi LX, Zhang R, Spencer J, et al. Emergence of plasmid-mediated colistin resistance mechanism MCR-1 in animals and human beings in China: a microbiological and molecular biological study. *Lancet Infect Dis*. 2016;16:161–8. [http://dx.doi.org/10.1016/S1473-3099\(15\)00424-7](http://dx.doi.org/10.1016/S1473-3099(15)00424-7)
8. Arcilla MS, van Hattem JM, Matamoros S, Melles DC, Penders J, de Jong MD, et al. Dissemination of the *mcr-1* colistin resistance gene. *Lancet Infect Dis*. 2016;16:147–9. [http://dx.doi.org/10.1016/S1473-3099\(15\)00541-1](http://dx.doi.org/10.1016/S1473-3099(15)00541-1)
9. Malhotra-Kumar S, Xavier BB, Das AJ, Lammens C, Hoang HT, Pham TP, et al. Colistin resistance gene *mcr-1* harboured on a multidrug resistant plasmid. *Lancet Infect Dis*. 2016;16:283–4. [http://dx.doi.org/10.1016/S1473-3099\(16\)00012-8](http://dx.doi.org/10.1016/S1473-3099(16)00012-8)
10. Poirel L, Kieffer N, Liassine N, Thanh D, Nordmann P. Plasmid-mediated carbapenem and colistin resistance in a clinical isolate of *Escherichia coli*. *Lancet Infect Dis*. 2016;16:281. [http://dx.doi.org/10.1016/S1473-3099\(16\)00006-2](http://dx.doi.org/10.1016/S1473-3099(16)00006-2)
11. Hugh R, Leifson E. The taxonomic significance of fermentative versus oxidative metabolism of carbohydrates by various gram negative bacteria. *J Bacteriol*. 1953;66:24–6.
12. Jayol A, Nordmann P, Brink A, Poirel L. Heteroresistance to colistin in *Klebsiella pneumoniae* associated with alterations in the PhoPQ regulatory system. *Antimicrob Agents Chemother*. 2015;59:2780–4. <http://dx.doi.org/10.1128/AAC.05055-14>
13. Clinical and Laboratory Standards Institute. Methods for dilution of antimicrobial susceptibility tests for bacteria that grow aerobically: approved standard. 9th ed. Document M07–A9. Wayne (PA): The Institute; 2012.
14. Clinical and Laboratory Standards Institute. Performance standards for antimicrobial susceptibility testing: 24th informational supplement. Document M100–S24. Wayne (PA): The Institute; 2014.
15. Humphries RM. Susceptibility testing of the polymyxins: where are we now? *Pharmacotherapy*. 2015;35:22–7. <http://dx.doi.org/10.1002/phar.1505>
16. European Committee on Antimicrobial Susceptibility Testing. Breakpoints tables for interpretation of MICs and zone diameters.

- Version 2.0. 2014 [cited 2016 Apr 5]. [http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST\\_files/Breakpoint\\_tables/v\\_6.0\\_Breakpoint\\_table.pdf](http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Breakpoint_tables/v_6.0_Breakpoint_table.pdf)
17. Jayol A, Poirel L, Brink A, Villegas MV, Yilmaz M, Nordmann P. Resistance to colistin associated with a single amino acid change in protein PmrB among *Klebsiella pneumoniae* isolates of worldwide origin. *Antimicrob Agents Chemother*. 2014;58:4762–6. <http://dx.doi.org/10.1128/AAC.00084-14>
  18. Jayol A, Poirel L, Villegas MV, Nordmann P. Modulation of *mgrB* gene expression as a source of colistin resistance in *Klebsiella oxytoca*. *Int J Antimicrob Agents*. 2015;46:108–10. <http://dx.doi.org/10.1016/j.ijantimicag.2015.02.015>
  19. Poirel L, Jayol A, Bontron S, Villegas MV, Ozdamar M, Turkoglu S, et al. The *mgrB* gene as a key target for acquired resistance to colistin in *Klebsiella pneumoniae*. *J Antimicrob Chemother*. 2015;70:75–80. <http://dx.doi.org/10.1093/jac/dku323>
  20. Carrër A, Lassel L, Fortineau N, Mansouri M, Anguel N, Richard C, et al. Outbreak of CTX-M-15-producing *Klebsiella pneumoniae* in the intensive care unit of a French hospital. *Microb Drug Resist*. 2009;15:47–54. <http://dx.doi.org/10.1089/mdr.2009.0868>
  21. He J, Ledesma KR, Lam WY, Figueroa DA, Lim TP, Chow DS, et al. Variability of polymyxin B major components in commercial formulations. *Int J Antimicrob Agents*. 2010;35:308–10. <http://dx.doi.org/10.1016/j.ijantimicag.2009.11.005>
  22. Capone A, Giannella M, Fortini D, Giordano A, Meledandri M, Ballardini M, et al. High rate of colistin resistance among patients with carbapenem-resistant *Klebsiella pneumoniae* infection accounts for an excess of mortality. *Clin Microbiol Infect*. 2013;19:E23–30. <http://dx.doi.org/10.1111/1469-0691.12070>

---

Address for correspondence: Patrice Nordmann, Medical and Molecular Microbiology, Department of Medicine, Faculty of Science, University of Fribourg, Rue Albert Gockel 3, CH-1700 Fribourg, Switzerland; email: [patrice.nordmann@unifr.ch](mailto:patrice.nordmann@unifr.ch)

# Rapid Detection of Polymyxin Resistance in *Enterobacteriaceae*

## Technical Appendix

**Technical Appendix Table.** Rapid polymyxin NP test results for polymyxin-resistant isolates with intrinsic resistance, chromosome- and plasmid-mediated acquired resistance, and for polymyxin-susceptible isolates\*

Strain	Species	Origin	Polymyxin phenotype	Rapid polymyxin NP test	Colistin MIC, µg/mL	Resistance mechanism	Strain reference
Strains intrinsically resistant to colistin							
FR-01†	<i>M. morgani</i>	France	R	+	>128	NA	Positive control
FR-02	<i>P. mirabilis</i>	France	R	+	>128	NA	Unpublished
FR-03	<i>P. vulgaris</i>	France	R	+	>128	NA	Unpublished
FR-04	<i>P. stuartii</i>	France	R	+	>128	NA	Unpublished
FR-05	<i>S. marcescens</i>	France	R	+	>128	NA	Unpublished
Strains resistant to colistin with an identified mechanism of resistance							
FR-06	<i>K. pneumoniae</i>	Colombia	R	+	32	PmrA G53C	Unpublished
FR-07	<i>K. pneumoniae</i>	France	R	+	32	PmrA G53S	Unpublished
FR-08	<i>K. pneumoniae</i>	South Africa	R	+	128	PmrA G53S	Unpublished
FR-09	<i>K. pneumoniae</i>	Turkey	R	+	32	PmrB L17Q	Unpublished
FR-10	<i>K. pneumoniae</i>	South Africa	R	+	16	PmrB T157P	Isolate AF1b (1)
FR-11	<i>K. pneumoniae</i>	Colombia	R	+	32	PmrB T157P	Isolate C3 (1)
FR-12	<i>K. pneumoniae</i>	Colombia	R	+	16	PmrB T157P	Isolate C19 (1)
FR-13	<i>K. pneumoniae</i>	Turkey	R	+	32	PmrB T157P	Isolate T2 (1)
FR-14	<i>K. pneumoniae</i>	Turkey	R	+	32	PmrB T157P	Unpublished
FR-15	<i>K. pneumoniae</i>	Turkey	R	+	16	PmrB T157P	Unpublished
FR-16	<i>K. pneumoniae</i>	South Africa	HR	+	128	PhoP D191Y±deletion of 25 nt	Isolate Kp75 (2)
FR-17	<i>K. pneumoniae</i>	Turkey	R	+	>128	PhoQ R16C	Unpublished
FR-18	<i>K. pneumoniae</i>	Turkey	R	+	32	MgrB W20R	Unpublished
FR-19	<i>K. pneumoniae</i>	France	R	+	32	MgrB M27K	Unpublished
FR-20	<i>K. pneumoniae</i>	France	R	+	64	MgrB C39Y	Unpublished
FR-21	<i>K. pneumoniae</i>	France	R	+	32	MgrB N42Y and K43I	Unpublished
FR-22	<i>K. pneumoniae</i>	Angola	R	+	64	MgrB I45T	Unpublished
FR-23	<i>K. pneumoniae</i>	France	R	+	64	MgrB P46S	Unpublished
FR-24	<i>K. pneumoniae</i>	Turkey	R	+	4	MgrB W47R	Unpublished
FR-25	<i>K. pneumoniae</i>	France	R	+	128	MgrB truncated (2 aa)	Unpublished
FR-26	<i>K. pneumoniae</i>	France	R	+	128	MgrB truncated (2 aa)	Unpublished
FR-27	<i>K. pneumoniae</i>	France	R	+	64	MgrB truncated (2 aa)	Unpublished
FR-28	<i>K. pneumoniae</i>	France	R	+	128	MgrB truncated (27 aa)	Unpublished
FR-29	<i>K. pneumoniae</i>	France	R	+	32	MgrB truncated (27 aa)	Unpublished
FR-30	<i>K. pneumoniae</i>	France	R	+	>128	MgrB truncated (27 aa)	Isolate Sa (3)
FR-31	<i>K. pneumoniae</i>	France	R	+	64	MgrB truncated (29 aa)	Isolate 1515 (3)
FR-32	<i>K. pneumoniae</i>	Switzerland	R	+	64	MgrB truncated (29 aa)	Unpublished
FR-33	<i>K. pneumoniae</i>	Spain	R	+	32	MgrB truncated (29 aa)	Unpublished
FR-34	<i>K. pneumoniae</i>	South Africa	R	+	64	MgrB truncated (29 aa)	Unpublished
FR-35	<i>K. pneumoniae</i>	South Africa	R	+	128	MgrB truncated (29 aa)	Unpublished
FR-36	<i>K. pneumoniae</i>	Colombia	R	+	128	MgrB truncated (29 aa)	Isolate C11 (3)
FR-37	<i>K. pneumoniae</i>	France	R	+	32	MgrB truncated (32 aa)	Unpublished
FR-38	<i>K. pneumoniae</i>	Turkey	R	+	32	MgrB truncated (46 aa)	Unpublished
FR-39	<i>K. pneumoniae</i>	France	R	+	64	<i>mgrB</i> IS 1R between +21 and +22	Unpublished
FR-40	<i>K. pneumoniae</i>	France	R	+	64	<i>mgrB</i> ISEcp11/blaCTX-M-15 between +21 and +22	Unpublished
FR-41	<i>K. pneumoniae</i>	France	R	+	32	<i>mgrB</i> IS102-like between +36 and +37	Unpublished

Strain	Species	Origin	Polymyxin phenotype	Rapid polymyxin NP test	Colistin MIC, $\mu\text{g/mL}$	Resistance mechanism	Strain reference
FR-42	<i>K. pneumoniae</i>	France	R	+	>128	<i>mgrB</i> IS102-like between +44 and +45	Unpublished
FR-43	<i>K. pneumoniae</i>	South Africa	R	+	64	<i>mgrB</i> IS903b between +44 and +45	Unpublished
FR-44	<i>K. pneumoniae</i>	Turkey	R	+	64	<i>mgrB</i> IS2 between +44 and +45	Unpublished
FR-45	<i>K. pneumoniae</i>	Turkey	R	+	128	<i>mgrB</i> IS1R between +44 and +45	Unpublished
FR-46	<i>K. pneumoniae</i>	South Africa	R	+	64	<i>mgrB</i> IS1R between +61 and +62	Unpublished
FR-47	<i>K. pneumoniae</i>	Turkey	R	+	64	<i>mgrB</i> IS903b-like between +69 and +70	Unpublished
FR-48	<i>K. pneumoniae</i>	Spain	R	+	128	<i>mgrB</i> IS903-like between +70 and +71	Unpublished
FR-49	<i>K. pneumoniae</i>	France	R	+	64	<i>mgrB</i> IS5-like between +74 and 75	Isolate 1118 (3)
FR-50	<i>K. pneumoniae</i>	France	R	+	16	<i>mgrB</i> IS5-like between +74 and 75	Isolate 20C9 (3)
FR-51	<i>K. pneumoniae</i>	Colombia	R	+	64	<i>mgrB</i> IS5-like between +74 and 75	Isolate C9 (3)
FR-52	<i>K. pneumoniae</i>	Turkey	R	+	128	<i>mgrB</i> IS5-like between +74 and 75	Isolate T1b (3)
FR-53	<i>K. pneumoniae</i>	France	R	+	64	<i>mgrB</i> IS5-like between +74 and +75	Unpublished
FR-54	<i>K. pneumoniae</i>	Colombia	R	+	128	<i>mgrB</i> ISKpn13 between +74 and +75	Isolate C21 (3)
FR-55	<i>K. pneumoniae</i>	France	R	+	>128	<i>mgrB</i> ISKpn26-like between +74 and +75	Unpublished
FR-56	<i>K. pneumoniae</i>	Spain	R	+	64	<i>mgrB</i> ISKpn26-like between +74 and +75	Unpublished
FR-57	<i>K. pneumoniae</i>	Turkey	R	+	128	<i>mgrB</i> ISKpn26-like between +74 and +75	Unpublished
FR-58	<i>K. pneumoniae</i>	South Africa	R	+	32	<i>mgrB</i> ISKpn26-like between +76 and +77	Unpublished
FR-59	<i>K. pneumoniae</i>	South Africa	R	+	32	<i>mgrB</i> IS903B between +76 and +77	Unpublished
FR-60	<i>K. pneumoniae</i>	Turkey	R	+	128	<i>mgrB</i> ISKpn14 between +77 and +78	Unpublished
FR-61	<i>K. pneumoniae</i>	France	R	+	128	<i>mgrB</i> duplication 19 nt between +84 and +85	Unpublished
FR-62	<i>K. pneumoniae</i>	South Africa	R	+	64	<i>mgrB</i> IS903b between +94 and +95	Unpublished
FR-63	<i>K. pneumoniae</i>	France	R	+	128	<i>mgrB</i> IS1R-like between +116 and +117	Unpublished
FR-64	<i>K. pneumoniae</i>	France	R	+	64	<i>mgrB</i> IS903b-like between +116 and +117	Unpublished
FR-65	<i>K. pneumoniae</i>	France	R	+	8	<i>mgrB</i> IS1R between +118 and +119	Unpublished
FR-66	<i>K. pneumoniae</i>	Turkey	R	+	32	<i>mgrB</i> IS1R between +123 and +124	Unpublished
FR-67	<i>K. pneumoniae</i>	France	R	+	128	<i>mgrB</i> ISKpn26-like between +125 and +126	Unpublished
FR-68	<i>K. pneumoniae</i>	Colombia	R	+	64	<i>mgrB</i> ISKpn14 between +127 and +128	Isolate C22 (3)
FR-69	<i>K. pneumoniae</i>	South Africa	R	+	128	<i>mgrB</i> IS1R between +131 and +132	Unpublished
FR-70	<i>K. pneumoniae</i>	Colombia	R	+	128	<i>mgrB</i> IS10R between -26 and -27	Isolate C1 (3)
FR-71	<i>K. pneumoniae</i>	Turkey	R	+	64	<i>mgrB</i> ISKpn14 between -27 and -28	Unpublished
FR-72	<i>K. pneumoniae</i>	Turkey	R	+	64	<i>mgrB</i> ISKpn14 between -28 and -29	Unpublished
FR-73	<i>K. pneumoniae</i>	France	R	+	64	<i>mgrB</i> IS1R between -36 and -37	Unpublished
FR-74	<i>K. pneumoniae</i>	France	R	+	128	<i>mgrB</i> IS1R between -45 and -46	Unpublished

Strain	Species	Origin	Polymyxin phenotype	Rapid polymyxin NP test	Colistin MIC, µg/mL	Resistance mechanism	Strain reference
FR-75	<i>K. pneumoniae</i>	France	R	+	64	<i>mgrB</i> IS1R between -45 and -46	Unpublished
FR-76	<i>K. pneumoniae</i>	Turkey	R	+	32	<i>mgrB</i> IS1R between -45 and -46	Unpublished
FR-77	<i>K. pneumoniae</i>	France	R	+	32	<i>mgrB</i> ISKpn14-like between -45 and -46	Unpublished
FR-78	<i>K. pneumoniae</i>	France	R	+	32	<i>mgrB</i> IS1R between -61 and -62	Unpublished
FR-79	<i>K. pneumoniae</i>	France	R	+	16	Full <i>mgrB</i> gene deletion	Unpublished
FR-80	<i>K. pneumoniae</i>	France	R	+	>128	Full <i>mgrB</i> gene deletion	Unpublished
FR-81	<i>K. pneumoniae</i>	South Africa	R	+	64	Full <i>mgrB</i> gene deletion	Unpublished
FR-82	<i>K. pneumoniae</i>	South Africa	R	+	64	Full <i>mgrB</i> gene deletion	Unpublished
FR-83	<i>K. pneumoniae</i>	France	R	+	32	Deletion nt 23 <i>mgrB</i>	Unpublished
FR-84	<i>K. pneumoniae</i>	South Africa	R	+	128	Deletion nt 70 <i>mgrB</i> and substitution nt 73	Unpublished
FR-85	<i>K. pneumoniae</i>	France	R	+	64	Deletion nt 74 <i>mgrB</i>	Unpublished
FR-86	<i>K. pneumoniae</i>	Spain	R	+	64	Deletion nt 100 <i>mgrB</i>	Unpublished
FR-87	<i>K. pneumoniae</i>	Spain	R	+	16	Deletion nt 100 <i>mgrB</i>	Unpublished
FR-88	<i>K. pneumoniae</i>	Turkey	R	+	>128	Deletion nt 22 to 32 <i>mgrB</i>	Unpublished
FR-89	<i>K. pneumoniae</i>	Colombia	R	+	>128	Deletion nt 23 to 33 <i>mgrB</i>	Unpublished
FR-90	<i>K. pneumoniae</i>	South Africa	R	+	64	Deletion nt 30 et 31 <i>mgrB</i>	Unpublished
FR-91	<i>K. pneumoniae</i>	South Africa	R	+	64	Deletion nt 48 to 57 <i>mgrB</i>	Unpublished
FR-92	<i>K. oxytoca</i>	Colombia	R	+	64	MgrB ISKpn26-like between -38 and -39	Isolate C24 (4)
FR-93	<i>E. coli</i>	Switzerland	R	+	4	Plasmid-mediated <i>mcr-1</i> gene	Isolate KRI (5)
FR-94	<i>E. coli</i>	South Africa	R	+	16	Plasmid-mediated <i>mcr-1</i> gene	Unpublished
FR-95	<i>E. coli</i>	South Africa	R	+	16	Plasmid-mediated <i>mcr-1</i> gene	Unpublished
FR-96	<i>E. coli</i>	South Africa	R	+	16	Plasmid-mediated <i>mcr-1</i> gene	Unpublished
FR-97	<i>E. coli</i>	South Africa	R	+	16	Plasmid-mediated <i>mcr-1</i> gene	Unpublished
FR-98	<i>E. coli</i>	South Africa	R	+	8	Plasmid-mediated <i>mcr-1</i> gene	Unpublished
FR-99	<i>E. coli</i>	South Africa	R	+	16	Plasmid-mediated <i>mcr-1</i> gene	Unpublished
Strains resistant to colistin without an identified mechanism of resistance							
FR-100	<i>K. pneumoniae</i>	France	R	+	32	Unknown	Unpublished
FR-101	<i>K. pneumoniae</i>	France	R	+	32	Unknown	Unpublished
FR-102	<i>K. pneumoniae</i>	France	R	+	32	Unknown	Unpublished
FR-103	<i>K. pneumoniae</i>	France	R	+	4	Unknown	Unpublished
FR-104	<i>K. pneumoniae</i>	France	R	+	64	Unknown	Unpublished
FR-105	<i>K. pneumoniae</i>	France	R	+	128	Unknown	Unpublished
FR-106	<i>K. pneumoniae</i>	France	R	+	>128	Unknown	Unpublished
FR-107	<i>K. pneumoniae</i>	Spain	R	+	32	Unknown	Unpublished
FR-108	<i>K. pneumoniae</i>	Spain	R	+	32	Unknown	Unpublished
FR-109	<i>K. pneumoniae</i>	South Africa	R	+	64	Unknown	Unpublished
FR-110	<i>K. pneumoniae</i>	South Africa	R	+	>128	Unknown	Unpublished
FR-111	<i>K. pneumoniae</i>	South Africa	R	+	32	Unknown	Unpublished
FR-112	<i>K. pneumoniae</i>	Colombia	R	+	>128	Unknown	Unpublished
FR-113	<i>K. pneumoniae</i>	Colombia	R	+	>128	Unknown	Unpublished
FR-114	<i>K. pneumoniae</i>	Colombia	R	+	64	Unknown	Unpublished
FR-115	<i>K. pneumoniae</i>	Colombia	R	+	64	Unknown	Unpublished
FR-116	<i>K. pneumoniae</i>	Colombia	R	+	32	Unknown	Unpublished
FR-117	<i>K. pneumoniae</i>	Turkey	R	+	32	Unknown	Unpublished
FR-118	<i>K. pneumoniae</i>	Turkey	R	+	4	Unknown	Unpublished
FR-119	<i>E. coli</i>	France	R	-	8	Unknown	Unpublished
FR-120	<i>E. coli</i>	France	R	+	8	Unknown	Unpublished
FR-121	<i>E. coli</i>	France	R	+	4	Unknown	Unpublished
FR-122	<i>E. cloacae</i>	Colombia	R	+	32	Unknown	Unpublished

Strain	Species	Origin	Polymyxin phenotype	Rapid polymyxin NP test	Colistin MIC, µg/mL	Resistance mechanism	Strain reference
FR-123	<i>E. cloacae</i>	Colombia	R	+	>128	Unknown	Unpublished
FR-124	<i>E. cloacae</i>	Colombia	R	+	>128	Unknown	Unpublished
FR-125	<i>E. cloacae</i>	France	R	+	>128	Unknown	Unpublished
FR-126	<i>E. cloacae</i>	France	R	+	>128	Unknown	Unpublished
FR-127	<i>E. cloacae</i>	France	R	+	64	Unknown	Unpublished
FR-128	<i>E. cloacae</i>	France	R	+	64	Unknown	Unpublished
FR-129	<i>E. cloacae</i>	France	R	+	32	Unknown	Unpublished
FR-130	<i>E. cloacae</i>	France	R	+	>128	Unknown	Unpublished
FR-131	<i>E. cloacae</i>	France	R	+	16	Unknown	Unpublished
FR-132	<i>E. cloacae</i>	France	R	+	>128	Unknown	Unpublished
FR-133	<i>E. cloacae</i>	France	R	+	>128	Unknown	Unpublished
FR-134	<i>E. asburiae</i>	France	R	+	>128	Unknown	Unpublished
FR-135	<i>H. alvei</i>	France	R	+	>128	Unknown	Unpublished
Strains susceptible to colistin							
FR-136 <sup>†</sup>	<i>E. coli</i>	USA	S	–	0.25	NA	ATCC 25922, Negative control
FR-137	<i>E. coli</i>	Colombia	S	–	0.12	NA	Unpublished
FR-138	<i>E. coli</i>	Colombia	S	–	0.12	NA	Unpublished
FR-139	<i>E. coli</i>	Switzerland	S	–	0.12	NA	Unpublished
FR-140	<i>E. coli</i>	Switzerland	S	–	0.12	NA	Unpublished
FR-141	<i>E. coli</i>	Switzerland	S	–	0.12	NA	Unpublished
FR-142	<i>E. coli</i>	France	S	–	0.12	NA	Unpublished
FR-143	<i>E. coli</i>	France	S	–	0.12	NA	Unpublished
FR-144	<i>E. coli</i>	France	S	–	0.12	NA	Unpublished
FR-145	<i>E. coli</i>	France	S	–	0.12	NA	Unpublished
FR-146	<i>E. coli</i>	France	S	–	0.12	NA	Unpublished
FR-147	<i>E. coli</i>	France	S	–	0.12	NA	Unpublished
FR-148	<i>E. coli</i>	France	S	–	0.12	NA	Unpublished
FR-149	<i>E. coli</i>	France	S	–	0.25	NA	Unpublished
FR-150	<i>E. coli</i>	France	S	–	0.25	NA	Unpublished
FR-151	<i>E. coli</i>	France	S	–	0.25	NA	Unpublished
FR-152	<i>E. coli</i>	France	S	–	0.25	NA	Unpublished
FR-153	<i>E. coli</i>	France	S	–	0.25	NA	Unpublished
FR-154	<i>E. coli</i>	France	S	–	0.25	NA	Unpublished
FR-155	<i>E. coli</i>	France	S	–	0.25	NA	Unpublished
FR-156	<i>K. pneumoniae</i>	USA	S	–	0.12	NA	ATCC 53153
FR-157	<i>K. pneumoniae</i>	South Africa	S	–	0.25	NA	Isolate AF1a (1)
FR-158	<i>K. pneumoniae</i>	Turkey	S	–	0.12	NA	Isolate T1a (3)
FR-159	<i>K. pneumoniae</i>	Spain	S	–	0.12	NA	Unpublished
FR-160	<i>K. pneumoniae</i>	Spain	S	–	0.25	NA	Unpublished
FR-161	<i>K. pneumoniae</i>	Spain	S	–	0.25	NA	Unpublished
FR-162	<i>K. pneumoniae</i>	Spain	S	–	0.5	NA	Unpublished
FR-163	<i>K. pneumoniae</i>	Spain	S	–	0.5	NA	Unpublished
FR-164	<i>K. pneumoniae</i>	Spain	S	–	0.5	NA	Unpublished
FR-165	<i>K. pneumoniae</i>	Spain	S	–	1	NA	Unpublished
FR-166	<i>K. pneumoniae</i>	Colombia	S	–	0.12	NA	Unpublished
FR-167	<i>K. pneumoniae</i>	Colombia	S	–	0.12	NA	Unpublished
FR-168	<i>K. pneumoniae</i>	Colombia	S	–	0.12	NA	Unpublished
FR-169	<i>K. pneumoniae</i>	Colombia	S	–	0.25	NA	Unpublished
FR-170	<i>K. pneumoniae</i>	Colombia	S	–	0.5	NA	Unpublished
FR-171	<i>K. pneumoniae</i>	Switzerland	S	–	0.12	NA	Unpublished
FR-172	<i>K. pneumoniae</i>	Switzerland	S	–	0.12	NA	Unpublished
FR-173	<i>K. pneumoniae</i>	Switzerland	S	–	0.25	NA	Unpublished
FR-174	<i>K. pneumoniae</i>	France	S	–	0.12	NA	Unpublished
FR-175	<i>K. pneumoniae</i>	France	S	–	0.12	NA	Unpublished
FR-176	<i>K. pneumoniae</i>	France	S	–	0.12	NA	Unpublished
FR-177	<i>K. pneumoniae</i>	France	S	–	0.25	NA	Unpublished
FR-178	<i>K. pneumoniae</i>	France	S	–	0.25	NA	Unpublished
FR-179	<i>K. pneumoniae</i>	France	S	–	0.25	NA	Unpublished
FR-180	<i>K. pneumoniae</i>	France	S	+	1	NA	Unpublished
FR-181	<i>K. pneumoniae</i>	France	S	+	2	NA	Unpublished
FR-182	<i>K. pneumoniae</i>	France	S	+	2	NA	Unpublished
FR-183	<i>K. oxytoca</i>	France	S	–	0.12	NA	Unpublished
FR-184	<i>K. oxytoca</i>	France	S	–	0.12	NA	Unpublished
FR-185	<i>K. oxytoca</i>	France	S	–	0.25	NA	Unpublished



Strain	Species	Origin	Polymyxin phenotype	Rapid polymyxin NP test	Colistin MIC, µg/mL	Resistance mechanism	Strain reference
FR-186	<i>E. cloacae</i>	Colombia	S	–	0.12	NA	Unpublished
FR-187	<i>E. cloacae</i>	Colombia	S	–	0.12	NA	Unpublished
FR-188	<i>E. cloacae</i>	France	S	–	0.12	NA	Unpublished
FR-189	<i>E. cloacae</i>	France	S	–	0.12	NA	Unpublished
FR-190	<i>E. cloacae</i>	France	S	–	0.25	NA	Unpublished
FR-191	<i>E. cloacae</i>	France	S	–	0.5	NA	Unpublished
FR-192	<i>E. aerogenes</i>	Switzerland	S	–	0.12	NA	Unpublished
FR-193	<i>E. aerogenes</i>	France	S	–	0.12	NA	Unpublished
FR-194	<i>E. aerogenes</i>	France	S	–	0.25	NA	Unpublished
FR-195	<i>C. freundii</i>	France	S	–	0.12	NA	Unpublished
FR-196	<i>C. freundii</i>	France	S	–	0.25	NA	Unpublished
FR-197	<i>C. freundii</i>	Colombia	S	–	0.25	NA	Unpublished
FR-198	<i>C. koseri</i>	France	S	–	0.12	NA	Unpublished
FR-199	<i>C. koseri</i>	France	S	–	0.12	NA	Unpublished
FR-200	<i>C. koseri</i>	France	S	–	0.25	NA	Unpublished

\*HR, heteroresistant; NA, not applicable; R, resistant; S, susceptible.

†Strains FR-01 and FR-136 were used as positive and negative controls, respectively.

## References

- Jayol A, Poirel L, Brink A, Villegas MV, Yilmaz M, Nordmann P. Resistance to colistin associated with a single amino acid change in protein PmrB among *Klebsiella pneumoniae* isolates of worldwide origin. *Antimicrob Agents Chemother.* 2014;58:4762–6. [PubMed](#)  
<http://dx.doi.org/10.1128/AAC.00084-14>
- Jayol A, Nordmann P, Brink A, Poirel L. Heteroresistance to colistin in *Klebsiella pneumoniae* associated with alterations in the PhoPQ regulatory system. *Antimicrob Agents Chemother.* 2015;59:2780–4. [PubMed](#) <http://dx.doi.org/10.1128/AAC.05055-14>
- Poirel L, Jayol A, Bontron S, Villegas MV, Ozdamar M, Turkoglu S, et al. The *mgrB* gene as a key target for acquired resistance to colistin in *Klebsiella pneumoniae*. *J Antimicrob Chemother.* 2015;70:75–80. [PubMed](#) <http://dx.doi.org/10.1093/jac/dku323>
- Jayol A, Poirel L, Villegas MV, Nordmann P. Modulation of *mgrB* gene expression as a source of colistin resistance in *Klebsiella oxytoca*. *Int J Antimicrob Agents.* 2015;46:108–10. [PubMed](#)  
<http://dx.doi.org/10.1016/j.ijantimicag.2015.02.015>
- Poirel L, Kieffer N, Liassine N, Thanh D, Nordmann P. Plasmid-mediated carbapenem and colistin resistance in a clinical isolate of *Escherichia coli*. *Lancet Infect Dis.* 2016;16:281. [PubMed](#)  
[http://dx.doi.org/10.1016/S1473-3099\(16\)00006-2](http://dx.doi.org/10.1016/S1473-3099(16)00006-2)



## **1.2. Détection rapide des BGN résistants à la colistine à partir d'hémocultures**

### **Contexte :**

La prévalence de la résistance aux carbapénèmes dans les réanimations de certains pays est supérieure à 50 % (111). La consommation de colistine dans ces pays augmente par conséquent mais des résistances à la colistine émergent. En Italie, une épidémie avec des souches de *K. pneumoniae* productrices de KPC et résistantes à la colistine responsables de 93 cas de septicémies a été décrite (112), soulignant l'importance de la détection de la résistance à la colistine à partir des flacons d'hémocultures.

### **Objectif de l'étude :**

L'objectif de cette étude était d'adapter le test Rapid Polymyxin NP pour la détection des souches résistantes à la colistine directement à partir des flacons d'hémocultures, sans passer par une étape de réisolement. Le but était de détecter plus rapidement ces souches résistantes, pour d'une part optimiser la prise en charge thérapeutique des patients bactériémiques et d'autre part, isoler les patients porteurs de souches résistantes pour éviter la dissémination de ces souches dans les unités de soins. Le test devait avoir une bonne sensibilité pour la détection des souches résistantes, être réalisable en un minimum de manipulations pour limiter le temps technique, et devait être rapide avec un rendu des résultats en moins de 4 heures.

### **Principaux résultats :**

Nous avons montré qu'il était possible de réaliser le test après une simple dilution au 1/10<sup>ème</sup> de l'hémoculture dans du NaCl 0,9 %. Nous avons testé 73 hémocultures positives avec des souches d'entérobactéries d'espèces variées, incluant 56 souches résistantes (résistances naturelles ou acquises, chromosomiques ou plasmidiques) et 17 souches sensibles à la colistine. Ce test a montré une excellente sensibilité et spécificité (98,2 et 100 %, respectivement) avec des résultats rapides (en moins de 4 h).

### **Conclusion :**

Nous avons montré que le Rapid Polymyxin NP test pouvait être réalisé directement à partir d'hémocultures pour détecter les souches d'entérobactéries résistantes à la colistine en moins de 4h. Comparé aux méthodes standards, ce test permet un raccourcissement du délai de rendu de la sensibilité à la colistine de plus de 40 h (24 h de culture bactérienne et 24 h additionnelles pour tester la sensibilité avec les méthodes standards). Il ne demande que très peu de temps technique car le test ne nécessite pas d'étape de subculture, de centrifugation ou de lavage. Dans les pays où les carbapénémases sont endémiques, une détection rapide de la résistance à la colistine chez les souches d'entérobactéries responsables de septicémies

pourra contribuer à l'initiation d'une antibiothérapie appropriée dans un délai très court (quelques heures après la positivité de l'hémoculture) et pourrait ainsi contribuer à diminuer la mortalité en cas de septicémie avec des souches multirésistantes aux antibiotiques. Ce test pourrait aussi permettre d'implémenter des précautions contacts pour isoler les patients infectés par des souches multirésistantes productrices de carbapénèmases et résistantes à la colistine pour éviter le développement d'épidémies hospitalières comme celles décrites en Italie.

**Ce travail a été publié dans *Journal of Clinical Microbiology* en juin 2016 (IF = 3.631).**

# Rapid Detection of Polymyxin-Resistant *Enterobacteriaceae* from Blood Cultures

Aurélie Jayol,<sup>a,b,c</sup> Véronique Dubois,<sup>c</sup> Laurent Poirel,<sup>a,b</sup> Patrice Nordmann<sup>a,b,d</sup>

Emerging Antibiotic Resistance Unit, Medical and Molecular Microbiology, Department of Medicine, University of Fribourg, Fribourg, Switzerland<sup>a</sup>; INSERM European Unit (LEA Paris, France), University of Fribourg, Fribourg, Switzerland<sup>b</sup>; CNRS UMR5234, University of Bordeaux, Bordeaux, France<sup>c</sup>; University of Lausanne and University Hospital Center, Lausanne, Switzerland<sup>d</sup>

**Enterobacterial strains resistant to polymyxins are being increasingly reported worldwide. The conventional methods for detection of colistin-resistant isolates such as broth microdilution remain time-consuming (24 to 48 h), and methods such as disc diffusion and Etest are not reliable. Recently, the rapid polymyxin NP test was developed for rapid identification of polymyxin-resistant *Enterobacteriaceae*. This test is based on the detection of glucose metabolism related to bacterial growth in the presence of a defined concentration of colistin (or polymyxin B). The formation of acid metabolites is evidenced by a color change of a pH indicator (red phenol) in less than 2 h. In this study, the polymyxin NP test was evaluated for detection of colistin-resistant *Enterobacteriaceae* directly from blood cultures. The test was performed with 73 blood culture sets (either spiked or clinical blood cultures) with various enterobacterial species. The test exhibited excellent discrimination between polymyxin-resistant and polymyxin-susceptible enterobacterial isolates, and results are obtained from blood cultures within 4 h. It is easy to perform and requires neither subculture nor a centrifugation step. This test is rapid, specific, and sensitive and allows early identification of polymyxin-resistant *Enterobacteriaceae* directly from blood cultures.**

The spread of carbapenemase-producing *Enterobacteriaceae* has now been reported worldwide (1). Considering the paucity of antibiotics that are effective against those multidrug-resistant bacteria, it is of utmost importance to preserve the clinical efficacy of last-resort antibiotics such as polymyxins (colistin and polymyxin B) (2). However, polymyxin-resistant *Enterobacteriaceae* are being increasingly reported worldwide, exhibiting either chromosomal mutations (3) or plasmid-mediated *mcr-1* gene acquisition (4). The methods available to determine polymyxin susceptibility require isolation and growth of the bacteria from the infected samples, and the susceptibility testing methods add, at least, an additional delay of 18 h to obtain the results (5). Moreover, routine methods used in the clinical microbiology laboratory (disk diffusion, Etest strips, and some automated systems such as the Vitek2 and the MicroScan) are not reliable for detecting polymyxin-resistant isolates (5–9). Molecular tests are not effective for determining polymyxin resistance due to the variety of genes that may be altered or truncated as a source of chromosomal resistance (3).

Rapid identification and antimicrobial susceptibility testing are essential for guiding clinicians in the selection of the most appropriate treatment for patients with bloodstream infections. The mortality rates from sepsis in intensive care units range from 20 to 60% worldwide. Inappropriate initial antimicrobial therapy for septic shock occurs in about 20% of patients, is associated with a 5-fold reduction in survival (10), and is responsible for an increased length of hospital stay (11). This is largely due to the fact that it takes several days to obtain the susceptibility pattern of the bacteria responsible for the septicemia. In Italy, a large hospital outbreak of colistin-resistant and carbapenemase (KPC)-producing *Klebsiella pneumoniae* isolates, correlating with the local increased colistin consumption, has been recently described (12). Therefore, there is a need for a rapid detection of polymyxin-resistant isolates from blood cultures.

Recently, a diagnostic test (the rapid polymyxin NP test) based on the detection of glucose metabolism related to bacterial growth

in the presence of colistin has been developed to rapidly identify (<2 h) polymyxin-resistant enterobacterial strains from colonies grown on solid media (13). This test yields sensitivity and specificity values of 99.3% and 95.4%, respectively. It has not yet been evaluated with clinical samples.

The aim of this study was to determine the ability of the rapid polymyxin NP test to detect polymyxin-resistant *Enterobacteriaceae* from blood cultures. First, we evaluated this test with blood cultures spiked with colistin-resistant enterobacterial strains exhibiting various mechanisms of resistance (intrinsic or acquired, chromosomal, and plasmid-mediated). Then, the test was performed prospectively on clinical blood cultures positive for Gram-negative bacteria.

## MATERIALS AND METHODS

**Spiked blood cultures.** The panel of strains used for spiking blood cultures included a single reference strain susceptible to colistin (*Escherichia coli* ATCC 25922) and 52 colistin-resistant enterobacterial strains (MICs of colistin ranging from 4 to >128 µg/ml). Among the latter strains, 5 strains exhibited an intrinsic polymyxin resistance trait (*Proteus* spp., *n* = 2; *Providencia* spp., *n* = 1; *Morganella* spp., *n* = 1; and *Serratia* spp., *n* = 1), and 47 strains presented an acquired resistance trait and belonged to various species (*E. coli*, *n* = 11; *Klebsiella* spp., *n* = 31; *Enterobacter* spp., *n* = 4; and *Hafnia alvei*, *n* = 1). Some of the strains had previously been

Received 27 April 2016 Returned for modification 18 May 2016

Accepted 8 June 2016

Accepted manuscript posted online 15 June 2016

Citation Jayol A, Dubois V, Poirel L, Nordmann P. 2016. Rapid detection of polymyxin-resistant *Enterobacteriaceae* from blood cultures. *J Clin Microbiol* 54:2273–2277. doi:10.1128/JCM.00918-16.

Editor: P. Bourbeau, St. James

Address correspondence to Patrice Nordmann, patrice.nordmann@unifr.ch.

Copyright © 2016, American Society for Microbiology. All Rights Reserved.

TABLE 1 Rapid polymyxin NP test results for spiked blood cultures and clinical blood cultures

Strain	Species	Origin	Phenotype <sup>a</sup>	Mechanism of resistance to colistin	MIC for colistin <sup>b</sup>	Rapid polymyxin NP test <sup>c</sup>	
						Aerobic vial	Anaerobic vial
Clinical blood cultures							
B1	<i>Escherichia coli</i>	France	S	NA <sup>d</sup>	0.12	– (4 h)	– (4 h)
B2	<i>Escherichia coli</i>	France	S	NA	0.12	– (4 h)	– (4 h)
B3	<i>Escherichia coli</i>	France	S	NA	0.12	– (4 h)	– (4 h)
B4	<i>Escherichia coli</i>	France	S	NA	0.12	– (4 h)	– (4 h)
B5	<i>Escherichia coli</i>	France	S	NA	0.25	– (4 h)	– (4 h)
B6	<i>Escherichia coli</i>	France	S	NA	0.5	– (4 h)	– (4 h)
B7	<i>Escherichia coli</i>	France	S	NA	0.12	– (4 h)	– (4 h)
B8	<i>Klebsiella pneumoniae</i>	France	S	NA	0.12	– (4 h)	– (4 h)
B9	<i>Klebsiella pneumoniae</i>	France	S	NA	0.12	– (4 h)	– (4 h)
B10	<i>Klebsiella pneumoniae</i>	France	S	NA	0.12	– (4 h)	– (4 h)
B11	<i>Klebsiella pneumoniae</i>	France	S	NA	0.25	– (4 h)	– (4 h)
B12	<i>Klebsiella oxytoca</i>	France	S	NA	0.12	– (4 h)	– (4 h)
B13	<i>Enterobacter cloacae</i>	France	S	NA	0.25	– (4 h)	– (4 h)
B14	<i>Enterobacter cloacae</i>	France	S	NA	0.5	– (4 h)	– (4 h)
B15	<i>Citrobacter koseri</i>	France	S	NA	0.12	– (4 h)	– (4 h)
B16	<i>Citrobacter koseri</i>	France	S	NA	0.12	– (4 h)	– (4 h)
B17	<i>Hafnia alvei</i>	France	R	Unknown	16	+ (3 h)	+ (3 h)
B18	<i>Serratia marcescens</i>	France	R	Intrinsic	128	+ (2 h)	+ (3 h)
B19	<i>Serratia marcescens</i>	France	R	Intrinsic	>128	+ (2 h)	+ (3 h)
B20	<i>Proteus vulgaris</i>	France	R	Intrinsic	>128	+ (2 h)	+ (2 h)
Spiked blood cultures							
FR-136	<i>Escherichia coli</i>	ATCC 25922	S	NA	0.25	– (4 h)	– (4 h)
FR-119	<i>Escherichia coli</i>	France	R	Unknown	<b>8</b>	<b>– (4 h)</b>	<b>– (4 h)</b>
FR-120	<i>Escherichia coli</i>	France	R	Unknown	16	+ (3 h)	+ (3 h)
FR-121	<i>Escherichia coli</i>	France	R	Unknown	4	+ (3 h)	+ (2 h)
B21	<i>Escherichia coli</i>	France	R	PmrB D152V	8	+ (4 h)	+ (4 h)
FR-93	<i>Escherichia coli</i>	Switzerland	R	MCR-1 plasmid	4	+ (4 h)	+ (3 h)
FR-94	<i>Escherichia coli</i>	South Africa	R	MCR-1 plasmid	8	+ (3 h)	+ (3 h)
FR-95	<i>Escherichia coli</i>	South Africa	R	MCR-1 plasmid	8	+ (4 h)	+ (3 h)
FR-96	<i>Escherichia coli</i>	South Africa	R	MCR-1 plasmid	8	+ (3 h)	+ (3 h)
FR-97	<i>Escherichia coli</i>	South Africa	R	MCR-1 plasmid	16	+ (3 h)	+ (3 h)
FR-98	<i>Escherichia coli</i>	South Africa	R	MCR-1 plasmid	8	+ (2 h)	+ (2 h)
FR-99	<i>Escherichia coli</i>	South Africa	R	MCR-1 plasmid	16	+ (4 h)	+ (3 h)
FR-100	<i>Klebsiella pneumoniae</i>	France	R	Unknown	32	+ (3 h)	+ (4 h)
FR-101	<i>Klebsiella pneumoniae</i>	France	R	Unknown	64	+ (3 h)	+ (3 h)
FR-103	<i>Klebsiella pneumoniae</i>	France	R	Unknown	4	+ (3 h)	+ (3 h)
FR-104	<i>Klebsiella pneumoniae</i>	France	R	Unknown	64	+ (2 h)	+ (3 h)
FR-106	<i>Klebsiella pneumoniae</i>	France	R	Unknown	>128	+ (3 h)	+ (3 h)
FR-107	<i>Klebsiella pneumoniae</i>	Spain	R	Unknown	32	+ (2 h)	+ (2 h)
FR-108	<i>Klebsiella pneumoniae</i>	Spain	R	Unknown	32	+ (2 h)	+ (2 h)
FR-115	<i>Klebsiella pneumoniae</i>	Colombia	R	Unknown	64	+ (3 h)	+ (3 h)
FR-118	<i>Klebsiella pneumoniae</i>	Turkey	R	Unknown	4	+ (2 h)	+ (2 h)
FR-06	<i>Klebsiella pneumoniae</i>	Colombia	R	PmrA G53C	64	+ (2 h)	+ (2 h)
FR-10	<i>Klebsiella pneumoniae</i>	South Africa	R	PmrB T157P	16	+ (4 h)	+ (2 h)
FR-11	<i>Klebsiella pneumoniae</i>	Colombia	R	PmrB T157P	32	+ (4 h)	+ (3 h)
FR-16b	<i>Klebsiella pneumoniae</i>	South Africa	R	PhoP D191Y	128	+ (3 h)	+ (3 h)
FR-21	<i>Klebsiella pneumoniae</i>	France	R	MgrB N42Y et K43I	64	+ (3 h)	+ (3 h)
FR-22	<i>Klebsiella pneumoniae</i>	Angola	R	MgrB I45T	64	+ (3 h)	+ (3 h)
FR-25	<i>Klebsiella pneumoniae</i>	France	R	MgrB truncated	128	+ (4 h)	+ (2 h)
FR-32	<i>Klebsiella pneumoniae</i>	Switzerland	R	MgrB truncated	64	+ (4 h)	+ (3 h)
FR-36	<i>Klebsiella pneumoniae</i>	Colombia	R	MgrB truncated	64	+ (2 h)	+ (3 h)
FR-40	<i>Klebsiella pneumoniae</i>	France	R	<i>bla</i> <sub>CTX-M-15</sub> / <i>ISEcp1</i> into <i>mgrB</i> gene	64	+ (2 h)	+ (2 h)
FR-42	<i>Klebsiella pneumoniae</i>	France	R	<i>IS102</i> into <i>mgrB</i> gene	>128	+ (3 h)	+ (3 h)
FR-47	<i>Klebsiella pneumoniae</i>	Turkey	R	<i>IS903b</i> into <i>mgrB</i> gene	64	+ (2 h)	+ (2 h)
FR-48	<i>Klebsiella pneumoniae</i>	Spain	R	<i>IS903</i> into <i>mgrB</i> gene	128	+ (3 h)	+ (3 h)
FR-49	<i>Klebsiella pneumoniae</i>	France	R	<i>IS5</i> into <i>mgrB</i> gene	64	+ (2 h)	+ (4 h)
FR-52	<i>Klebsiella pneumoniae</i>	Turkey	R	<i>IS5</i> into <i>mgrB</i> gene	128	+ (2 h)	+ (2 h)

(Continued on following page)

TABLE 1 (Continued)

Strain	Species	Origin	Phenotype <sup>a</sup>	Mechanism of resistance to colistin	MIC for colistin <sup>b</sup>	Rapid polymyxin NP test <sup>c</sup>	
						Aerobic vial	Anaerobic vial
FR-54	<i>Klebsiella pneumoniae</i>	Colombia	R	ISKpn13 into <i>mgrB</i> gene	128	+ (2 h)	+ (3 h)
FR-56	<i>Klebsiella pneumoniae</i>	Spain	R	ISKpn26 into <i>mgrB</i> gene	64	+ (2 h)	+ (2 h)
FR-68	<i>Klebsiella pneumoniae</i>	Colombia	R	ISKpn14 into <i>mgrB</i> gene	32	+ (3 h)	+ (3 h)
FR-70	<i>Klebsiella pneumoniae</i>	Colombia	R	IS10R into <i>mgrB</i> promoter	128	+ (3 h)	+ (3 h)
FR-71	<i>Klebsiella pneumoniae</i>	Turkey	R	ISKpn14 into <i>mgrB</i> promoter	32	+ (2 h)	+ (2 h)
FR-89	<i>Klebsiella pneumoniae</i>	Colombia	R	Deletion of 11 bp into <i>mgrB</i>	>128	+ (3 h)	+ (3 h)
FR-92	<i>Klebsiella oxytoca</i>	Colombia	R	ISKpn26 into <i>mgrB</i> promoter	64	+ (2 h)	+ (2 h)
FR-122	<i>Enterobacter cloacae</i>	Colombia	R	Unknown	32	+ (3 h)	+ (3 h)
FR-123	<i>Enterobacter cloacae</i>	Colombia	R	Unknown	>128	+ (4 h)	+ (4 h)
FR-125	<i>Enterobacter cloacae</i>	France	R	Unknown	>128	+ (2 h)	+ (2 h)
FR-129	<i>Enterobacter cloacae</i>	France	R	Unknown	32	+ (3 h)	+ (2 h)
FR-135	<i>Hafnia alvei</i>	France	R	Unknown	16	+ (2 h)	+ (2 h)
FR-01	<i>Morganella morganii</i>	France	R	Intrinsic	>128	+ (2 h)	+ (2 h)
FR-02	<i>Proteus mirabilis</i>	France	R	Intrinsic	>128	+ (3 h)	+ (3 h)
FR-03	<i>Proteus vulgaris</i>	France	R	Intrinsic	>128	+ (3 h)	+ (2 h)
FR-04	<i>Providencia stuartii</i>	France	R	Intrinsic	>128	+ (2 h)	+ (3 h)
FR-05	<i>Serratia marcescens</i>	France	R	Intrinsic	>128	+ (3 h)	+ (3 h)

<sup>a</sup> S, susceptible; R, resistant.

<sup>b</sup> MIC values of colistin: the breakpoint value for susceptible is  $\leq 2$   $\mu\text{g/ml}$  and for resistance is  $> 2$   $\mu\text{g/ml}$  for *Enterobacteriaceae* according to the EUCAST (23).

<sup>c</sup> Discrepant results obtained with the rapid polymyxin NP test compared to the broth dilution method are in bold. -, negative; +, positive.

<sup>d</sup> NA, not applicable.

characterized for their polymyxin resistance mechanisms at the molecular level (13–19).

To spike the blood cultures, the colistin-resistant enterobacterial strains were subcultured onto Luria-Bertani agar overnight. Then, aerobic and anaerobic blood culture vials without charcoal (BacT/Alert FA Plus and BacT/Alert FN Plus, respectively; bioMérieux, Marcy l'Etoile, France) containing 10 ml of sterile human blood were inoculated with  $10^3$  CFU/ml of the bacterial strain to be tested. This inoculum was prepared by diluting a 0.5 McFarland standard suspension (about  $10^8$  CFU/ml) in 0.9% NaCl. Then, blood culture vials were incubated until a positive blood culture was detected by the BacT/Alert 3D blood culture system. With this protocol, the time for detecting the positivity of the blood cultures ranged from 6 to 15 h, and the final inoculum ranged from  $10^7$  to  $10^9$  CFU/ml, as previously reported (20).

**Clinical blood cultures.** All sets of positive blood cultures recovered during the November 2015 to February 2016 period of time from nonduplicate patients that were detected as positive by the BacT/Alert 3D blood culture system (bioMérieux) and stained as Gram-negative were included. Both aerobic and anaerobic vials were tested. Species identification was carried out with the matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) Biotyper system (Bruker Corporation, Bremen, Germany) after 3 h of incubation of the blood culture on chocolate agar. Positive blood cultures with nonenterobacterial isolates or with a mix of bacteria were excluded from the testing. Overall, a total of 20 monomicrobial blood cultures positive for enterobacterial isolates were tested (Table 1).

**Colistin susceptibility testing.** Colistin susceptibility testing was performed by determination of MICs using the broth microdilution method (BMD) according to the CLSI guidelines (21, 22). The BMD method was carried out with colonies grown on chocolate agar after incubation of the positive blood cultures. The EUCAST guidelines (version 2015) were used for categorical agreement (23).

**Detection of colistin resistance from positive blood cultures using the rapid polymyxin NP test.** For each positive blood culture tested (spiked or clinical blood culture), 50  $\mu\text{l}$  of blood culture was mixed with

450  $\mu\text{l}$  of sterile 0.9% NaCl (10-fold dilution). The rapid polymyxin NP test was then performed as described with bacterial colonies (13). Briefly, 50  $\mu\text{l}$  of the 10-fold dilution of the blood culture was mixed with (i) 150  $\mu\text{l}$  of colistin-free polymyxin NP solution and (ii) 150  $\mu\text{l}$  of polymyxin NP solution containing 5  $\mu\text{g/ml}$  of colistin sulfate (Sigma-Aldrich, Buchs, Switzerland) in a 96-well polystyrene plate (reference no. 82.1582.001; Sarstedt, Nümbrecht, Germany) (Fig. 1). The final concentration of colistin was 3.75  $\mu\text{g/ml}$  in each well. In parallel, 50  $\mu\text{l}$  of 0.9% NaCl was mixed with the polymyxin NP solution to verify the absence of glucose contamination. The plate was incubated at  $35 \pm 2^\circ\text{C}$  in ambient air for 4 h, and visual inspection was performed each hour to observe the color change. Test results were interpreted by technicians who were blinded to the sample identity. The test was considered negative when the well containing the mix with colistin remained orange and positive when it turned from orange to yellow (Fig. 1). The test was considered noninterpretable when the colistin-free well remained orange (absence of glucose metabolism) (Fig. 1). *E. coli* ATCC 25922 and a *Morganella morganii* strain were used as negative and positive controls, respectively.

## RESULTS AND DISCUSSION

To determine the performance of the rapid polymyxin NP test directly from blood cultures, 73 blood culture sets (aerobic and anaerobic vials) positive for enterobacterial strains were tested, including monomicrobial clinical samples and blood cultures spiked with colistin-resistant isolates presenting various mechanisms of resistance (intrinsic, acquired chromosomally encoded, plasmid-mediated, and unknown mechanisms). The results for the rapid polymyxin NP test were compared with the MICs determined by the BMD method taken as the gold standard.

The 52 blood cultures spiked with enterobacterial strains resistant to colistin gave positive results in 2 to 4 h (Table 1), except for a single colistin-resistant *E. coli* isolate that also gave a negative result by using the rapid polymyxin NP test performed from col-

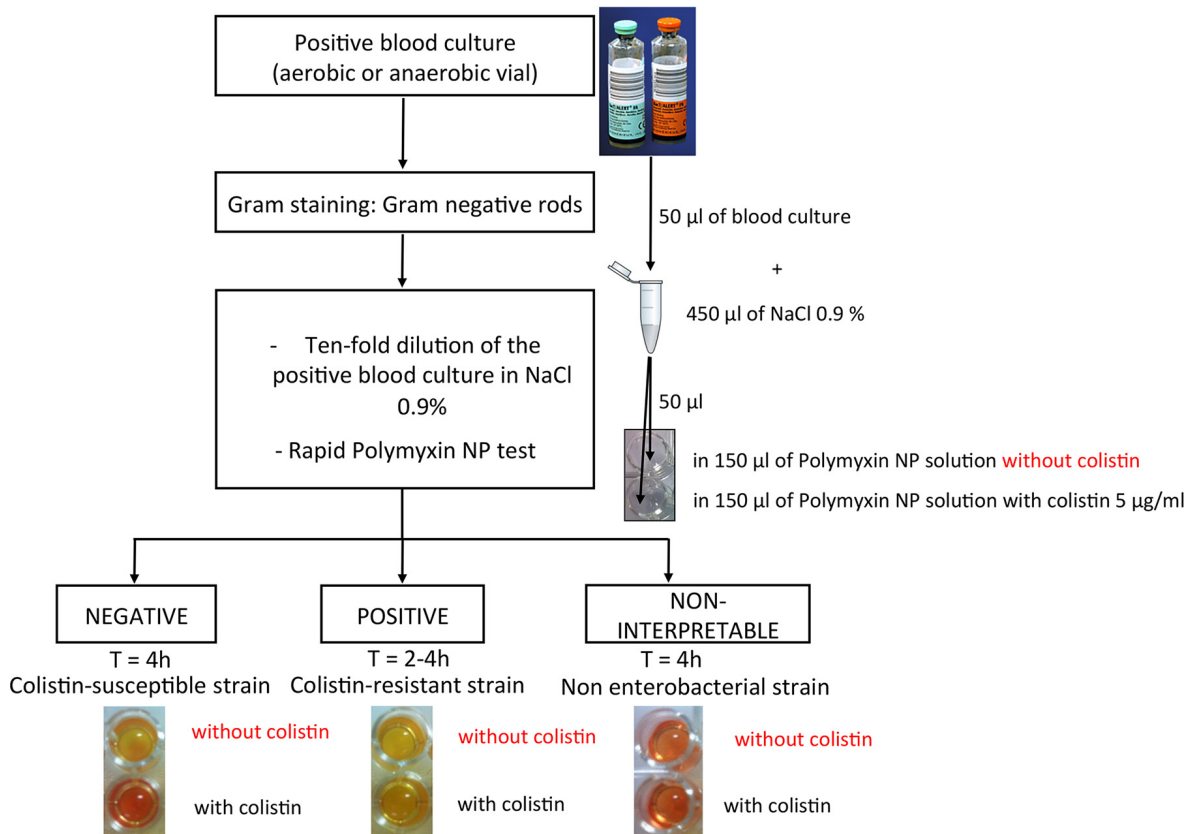


FIG 1 Strategy for identification of colistin-resistant *Enterobacteriaceae* from blood cultures using the rapid polymyxin NP test. T, time.

onies (13). The mechanism of colistin resistance of this single isolate remains unknown.

Twenty positive clinical blood culture sets detected by the BacT/Alert 3D blood culture system were also tested. Among them, four were positive with colistin-resistant enterobacterial isolates in 2 to 4 h of incubation: three isolates belonged to species that are naturally resistant to colistin (two *Serratia marcescens* isolates and a single *Proteus vulgaris* isolate) and a single *H. alvei* isolate showed acquired resistance to colistin. The rapid polymyxin NP test gave positive results for all of those isolates (Table 1). Sixteen blood cultures were grown with enterobacterial isolates belonging to genera naturally susceptible to colistin (*E. coli*, *Klebsiella*, *Enterobacter*, and *Citrobacter*). None of them presented acquired resistance to colistin (the MICs of colistin ranging from 0.125 to 0.5 µg/ml) and the rapid polymyxin NP test gave negative results. Positive blood cultures with nonfermenting bacterial isolates (*Acinetobacter* spp. and *Pseudomonas* spp.) gave noninterpretable results (Fig. 1).

A series of other conditions (variable blood culture dilutions or conditions of atmosphere incubation) were tested but gave less optimal results. Indeed, the test performed without blood culture dilution revealed that the excess of blood prevented the optimal reading of color changes. We did not observe spontaneous lysis of the blood cultures that might possibly have interfered with the results of the test. Other blood culture dilutions (1/5 or 1/100) gave less rapid positive results (data not shown). Incubation under CO<sub>2</sub> gave false-positive results because of excessive acidification of the polymyxin NP solution, as opposed to results obtained with

the rapid polymyxin NP test performed with bacterial colonies (13).

This test performed from blood cultures may have the same limitation as the rapid polymyxin NP test performed from bacterial colonies (13), i.e., the lack of detection of heteroresistant isolates with low MICs to polymyxins by BMD. This limitation has to be further explored.

In conclusion, the rapid polymyxin NP test from positive blood cultures showed good performances for the detection of enterobacterial isolates resistant to colistin. It shortens the delay in obtaining results to ca. 4 h versus 48 h for standard antibiotic susceptibility testing (24 h for obtaining a bacterial culture and 24 additional hours for susceptibility testing). This test may be implemented for routine testing of blood cultures and is best when combined with a rapid identification technique such as MALDI-TOF technology. It is cost-effective since it avoids in particular the time-consuming steps of manual determination of MIC values (e.g., powder weighing and dilution). As highlighted above, this test allows rapid identification of polymyxin-resistant *Enterobacteriaceae* responsible for bacteremia, which is not possible with molecular techniques, considering the complexity and the diversity of the polymyxin resistance mechanisms.

Early detection of colistin-resistant strains might contribute to early initiation of appropriate antibiotic therapy for septic patients infected with multidrug-resistant *Enterobacteriaceae*. In countries where carbapenemase-producing enterobacterial isolates are endemic (e.g., Greece and Italy), implementation of the test might contribute to early initiation of appropriate antibiotic



therapy. Its use may preserve polymyxin as the last-resort antibiotic. In addition, implementation of contact isolation precautions for carriers of polymyxin-resistant isolates could prevent the development of outbreaks with those multidrug-resistant isolates.

## ACKNOWLEDGMENTS

This work was financed by the University of Fribourg, Switzerland.

An international patent form has been filed on behalf of the University of Fribourg (Switzerland) corresponding to the rapid polymyxin NP test.

## FUNDING INFORMATION

This work, including the efforts of Aurelie Jayol, Véronique Dubois, Laurent Poirel, and Patrice Nordmann, was funded by University of Fribourg, Switzerland.

## REFERENCES

- Nordmann P, Poirel L. 2014. The difficult-to-control spread of carbapenemase producers among *Enterobacteriaceae* worldwide. *Clin Microbiol Infect* 20:821–830. <http://dx.doi.org/10.1111/1469-0691.12719>.
- Livermore DM, Warner M, Mushtaq S, Doumith M, Zhang J, Woodford N. 2011. What remains against carbapenem-resistant *Enterobacteriaceae*? Evaluation of chloramphenicol, ciprofloxacin, colistin, fosfomycin, minocycline, nitrofurantoin, temocillin and tigecycline. *Int J Antimicrob Agents* 37:415–419. <http://dx.doi.org/10.1016/j.ijantimicag.2011.01.012>.
- Olaitan AO, Morand S, Rolain JM. 2014. Mechanisms of polymyxin resistance: acquired and intrinsic resistance in bacteria. *Front Microbiol* 5:643. <http://dx.doi.org/10.3389/fmicb.2014.00643>.
- Liu YY, Wang Y, Walsh TR, Yi LX, Zhang R, Spencer J, Doi Y, Tian G, Dong B, Huang X, Yu LF, Gu D, Ren H, Chen X, Lv L, He D, Zhou H, Liang Z, Liu JH, Shen J. 2016. Emergence of plasmid-mediated colistin resistance mechanism MCR-1 in animals and human beings in China: a microbiological and molecular biological study. *Lancet Infect Dis* 16:161–168. [http://dx.doi.org/10.1016/S1473-3099\(15\)00424-7](http://dx.doi.org/10.1016/S1473-3099(15)00424-7).
- Humphries RM. 2015. Susceptibility testing of the polymyxins: where are we now? *Pharmacotherapy* 35:22–27. <http://dx.doi.org/10.1002/phar.1505>.
- Lo-Ten-Foe JR, de Smet AM, Diederer BM, Kluytmans JA, van Keulen PH. 2007. Comparative evaluation of the Vitek 2, disk diffusion, Etest, broth microdilution, and agar dilution susceptibility testing methods for colistin in clinical isolates, including heteroresistant *Enterobacter cloacae* and *Acinetobacter baumannii* strains. *Antimicrob Agents Chemother* 51:3726–3730. <http://dx.doi.org/10.1128/AAC.01406-06>.
- Maalej SM, Meziou MR, Rhimi FM, Hammami A. 2011. Comparison of disc diffusion, Etest and agar dilution for susceptibility testing of colistin against *Enterobacteriaceae*. *Lett Appl Microbiol* 53:546–551. <http://dx.doi.org/10.1111/j.1472-765X.2011.03145.x>.
- Tan TY, Ng SY. 2007. Comparison of Etest, Vitek and agar dilution for susceptibility testing of colistin. *Clin Microbiol Infect* 13:541–544. <http://dx.doi.org/10.1111/j.1469-0691.2007.01708.x>.
- Perez LR. 2015. Evaluation of polymyxin susceptibility profile among KPC-producing *Klebsiella pneumoniae* using Etest and MicroScan Walk-Away automated system. *APMIS* 123:951–954. <http://dx.doi.org/10.1111/apm.12438>.
- Kumar A, Ellis P, Arabi Y, Roberts D, Light B, Parrillo JE, Dodek P, Wood G, Kumar A, Simon D, Peters C, Ahsan M, Chateau D, Cooperative Antimicrobial Therapy of Septic Shock Database Research Group. 2009. Initiation of inappropriate antimicrobial therapy results in a fivefold reduction of survival in human septic shock. *Chest* 136:1237–1248. <http://dx.doi.org/10.1378/chest.09-0087>.
- Garnacho-Montero J, Ortiz-Leyba C, Herrera-Melero I, Aldabó-Pallás T, Cayuela-Dominguez A, Marquez-Vacaro JA, Carbajal-Guerrero J, Garcia-Garmendia JL. 2008. Mortality and morbidity attributable to inadequate empirical antimicrobial therapy in patients admitted to the ICU with sepsis: a matched cohort study. *J Antimicrob Chemother* 61:436–441. <http://dx.doi.org/10.1093/jac/dkm460>.
- Giani T, Arena F, Vaggelli G, Conte V, Chiarelli A, Henrici De Angelis L, Fornaini R, Grazzini M, Niccolini F, Pecile P, Rossolini GM. 2015. Large nosocomial outbreak of colistin-resistant, carbapenemase-producing *Klebsiella pneumoniae* traced to clonal expansion of an *mgrB* deletion mutant. *J Clin Microbiol* 53:3341–2234. <http://dx.doi.org/10.1128/JCM.01017-15>.
- Nordmann P, Jayol A, Poirel L. 2016. Rapid detection of polymyxin resistance in *Enterobacteriaceae*. *Emerg Infect Dis* 22:1038–1043. <http://dx.doi.org/10.3201/eid2206.151840>.
- Jayol A, Nordmann P, Brink A, Poirel L. 2015. Heteroresistance to colistin in *Klebsiella pneumoniae* associated with alterations in the PhoPQ regulatory system. *Antimicrob Agents Chemother* 59:2780–2784. <http://dx.doi.org/10.1128/AAC.05055-14>.
- Jayol A, Nordmann P, Desroches M, Decusser JW, Poirel L. 2016. Acquisition of broad-spectrum cephalosporin resistance leading to colistin resistance in *Klebsiella pneumoniae*. *Antimicrob Agents Chemother* 60:3199–3201. <http://dx.doi.org/10.1128/AAC.00237-16>.
- Jayol A, Poirel L, Brink A, Villegas MV, Yilmaz M, Nordmann P. 2014. Resistance to colistin associated with a single amino acid change in protein PmrB among *Klebsiella pneumoniae* isolates of worldwide origin. *Antimicrob Agents Chemother* 58:4762–4766. <http://dx.doi.org/10.1128/AAC.00084-14>.
- Jayol A, Poirel L, Villegas MV, Nordmann P. 2015. Modulation of *mgrB* gene expression as a source of colistin resistance in *Klebsiella oxytoca*. *Int J Antimicrob Agents* 46:108–110. <http://dx.doi.org/10.1016/j.ijantimicag.2015.02.015>.
- Poirel L, Jayol A, Bontron S, Villegas MV, Ozdamar M, Türkoglu S, Nordmann P. 2015. The *mgrB* gene as a key target for acquired resistance to colistin in *Klebsiella pneumoniae*. *J Antimicrob Chemother* 70:75–80. <http://dx.doi.org/10.1093/jac/dku323>.
- Poirel L, Kieffer N, Liassine N, Thanh D, Nordmann P. 2016. Plasmid-mediated carbapenem and colistin resistance in a clinical isolate of *Escherichia coli*. *Lancet Infect Dis* 16:281–282. [http://dx.doi.org/10.1016/S1473-3099\(16\)00007-4](http://dx.doi.org/10.1016/S1473-3099(16)00007-4).
- Dortet L, Bréchard L, Poirel L, Nordmann P. 2014. Rapid detection of carbapenemase-producing *Enterobacteriaceae* from blood cultures. *Clin Microbiol Infect* 20:340–344. <http://dx.doi.org/10.1111/1469-0691.12318>.
- Clinical and Laboratory Standards Institute. 2012. Methods for dilution of antimicrobial susceptibility tests for bacteria that grow aerobically; approved standard—9th ed. CLSI document M07-A9. Clinical and Laboratory Standards Institute, Wayne, PA.
- Clinical and Laboratory Standards Institute. 2015. Performance standards for antimicrobial susceptibility testing. CLSI document M100-S25. Clinical and Laboratory Standards Institute, Wayne, PA.
- European Committee on Antimicrobial Susceptibility Testing. 2014. Breakpoint tables for interpretation of MICs and zone diameters, version 2.0. European Committee on Antimicrobial Susceptibility Testing, Basel, Switzerland.

## **2. Développement d'une gélose sélective pour le screening des BGN résistants à la colistine**

### **Contexte :**

La colistine étant très utilisée en milieu vétérinaire et de plus en plus employée pour le traitement des infections à BGN multirésistants en médecine humaine, la mise au point d'un milieu sélectif pour isoler les souches de BGN résistants à la colistine à partir de prélèvements humains, animaux ou environnementaux était devenue nécessaire afin de détecter l'émergence et la diffusion de résistances. Par ailleurs, l'identification récente de résistances plasmidiques rendait encore plus importante la nécessité de surveillance de l'émergence de résistances car elle faisait craindre la diffusion rapide de la résistance à la colistine.

Lorsque nous avons débuté ce projet, il n'existait pas de milieu sélectif pour détecter les souches présentant une résistance acquise à la colistine. Seuls certains milieux permettant l'isolement de souches de BGN naturellement résistantes à la colistine comme *Burkholderia* ou encore *Serratia* étaient disponibles mais ils contenaient de fortes concentrations de colistine ne permettant pas la détection de souches présentant des faibles niveaux de résistance à la colistine (CMI entre 4 et 16 mg/L), en particulier les souches exprimant des résistances plasmidiques.

### **Objectif de cette étude :**

L'objectif de cette étude était de développer un milieu sélectif pour les BGN résistants à la colistine. Il devait permettre le screening des BGN résistants à la colistine à partir de prélèvements cliniques et à partir de colonies bactériennes, quel que soit le mécanisme de résistance à la colistine. Ce milieu devait si possible permettre la différenciation ou l'orientation vers les différentes espèces bactériennes (milieu chromogénique). Il devait également éviter l'envahissement de la gélose par les *Proteus* naturellement résistants à la colistine et ne devait pas contenir de sels biliaires qui inhibent la croissance de certaines souches résistantes à la colistine (113). Il devait inhiber la croissance des levures et des bactéries à Gram positif naturellement résistantes à la colistine.

### **Principaux résultats :**

Lors de la mise au point de ce milieu, nous avons testé différentes bases de géloses (Eosine-Bleu de Méthylène, MH, LB, UriSelect<sup>®</sup>4), différents antibiotiques anti-Gram positif (daptomycine, linézolide et vancomycine) et différentes concentrations finales de colistine (1 à 5 mg/L).



La composition finale de la gélose SuperPolymyxin qui a été retenue est :

- Gélose : Eosine-Méthylène Blue (EMB)
- Antibiotique pour inhiber la croissance des bactéries Gram positif : Daptomycine à une concentration de 10 mg/L
- Antifongique pour inhiber la croissance des levures : Amphotéricine B à une concentration de 5 mg/L
- Sulfate de colistine à une concentration de 3,5 mg/L

La gélose EMB utilisée comme base pour la préparation de cette gélose sélective inhibe la diffusion des *Proteus* sur la gélose et permet de différencier les BGN fermentants le lactose (marron) des BGN ne fermentants pas le lactose (couleur gélose, c'est-à-dire bordeaux). Par ailleurs, les souches de *E. coli* présentent une couleur vert métallique caractéristique sur cette gélose.

La vancomycine n'a pas été retenue comme antibiotique anti-Gram positif car des synergies entre la colistine et la vancomycine ont été observées. En effet, certaines souches d'entérobactéries résistantes à la colistine ne cultivaient pas sur les géloses contenant ces deux antibiotiques.

Pour évaluer les performances de la gélose SuperPolymyxin dans sa version finalisée, nous avons testé 4 souches de cocci Gram positif (2 staphylocoques et 2 entérocoques) et une souche de levure qui ne cultivaient pas sur la gélose confirmant sa sélectivité pour les Gram négatif. Nous avons également testé 83 souches de BGN d'espèces variées, incluant 52 souches résistantes (résistances naturelles ou acquises, chromosomiques ou plasmidiques) et 31 souches sensibles à la colistine. La limite de détection pour les souches sensibles à la colistine s'étendait de  $10^6$  à  $>10^7$  UFC/mL et pour les souches résistantes de  $10^1$  à  $5.10^2$  UFC/mL. Le milieu a montré une excellente sensibilité (100 %) pour détecter les souches résistantes quel que soit le mécanisme de résistance et une excellente spécificité (100 %). Par ailleurs, l'évaluation de la gélose a été réalisée pour la détection des souches résistantes à la colistine directement à partir des selles et toutes les souches résistantes à la colistine ont poussé avec une limite de détection allant de  $10^1$  à  $10^2$  UFC/mL.

### **Conclusion :**

Nous avons développé une gélose sélective, la gélose SuperPolymyxin, pour isoler les BGN résistants à la colistine à partir de prélèvements cliniques (écouvillons rectaux, selles...). Cette gélose représente un outil important pour la lutte contre la diffusion de la résistance à la colistine chez les BGN car il permet un screening des patients porteurs de BGN résistants à cet antibiotique. Couplé avec les tests moléculaires (PCR pour identifier les gènes plasmidiques *mcr*), il permet la détection des porteurs de souches d'entérobactéries présentant une résistance plasmidique à la colistine. Ce dépistage pourrait permettre

l'isolement précoce des patients porteurs pour éviter la diffusion de la résistance à la colistine dans les hôpitaux. Cette gélose pourrait aussi permettre d'effectuer des études épidémiologiques (screening de la résistance à la colistine chez l'homme, l'animal et dans l'environnement, par exemple l'eau) afin d'identifier les réservoirs de BGN résistants à la colistine.

**Ce travail a fait l'objet d'une demande de dépôt de brevet auprès de l'Office européen des brevets en mars 2015 et a été publié dans *Journal of Clinical Microbiology* en mars 2016 (IF = 3.631).**

# A Universal Culture Medium for Screening Polymyxin-Resistant Gram-Negative Isolates

Patrice Nordmann,<sup>a,b</sup> Aurélie Jayol,<sup>a</sup> Laurent Poirel<sup>a</sup>

Emerging Antibiotic Resistance Unit, Medical and Molecular Microbiology, Department of Medicine, Faculty of Science, University of Fribourg,<sup>a</sup> and HFR-Hôpital Cantonal,<sup>b</sup> Fribourg, Switzerland

**The colistin-containing SuperPolymyxin medium was developed for screening polymyxin-resistant Gram-negative bacteria. It was evaluated with 88 polymyxin-susceptible or polymyxin-resistant cultured Gram-negative isolates. Its sensitivity and specificity of detection were ca. 100%. The SuperPolymyxin medium is the first screening medium that is able to detect intrinsic and acquired polymyxin-resistant bacteria.**

Emergence of polymyxin resistance (PR) is increasingly observed among Gram-negative bacteria (1). The identification of the plasmid-mediated colistin resistance determinant MCR-1 is an additional source of concern (2). Therefore, an early detection of carriers of PR isolates is becoming important.

Polymyxin-containing culture media are known for screening intrinsic PR bacterial species, such as *Serratia marcescens* and *Burkholderia cepacia* (3, 4). Those media containing high concentrations of colistin are not adapted for screening isolates with acquired PR that may be of a low level and may contain deoxycholic acids that may interfere with the growth of PR bacteria (18).

Therefore, our aim was to develop a selective culture medium for screening any type of PR Gram-negative bacteria. The design of this medium took into account the necessity to avoid swarming of *Proteus* spp. (naturally PR) and to prevent contamination by Gram-positive bacteria and fungi. The optimal screening medium retained was based on the eosin methylene blue (EMB) medium (product no. 70186; EMB Fluka, St. Louis, MO, USA) (5), which is selective for Gram-negative bacteria. This medium may also contribute to species identification by differentiating lactose fermenters (dark blue-brown colonies) from nonfermenters (colorless or light lavender) (Fig. 1). Moreover, differentiation of lactose fermenters was possible with *Escherichia coli* colonies displaying a characteristic metallic green sheen (Fig. 1A) and *Enterobacter* spp. and *Klebsiella* spp. giving brown, dark-centered, and mucoid colonies (Fig. 1B). The optimal colistin (Sigma-Aldrich, St. Louis, MO, USA) concentration was retained at 3.5 µg/ml. Since some *Streptococcus* and *Staphylococcus* strains may still grow on EBM medium, daptomycin (Novartis, Horsham, United Kingdom) was added at a concentration of 10 µg/ml. It is noteworthy that daptomycin and not vancomycin was added since vancomycin poten-

TABLE 1 Preparation of the SuperPolymyxin medium

Compound	Stock solution (mg/ml)	Quantity or vol to add <sup>a</sup>	Final concn <sup>b</sup>
EMB agar powder		15 g	3.75%
Distilled water		400 ml	
Colistin sulfate	20 In water in glass tubes	70 µl	3.5
Daptomycin	20 In water	200 µl	10
Amphotericin B	20 In D-(+)-glucose 10%	100 µl	5

<sup>a</sup> The volume of 400 ml of SuperPolymyxin medium was for, i.e., 20 plates.

<sup>b</sup> Concentrations are in micrograms per milliliter unless noted otherwise.

tates the activity of colistin against several Gram-negative bacteria (6, 7). Amphotericin B (Bristol-Myers Squibb, Rueil-Malmaison, France) was also added as an anti-fungi molecule at a concentration of 5 µg/ml.

The stock solutions of colistin, daptomycin, and amphotericin B were prepared as indicated in Table 1 and may be kept at -20°C for 1 year. Notably, glass tubes were used to prepare colistin stock solutions to avoid its binding to polystyrene. Colistimethate sulfate, a therapeutic prodrug of colistin, cannot be used. The diluted powder of EMB was autoclaved at 121°C for 15 min. After cooling

Received 29 February 2016 Accepted 7 March 2016

Accepted manuscript posted online 16 March 2016

Citation Nordmann P, Jayol A, Poirel L. 2016. A universal culture medium for screening polymyxin-resistant Gram-negative isolates. J Clin Microbiol 54:1395–1399. doi:10.1128/JCM.00446-16.

Editor: B. A. Forbes

Address correspondence to Patrice Nordmann, patrice.nordmann@unifr.ch.

Copyright © 2016, American Society for Microbiology. All Rights Reserved.

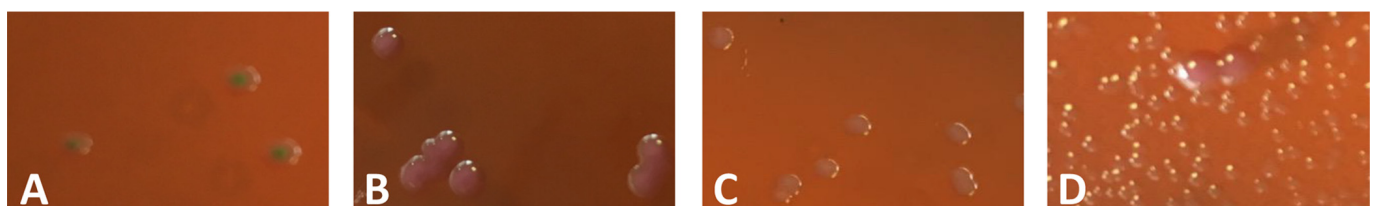


FIG 1 Polymyxin-resistant lactose-positive *E. coli* (A), polymyxin-resistant *K. pneumoniae* (B), polymyxin-resistant lactose-negative *E. coli* (C), and a mix of a heavy inoculum of *P. mirabilis* and a low inoculum of polymyxin-resistant *K. pneumoniae* (D) growing on the SuperPolymyxin medium.

TABLE 2 MICs of polymyxins for the studied strains and limits of detection of the SuperPolymyxin culture medium

Strain <sup>a</sup>	Species	Origin	MIC <sup>b</sup>		Polymyxin resistance <sup>c</sup>	Mechanism of polymyxin resistance	Lowest detection limit (CFU/ml) <sup>d</sup>	Lowest detection limit in stools (CFU/ml) <sup>d</sup>
			Colistin	Polymyxin B				
Yeast isolates								
FR-A	<i>C. albicans</i>	France	NA	NA	NA	NA	≥10 <sup>7</sup>	ND
Gram-positive cocci isolates								
FR-B	<i>S. aureus</i>	France	NA	NA	NA	NA	≥10 <sup>7</sup>	ND
FR-C	<i>Staphylococcus epidermidis</i>	France	NA	NA	NA	NA	≥10 <sup>7</sup>	ND
FR-D	<i>Enterococcus faecium</i>	France	NA	NA	NA	NA	≥10 <sup>7</sup>	ND
FR-E	<i>Enterococcus faecalis</i>	France	NA	NA	NA	NA	≥10 <sup>7</sup>	ND
Gram-negative rod isolates naturally resistant to polymyxins								
FR-01	<i>M. morgani</i>	France	>128	>128	R	Intrinsic	10 <sup>1</sup>	10 <sup>1</sup>
FR-02	<i>P. mirabilis</i>	France	>128	>128	R	Intrinsic	10 <sup>1</sup>	ND
FR-03	<i>Proteus vulgaris</i>	France	>128	>128	R	Intrinsic	10 <sup>1</sup>	ND
FR-04	<i>Providencia stuartii</i>	France	>128	>128	R	Intrinsic	10 <sup>1</sup>	ND
FR-05	<i>S. marcescens</i>	France	>128	>128	R	Intrinsic	10 <sup>1</sup>	ND
FR-201	<i>B. cepacia</i>	France	>128	>128	R	Intrinsic	10 <sup>1c</sup>	ND
FR-202	<i>Burkholderia gladioli</i>	France	>128	>128	R	Intrinsic	10 <sup>1</sup>	ND
Gram-negative rod isolates with an acquired mechanism of resistance to polymyxins								
FR-06	<i>K. pneumoniae</i>	France	32	64	R	PmrA G53C	10 <sup>1</sup>	10 <sup>1</sup>
FR-07	<i>K. pneumoniae</i>	France	32	32	R	PmrA G53S	10 <sup>1</sup>	10 <sup>1</sup>
FR-09	<i>K. pneumoniae</i>	Turkey	32	64	R	PmrB L17Q	10 <sup>1</sup>	10 <sup>1</sup>
FR-10	<i>K. pneumoniae</i>	South Africa	16	8	R	PmrB T157P	10 <sup>1</sup>	10 <sup>1</sup>
FR-17	<i>K. pneumoniae</i>	Turkey	<128	128	R	PhoQ R16C	10 <sup>1</sup>	10 <sup>1</sup>
FR-21	<i>K. pneumoniae</i>	France	32	64	R	MgrB N42Y/K43I	10 <sup>1</sup>	10 <sup>1</sup>
FR-30	<i>K. pneumoniae</i>	France	>128	64	R	MgrB truncated	10 <sup>1</sup>	10 <sup>1</sup>
FR-31	<i>K. pneumoniae</i>	France	64	32	R	MgrB truncated	10 <sup>1</sup>	ND
FR-36	<i>K. pneumoniae</i>	Colombia	128	128	R	MgrB truncated	10 <sup>1</sup>	ND
FR-40	<i>K. pneumoniae</i>	France	64	64	R	MgrB <i>ISEcp1-bla</i> <sub>CTX-M-15</sub>	10 <sup>1</sup>	10 <sup>1</sup>
FR-41	<i>K. pneumoniae</i>	France	>128	128	R	MgrB IS102-like	10 <sup>1</sup>	ND
FR-47	<i>K. pneumoniae</i>	Turkey	64	32	R	MgrB I3903b-like	10 <sup>1</sup>	ND
FR-48	<i>K. pneumoniae</i>	Spain	128	128	R	MgrB IS903-like	10 <sup>1</sup>	ND
FR-49	<i>K. pneumoniae</i>	France	64	32	R	MgrB IS5-like	10 <sup>1</sup>	ND
FR-54	<i>K. pneumoniae</i>	Colombia	128	128	R	MgrB ISKpn13	10 <sup>1</sup>	10 <sup>1</sup>
FR-56	<i>K. pneumoniae</i>	Spain	64	64	R	MgrB ISKpn26	10 <sup>1</sup>	ND
FR-68	<i>K. pneumoniae</i>	Colombia	64	64	R	MgrB ISKpn14	10 <sup>1</sup>	10 <sup>1</sup>
FR-70	<i>K. pneumoniae</i>	Colombia	128	128	R	<i>mgrB</i> promoter IS10R	10 <sup>1</sup>	ND
FR-71	<i>K. pneumoniae</i>	Turkey	32	32	R	<i>mgrB</i> promoter ISKpn14	10 <sup>1</sup>	ND
FR-86	<i>K. pneumoniae</i>	Spain	64	64	R	<i>mgrB</i> deletion nt 100	10 <sup>2</sup>	ND
FR-89	<i>K. pneumoniae</i>	Colombia	>128	>128	R	<i>mgrB</i> deletion nt 23 to 33	10 <sup>1</sup>	ND
FR-92	<i>K. oxytoca</i>	Colombia	64	64	R	<i>mgrB</i> promoter ISKpn26-like	10 <sup>1</sup>	10 <sup>1</sup>
FR-93	<i>E. coli</i>	France	4	4	R	Plasmid-mediated <i>mcr-1</i> gene	10 <sup>2</sup>	10 <sup>1</sup>
FR-94	<i>E. coli</i>	South Africa	16	16	R	Plasmid-mediated <i>mcr-1</i> gene	10 <sup>1</sup>	10 <sup>1</sup>
FR-98	<i>E. coli</i>	South Africa	8	8	R	Plasmid-mediated <i>mcr-1</i> gene	10 <sup>1</sup>	ND
FR-203	<i>A. baumannii</i>	Switzerland	128	128	R	PmrB G260D	10 <sup>2</sup>	10 <sup>1</sup>
FR-100	<i>K. pneumoniae</i>	France	64	64	R	Unknown	10 <sup>1</sup>	10 <sup>1</sup>
FR-102	<i>K. pneumoniae</i>	France	32	32	R	Unknown	10 <sup>1</sup>	ND
FR-113	<i>K. pneumoniae</i>	France	>128	>128	R	Unknown	10 <sup>1</sup>	ND
FR-114	<i>K. pneumoniae</i>	Colombia	64	128	R	Unknown	10 <sup>1</sup>	ND
FR-116	<i>K. pneumoniae</i>	France	64	32	R	Unknown	10 <sup>1</sup>	ND
FR-119	<i>E. coli</i>	France	8	4	R	Unknown	10 <sup>1</sup>	ND
FR-120	<i>E. coli</i>	France	8	4	R	Unknown	10 <sup>1</sup>	10 <sup>1</sup>
FR-121	<i>E. coli</i>	France	4	4	R	Unknown	10 <sup>1</sup>	ND

(Continued on following page)

TABLE 2 (Continued)

Strain <sup>a</sup>	Species	Origin	MIC <sup>b</sup>		Polymyxin resistance <sup>c</sup>	Mechanism of polymyxin resistance	Lowest detection limit (CFU/ml) <sup>d</sup>	Lowest detection limit in stools (CFU/ml) <sup>d</sup>
			Colistin	Polymyxin B				
FR-122	<i>E. cloacae</i>	Colombia	32	16	R	Unknown	10 <sup>1</sup>	10 <sup>1</sup>
FR-126	<i>E. cloacae</i>	France	>128	>128	R	Unknown	10 <sup>1</sup>	10 <sup>2</sup>
FR-135	<i>H. alvei</i>	France	16	8	R	Unknown	10 <sup>1</sup>	10 <sup>1</sup>
FR-204	<i>A. baumannii</i>	USA	>128	>128	R	Unknown	10 <sup>2</sup>	10 <sup>1</sup>
FR-205	<i>A. baumannii</i>	USA	8	8	R	Unknown	10 <sup>2</sup>	ND
FR-206	<i>A. baumannii</i>	USA	>128	>128	R	Unknown	10 <sup>2</sup>	ND
FR-207	<i>P. aeruginosa</i>	Colombia	64	64	R	Unknown	10 <sup>2f</sup>	10 <sup>1</sup>
FR-208	<i>P. aeruginosa</i>	France	>128	>128	R	Unknown	10 <sup>1e</sup>	ND
FR-209	<i>P. aeruginosa</i>	France	>128	>128	R	Unknown	10 <sup>2f</sup>	ND
FR-210	<i>S. maltophilia</i>	France	>128	>128	R	Unknown	10 <sup>1e</sup>	ND
FR-211	<i>S. maltophilia</i>	France	32	32	R	Unknown	5.10 <sup>2e</sup>	ND
Gram-negative rod isolates susceptible to polymyxins								
FR-136	<i>E. coli</i>	ATCC 25922	0.25	0.25	S	NA	<u>≥10<sup>7</sup></u>	ND
FR-212	<i>E. coli</i>	USA	0.12	0.12	S	NA	<u>≥10<sup>7</sup></u>	ND
FR-213	<i>E. coli</i>	Colombia	0.12	0.12	S	NA	<u>≥10<sup>7</sup></u>	ND
FR-214	<i>E. coli</i>	France	0.12	0.12	S	NA	<u>≥10<sup>7</sup></u>	ND
FR-215	<i>E. coli</i>	France	0.25	0.12	S	NA	<u>≥10<sup>7</sup></u>	ND
FR-216	<i>E. coli</i>	France	0.25	0.12	S	NA	<u>≥10<sup>7</sup></u>	ND
FR-217	<i>K. pneumoniae</i>	USA	0.12	0.12	S	NA	<u>5 × 10<sup>6</sup></u>	ND
FR-218	<i>K. pneumoniae</i>	Colombia	0.12	0.5	S	NA	<u>≥10<sup>7</sup></u>	ND
FR-219	<i>K. pneumoniae</i>	Colombia	0.12	0.12	S	NA	<u>≥10<sup>7</sup></u>	ND
FR-220	<i>K. pneumoniae</i>	Colombia	0.5	0.25	S	NA	<u>5 × 10<sup>6</sup></u>	ND
FR-221	<i>K. pneumoniae</i>	Colombia	0.5	0.5	S	NA	<u>≥10<sup>7</sup></u>	ND
FR-222	<i>K. pneumoniae</i>	France	0.12	0.25	S	NA	<u>≥10<sup>7</sup></u>	ND
FR-223	<i>K. pneumoniae</i>	France	0.12	0.12	S	NA	<u>≥10<sup>6</sup></u>	ND
FR-224	<i>K. pneumoniae</i>	France	0.25	0.5	S	NA	<u>≥10<sup>6</sup></u>	ND
FR-225	<i>K. pneumoniae</i>	France	0.5	0.5	S	NA	<u>≥10<sup>7</sup></u>	ND
FR-226	<i>K. pneumoniae</i>	Spain	0.5	0.5	S	NA	<u>10<sup>6</sup></u>	ND
FR-227	<i>K. pneumoniae</i>	Spain	0.5	0.5	S	NA	<u>10<sup>7</sup></u>	ND
FR-228	<i>E. cloacae</i>	Colombia	0.12	0.25	S	NA	<u>≥10<sup>7</sup></u>	ND
FR-229	<i>E. cloacae</i>	Colombia	0.12	0.12	S	NA	<u>≥10<sup>7</sup></u>	ND
FR-230	<i>E. cloacae</i>	France	0.12	0.25	S	NA	<u>5 × 10<sup>6</sup></u>	ND
FR-231	<i>Enterobacter aerogenes</i>	France	0.12	0.12	S	NA	<u>≥10<sup>7</sup></u>	ND
FR-232	<i>Citrobacter freundii</i>	Colombia	0.25	0.25	S	NA	<u>≥10<sup>7</sup></u>	ND
FR-233	<i>C. freundii</i>	France	0.12	0.5	S	NA	<u>5 × 10<sup>6</sup></u>	ND
FR-234	<i>P. aeruginosa</i>	ATCC 27853	0.5	0.5	S	NA	<u>5 × 10<sup>6</sup></u>	ND
FR-235	<i>P. aeruginosa</i>	Colombia	1	0.25	S	NA	<u>≥10<sup>7</sup></u>	ND
FR-236	<i>P. aeruginosa</i>	Colombia	2	0.5	S	NA	<u>10<sup>6</sup></u>	ND
FR-237	<i>P. aeruginosa</i>	Colombia	1	0.25	S	NA	<u>≥10<sup>7</sup></u>	ND
FR-238	<i>P. aeruginosa</i>	France	2	0.25	S	NA	<u>10<sup>6</sup></u>	ND
FR-239	<i>A. baumannii</i>	Colombia	0.5	0.12	S	NA	<u>≥10<sup>7</sup></u>	ND
FR-240	<i>A. baumannii</i>	France	0.5	0.25	S	NA	<u>10<sup>6</sup></u>	ND
FR-241	<i>A. baumannii</i>	USA	0.25	0.25	S	NA	<u>10<sup>6</sup></u>	ND

<sup>a</sup> The strains FR-01 and FR-136 were used as positive and negative controls, respectively.

<sup>b</sup> MICs of colistin and polymyxin B were determined using the broth microdilution method. NA, not available.

<sup>c</sup> R, resistant; S, susceptible.

<sup>d</sup> Underlined CFU counts are considered to be results below cutoff values set at  $\geq 1 \times 10^3$  CFU/ml. ND, not determinate.

<sup>e</sup> Positive culture after 36 h.

<sup>f</sup> Positive culture after 48 h.

the EMB medium for 1 h at 56°C, the antibiotic stock solutions were added. Poured plates were stored at 4°C, protected from direct light exposure for up to 1 week.

A total of 88 isolates were included in the study to evaluate the performance of the SuperPolymyxin medium that included 52 PR Gram-negative bacteria of worldwide origin (Table 2). Seven strains were intrinsic PR genders (*Morganella*, *Proteus*, *Providencia*, *Serratia*, and *Burkholderia*). Forty-five isolates with acquired

PR were also included and consisted of 36 enterobacterial strains (*E. coli*, *Klebsiella* spp., *Enterobacter cloacae*, and *Hafnia alvei*) and 9 nonfermenters (*Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Stenotrophomonas maltophilia*). Thirty-one polymyxin-susceptible strains were included with 23 enterobacterial strains and 8 nonfermenters (Table 2).

MICs of polymyxins (colistin and polymyxin B) were determined using the broth microdilution method in cation-adjusted

Mueller-Hinton broth as recommended by the CLSI (8). A final inoculum of  $5 \times 10^5$  CFU/ml of each strain was distributed in the 96-polystyrene-well tray (Sarstedt, Nümbrecht, Germany). *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 were used as polymyxin-susceptible control strains (9), and all experiments were repeated in triplicate. The results were interpreted according to the CLSI breakpoints (9) for *A. baumannii* (susceptible [S],  $\leq 2$   $\mu\text{g/ml}$ ; resistant [R],  $\geq 4$   $\mu\text{g/ml}$ ) and for *Pseudomonas* spp. (S,  $\leq 2$   $\mu\text{g/ml}$ ; R,  $\geq 8$   $\mu\text{g/ml}$ ) and according to the EUCAST breakpoints for *Enterobacteriaceae* (i.e., S,  $\leq 2$   $\mu\text{g/ml}$ ; R,  $> 2$   $\mu\text{g/ml}$ ) (10). MIC values for colistin and polymyxin B were superimposable, permitting an easy classification between PR and polymyxin-susceptible strains (Table 2).

The known genes possibly involved in chromosomally encoded PR in *E. coli*, *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *P. aeruginosa*, and *A. baumannii* were sequenced as described previously (11–15). The PCR detection of the *mcr-1* gene was carried out as described previously (2) (Table 2). MIC values of polymyxins for strains with acquired resistance were variable and were lower than those for intrinsically PR strains (Table 2).

Using an inoculum with an optical density of 0.5 McFarland standard (inoculum of  $\sim 10^8$  CFU/ml), serial 10-fold dilutions of the isolates were made in normal saline, and 100- $\mu\text{l}$  portions were plated onto the SuperPolymyxin medium. To quantify the viable bacteria in each dilution, Trypticase soy agar was inoculated concomitantly with 100  $\mu\text{l}$  of suspension and was incubated overnight at 37°C. The number of viable colonies was counted after 24 h of culture at 37°C (and after 36 and 48 h for *B. cepacia*, *P. aeruginosa*, and *S. maltophilia*). PR and polymyxin-susceptible control strains were FR-01 (*Morganella morganii*) and FR-136 (*E. coli* ATCC 25922), respectively (Table 2). The lowest limit of detection for the tested strains was determined using the SuperPolymyxin medium. The sensitivity and specificity cutoff values were set at  $1 \times 10^3$  CFU/ml, i.e., a limit value of  $1 \times 10^3$  CFU/ml and above was considered not efficiently detected (16). All of the PR strains grew on the SuperPolymyxin medium in 24 h except *P. aeruginosa*, *S. maltophilia*, and the intrinsically PR *Burkholderia* genus that grew in 24 to 48 h (Table 2). The lowest limit of detection was below the cutoff value for all PR strains, whereas the limit of detection of the polymyxin-susceptible strains was above the cutoff value at  $\geq 1 \times 10^6$  CFU/ml (Table 1). The sensitivity and specificity of the SuperPolymyxin medium for selecting PR isolates were 100% in both cases. Moreover, this medium was tested with a light growth of a PR *K. pneumoniae* isolate (FR-10) among a heavier growth of *Proteus mirabilis* (FR-02) and revealed that it nicely discriminated between the two species (Fig. 1D).

Spiked stools were also tested with a representative collection of 22 PR isolates of various species with various levels and mechanisms of PR (Table 2). Spiked fecal samples were made by adding 100  $\mu\text{l}$  of each strain dilution to 900  $\mu\text{l}$  of fecal suspension that was obtained by suspending 5 g of freshly pooled feces from five healthy volunteers in 50 ml of distilled water as described previously (17). A nonspiked fecal suspension was used as a negative control. The lowest detection limit of the PR isolate was determined by plating 100  $\mu\text{l}$  of each dilution on the medium. The sensitivity and specificity were determined using the same cutoff value set at  $\geq 10^3$  CFU/ml (17). This value may correspond to a low-level carriage of PR bacteria in stools. All of the PR isolates spiked in stools grew with a lowest detection limit ranging from  $10^1$  to  $10^2$  CFU/ml (Table 2).

Similar results were obtained with 20 colistin-susceptible and polymyxin B-susceptible strains and 20 colistin-resistant and polymyxin B-resistant strains by using polymyxin B at the same concentration instead of colistin sulfate.

Finally, to assess the storage stability of the SuperPolymyxin medium, *Candida albicans*, *Staphylococcus aureus*, and colistin-susceptible *E. coli* ATCC 25955 were subcultured daily onto SuperPolymyxin agar plates from a single batch of medium stored at 4°C. Growth of those isolates were consistently inhibited during at least a 7-day period.

The SuperPolymyxin medium constitutes a screening medium aimed to detect any PR bacteria regardless of its resistance mechanism and of its level. This medium may be used in human medicine for detecting carriers and in veterinary medicine for surveillance surveys.

## ACKNOWLEDGMENTS

We are in debt to several colleagues who sent us polymyxin-resistant isolates, i.e., A. Brink, Y. Doi, J. Fernandez-Dominguez, J.-W. Decusser, J.-Y. Madec, P. Plésiat, and M.-V. Villegas.

This work was financed by the University of Fribourg, Switzerland. An international patent form has been filed on behalf of the University of Fribourg (Switzerland) corresponding to this selective culture medium.

## REFERENCES

- Bialvaei AZ, Samadi Kafil H. 2015. Colistin, mechanisms and prevalence of resistance. *Curr Med Res Opin* 31:707–721. <http://dx.doi.org/10.1185/03007995.2015.1018989>.
- Liu YY, Wang Y, Walsh TR, Yi LX, Zhang R, Spencer J, Doi Y, Tian G, Dong B, Huang X, Yu LF, Gu D, Ren H, Chen X, Lv L, He D, Zhou H, Liang Z, Liu JH, Shen J. 2016. Emergence of plasmid-mediated colistin resistance mechanism MCR-1 in animals and human beings in China: a microbiological and molecular biological study. *Lancet Infect Dis* 16:161–168. [http://dx.doi.org/10.1016/S1473-3099\(15\)00424-7](http://dx.doi.org/10.1016/S1473-3099(15)00424-7).
- Grasso GM, D'Errico MM, Schioppa F, Romano F, Montanaro D. 1988. Use of colistin and sorbitol for better isolation of *Serratia marcescens* in clinical samples. *Epidemiol Infect* 101:315–320. <http://dx.doi.org/10.1017/S0950268800054248>.
- Gilligan PH, Gage PA, Bradshaw LM, Schidlow DV, DeCicco BT. 1985. Isolation medium for the recovery of *Pseudomonas cepacia* from respiratory secretions of patients with cystic fibrosis. *J Clin Microbiol* 22:5–8.
- Levine M. 1943. The effect of concentration of dyes on differentiation of enteric bacteria on eosin-methylene-blue agar. *J Bacteriol* 45:471–475.
- Gordon NC, Png K, Wareham DW. 2010. Potent synergy and sustained bactericidal activity of a vancomycin-colistin combination versus multi-drug-resistant strains of *Acinetobacter baumannii*. *Antimicrob Agents Chemother* 54:5316–5322. <http://dx.doi.org/10.1128/AAC.00922-10>.
- Randall CP, Mariner KR, Chopra I, O'Neill AJ. 2013. The target of daptomycin is absent from *Escherichia coli* and other Gram-negative pathogens. *Antimicrob Agents Chemother* 57:637–639. <http://dx.doi.org/10.1128/AAC.02005-12>.
- Clinical and Laboratory Standards Institute. 2012. Methods for dilution of antimicrobial susceptibility tests for bacteria that grow aerobically; approved standard—9th ed. CLSI document M07-A9. Clinical and Laboratory Standards Institute, Wayne, PA.
- Clinical and Laboratory Standards Institute. 2014. Performance standards for antimicrobial susceptibility testing; 24th informational supplement. CLSI M100-S24. Clinical and Laboratory Standards Institute, Wayne, PA.
- EUCAST. 2014. Breakpoint tables for interpretation of MICs and zone diameters. [http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST\\_files/Breakpoint\\_tables/Breakpoint\\_table\\_v\\_4.0.pdf](http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Breakpoint_tables/Breakpoint_table_v_4.0.pdf).
- Adams MD, Nickel GC, Bajaksouzian S, Lavender H, Murthy AR, Jacobs MR, Bonomo RA. 2009. Resistance to colistin in *Acinetobacter baumannii* associated with mutations in the PmrAB two-component system. *Antimicrob Agents Chemother* 53:3628–3634. <http://dx.doi.org/10.1128/AAC.00284-09>.
- Jayol A, Poirel L, Villegas MV, Nordmann P. 2015. Modulation of *mgrB*



- gene expression as a source of colistin resistance in *Klebsiella oxytoca*. *Int J Antimicrob Agents* 46:108–110. <http://dx.doi.org/10.1016/j.ijantimicag.2015.02.015>.
13. Lee JY, Ko KS. 2014. Mutations and expression of PmrAB and PhoPQ related with colistin resistance in *Pseudomonas aeruginosa* clinical isolates. *Diagn Microbiol Infect Dis* 78:271–276. <http://dx.doi.org/10.1016/j.diagmicrobio.2013.11.027>.
  14. Poirel L, Jayol A, Bontron S, Villegas MV, Ozdamar M, Turkoglu S, Nordmann P. 2015. The *mgrB* gene as a key target for acquired resistance to colistin in *Klebsiella pneumoniae*. *J Antimicrob Chemother* 70:75–80. <http://dx.doi.org/10.1093/jac/dku323>.
  15. Quesada A, Porrero MC, Tellez S, Palomo G, Garcia M, Dominguez L. 2015. Polymorphism of genes encoding PmrAB in colistin-resistant strains of *Escherichia coli* and *Salmonella enterica* isolated from poultry and swine. *J Antimicrob Chemother* 70:71–74. <http://dx.doi.org/10.1093/jac/dku320>.
  16. Nordmann P, Girlich D, Poirel L. 2012. Detection of carbapenemase producers in *Enterobacteriaceae* by use of a novel screening medium. *J Clin Microbiol* 50:2761–2766. <http://dx.doi.org/10.1128/JCM.06477-11>.
  17. Naas T, Ergani A, Carrère A, Nordmann P. 2011. Real-time PCR for detection of NDM-1 carbapenemase genes from spiked stool samples. *Antimicrob Agents Chemother* 55:4038–4043. <http://dx.doi.org/10.1128/AAC.01734-10>.
  18. Froelich JM, Tran K, Wall D. 2006. A *pmrA* constitutive mutant sensitizes *Escherichia coli* to deoxycholic acid. *J Bacteriol* 188:1180–1183. <http://dx.doi.org/10.1128/JB.188.3.1180-1183.2006>.

## **B. Evaluation des tests phénotypiques et des tests automatisés pour la détection de la résistance à la colistine**

### **1. Evaluation de l'automate BD Phoenix<sup>®</sup>**

#### **Contexte :**

Le 22 mars 2016, l'EUCAST a publié de nouvelles recommandations pour la détermination des CMI de la colistine ([www.eucast.org](http://www.eucast.org)). Ces recommandations indiquaient que seule la méthode de dilutions en milieu liquide devait être utilisée pour la détermination des CMI et que la méthode des disques et les méthodes de diffusion type bandelettes Etest<sup>®</sup> devaient être abandonnées. La méthode de dilutions en milieu liquide n'étant pas réalisable en routine à l'hôpital, les automates permettant la détermination des CMI par dilutions en milieu liquide comme le système automatisé BD Phoenix<sup>®</sup> (Becton Dickinson) semblaient être une alternative intéressante. Cependant, leurs performances pour la détection de la résistance à la colistine étaient peu connues.

#### **Objectif de l'étude :**

L'objectif de cette étude était donc de tester les performances du système BD Phoenix<sup>®</sup> pour la détection de la résistance à la colistine chez les BGN et de comparer les résultats obtenus à ceux du test Rapid Polymyxin NP et à ceux de la méthode de dilutions en milieu liquide de référence.

#### **Matériel et méthodes :**

Nous avons testé 123 souches d'entérobactéries d'espèces variées, incluant 83 souches résistantes (résistances naturelles ou acquises, chromosomiques ou plasmidiques) et 40 souches sensibles à la colistine. Les résultats obtenus avec le système BD Phoenix<sup>®</sup> et le Rapid Polymyxin NP test ont été comparés aux résultats obtenus avec la méthode de référence (dilutions en milieu liquide manuelles).

#### **Principaux résultats :**

Parmi les 40 souches d'entérobactéries sensibles à la colistine, aucun résultat de fausse résistance n'a été identifié avec le système BD Phoenix<sup>®</sup> (spécificité = 100 %), tandis qu'une seule souche de *K. pneumoniae* avec une CMI de la colistine égale à 2 mg/L (juste en dessous de la valeur seuil EUCAST) a été faussement identifiée comme résistante avec le Rapid Polymyxin NP test (spécificité = 97,5 %). Parmi les 83 souches résistantes à la colistine, l'automate BD Phoenix<sup>®</sup> et le Rapid Polymyxin NP test ont montré une excellente sensibilité pour la détection des 16 souches présentant une résistance plasmidique à la colistine (gènes *mcr-1* et *mcr-2*). Cependant, le BD Phoenix<sup>®</sup> n'a pas détecté la résistance de 7 souches

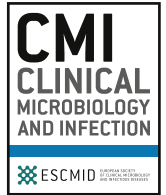


de *Enterobacter* spp., d'une souche de *K. pneumoniae*, d'une souche de *Salmonella enterica* et d'une souche de *E. coli* (sensibilité = 88 %), tandis qu'un seul échec de détection de la résistance pour la souche de *E. coli* a été attribué au Rapid Polymyxin NP test (sensibilité = 98,8 %). La souche de *E. coli* dont la résistance n'a pas été détectée par les deux systèmes et la souche de *S. enterica* dont la résistance n'a pas été détectée par le système BD Phoenix<sup>®</sup> présentaient un faible niveau de résistance (CMI = 8 et 4 mg/L, respectivement). Le mécanisme de résistance de ces deux souches reste inconnu. La souche de *K. pneumoniae* et les 7 souches de *Enterobacter* spp. dont la résistance n'a pas été détectée par le BD Phoenix<sup>®</sup> présentaient des CMIs comprises entre 16 et > 128 mg/L. Lors de la lecture des CMIs déterminées par la méthode de dilutions en milieu liquide, des « puits manqués » ont été observés pour 88 % de ces souches (absence de croissance à des concentrations de colistine entre 0,12 et 4 mg/L, alors qu'une croissance de la souche a été observée à des concentrations de colistine plus élevées). Cette observation suggère que l'échec de détection de ces souches serait dû à un phénotype d'hétérorésistance (85).

### **Conclusion :**

Cette étude a permis de déterminer les performances du BD Phoenix<sup>®</sup> pour la détection de la résistance à la colistine chez les entérobactéries. Nous avons montré la fiabilité de cet automate pour la détection des résistances plasmidiques mais avons montré ses limites pour la détection de l'hétérorésistance.

**Ce travail a été publié dans *Clinical Microbiology and Infection* en février 2018 (IF = 4.575).**



## Original article

Comparison of methods for detection of plasmid-mediated and chromosomally encoded colistin resistance in *Enterobacteriaceae*A. Jayol<sup>1,2,3,4</sup>, P. Nordmann<sup>3,4,5</sup>, P. Lehours<sup>1</sup>, L. Poirel<sup>3,4</sup>, V. Dubois<sup>1,2,\*</sup><sup>1</sup> Laboratory of Bacteriology, Bordeaux University Hospital, France<sup>2</sup> CNRS UMR5234, University of Bordeaux, Bordeaux, France<sup>3</sup> Emerging Antibiotic Resistance Unit, Medical and Molecular Microbiology, Department of Medicine, University of Fribourg, Fribourg, Switzerland<sup>4</sup> INSERM European Unit (LEA, IAME), Paris, France<sup>5</sup> University of Lausanne and University Hospital Center, Lausanne, Switzerland

## ARTICLE INFO

## Article history:

Received 22 December 2016

Received in revised form

3 May 2017

Accepted 1 June 2017

Available online xxx

Editor: P. Tassios

## Keywords:

Colistin

*mcr-1*

BD Phoenix automated system

Polymyxin B

Rapid Polymyxin NP test

Resistance

Susceptibility testing

## ABSTRACT

**Objectives:** Because of the emergence of plasmid-mediated (*mcr-1* and *mcr-2* genes) and chromosomally encoded colistin resistance, reliable methods for detecting colistin resistance/susceptibility in routine laboratories are required. We evaluated the respective performances of the BD Phoenix automated system, the newly developed Rapid Polymyxin NP test and the broth microdilution (BMD) reference method to detect colistin resistance in *Enterobacteriaceae*, and particularly those producing *mcr-1* and *mcr-2*.

**Methods:** Colistin susceptibility of 123 enterobacterial clinical isolates (40 colistin-susceptible and 83 colistin-resistant isolates) was tested with the BD Phoenix automated system, the Rapid Polymyxin NP test and the BMD method. Molecular mechanisms responsible for plasmid-mediated and chromosomally encoded colistin resistance mechanisms were investigated by PCR and sequencing.

**Results:** Considering BMD as a reference method, the BD Phoenix system failed to detect ten colistin-resistant isolates (one *Escherichia coli*, one *Klebsiella pneumoniae*, seven *Enterobacter* species and one *Salmonella enterica*). The Rapid Polymyxin NP test failed to detect the same single *E. coli* isolate. Those two latter methods detected the 16 *E. coli*, *K. pneumoniae* and *S. enterica* isolates producing the plasmid-encoded *mcr-1* and *mcr-2*.

**Conclusions:** The BD Phoenix system and the Rapid Polymyxin NP test are reliable techniques for detecting plasmid-mediated *mcr-1* and *mcr-2*-related colistin resistance. However, a high rate of false susceptibility was observed with the BD Phoenix system, indicating that susceptibility results obtained with that system should be confirmed by BMD method. By contrast, the Rapid Polymyxin NP test showed a good agreement with the BMD method, and results were obtained rapidly (within 2 hours). The BMD method should be performed if minimum inhibitory concentration values are needed. **A. Jayol, Clin Microbiol Infect 2017;■:1**

© 2017 European Society of Clinical Microbiology and Infectious Diseases. Published by Elsevier Ltd. All rights reserved.

## Introduction

The increasing use of colistin in human medicine, as well as the recent discovery of plasmid-mediated polymyxin resistance [1–4], highlight the need for reliable methods for polymyxin susceptibility testing.

The Clinical Laboratory Standard Institute (CLSI) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST), recently gathered in a joint subcommittee, chose the broth microdilution (BMD) method as the reference method (<http://www.eucast.org/>). It must be performed with sulfate salts of polymyxins (colistimethate used in human medicine ought not be used), with cation-adjusted Mueller-Hinton broth, without additives (in particular without polysorbate 80) and without treated polystyrene trays. Other methods, such as agar dilution, disk diffusion and gradient diffusion (Etest), have been ruled out. However, the reference standard—the BMD method—is difficult to

\* Corresponding author. V. Dubois, MFP Laboratory CNRS UMR-5234, 146 rue Léo Saignat, Batiment 3A, 33076 Bordeaux Cedex, France.

E-mail address: [veronique.dubois@u-bordeaux.fr](mailto:veronique.dubois@u-bordeaux.fr) (V. Dubois).

**Table 1**  
MICs of colistin (mg/L) by several tests

Isolate	Species (no. of isolates)	Phenotype	Mechanism of resistance to colistin <sup>a</sup>	BMD	Phoenix	Discrepancies	Rapid Polymyxin NP test	
				MIC colistin (mg/L)	MIC colistin (mg/L)		Result	Discrepancies
<b>Isolates susceptible to colistin</b>								
ATCC 25922	<i>Escherichia coli</i>	S	NA	0.25	≤0.5	No	–	No
2 to 15	<i>E. coli</i> (n = 14)	S	NA	0.12 to 0.5	≤0.5	No	–	No
16 to 26	<i>Klebsiella pneumoniae</i> (n = 11)	S	NA	0.12 to 2	≤0.5	No	–	Yes, ME (n = 1)
27 to 29	<i>Klebsiella oxytoca</i> (n = 3)	S	NA	0.12 to 0.25	≤0.5	No	–	No
30 to 32	<i>Enterobacter cloacae</i> (n = 3)	S	NA	0.12 to 0.25	≤0.5	No	–	No
33	<i>Enterobacter asburiae</i>	S	NA	0.12	≤0.5	No	–	No
34	<i>Enterobacter aerogenes</i>	S	NA	0.12	≤0.5	No	–	No
35 to 37	<i>Citrobacter freundii</i> (n = 3)	S	NA	0.25	≤0.5	No	–	No
38 to 40	<i>Citrobacter koseri</i> (n = 3)	S	NA	0.12 to 0.25	≤0.5	No	–	No
<b>Isolates resistant to colistin</b>								
41	<i>Morganella morganii</i>	R	Intrinsic	>128	>4	No	+	No
42–43	<i>Proteus mirabilis</i> (n = 2)	R	Intrinsic	>128	>4	No	+	No
44	<i>Proteus vulgaris</i>	R	Intrinsic	>128	>4	No	+	No
45	<i>Providencia stuartii</i>	R	Intrinsic	>128	>4	No	+	No
46 to 48	<i>Serratia marcescens</i> (n = 3)	R	Intrinsic	>128	>4	No	+	No
49 to 52	<i>Hafnia alvei</i> (n = 4)	R	Intrinsic	8 or 16	4 or >4	No	+	No
53 to 56	<i>Hafnia paralvei</i> (n = 4)	R	Intrinsic	8	4 or >4	No	+	No
57 to 68	<i>E. coli</i> (n = 11)	R	Plasmid-mediated <i>mcr-1</i> gene	4 or 8	4 or >4	No	+	No
69	<i>E. coli</i>	R	Plasmid-mediated <i>mcr-1</i> gene	64	>4	No	+	No
70	<i>E. coli</i>	R	Plasmid-mediated <i>mcr-2</i> gene	4	4	No	+	No
71	<i>K. oxytoca</i>	R	ISKpn26 into <i>mgrB</i> promotor	64	>4	No	+	No
72	<i>E. coli</i>	R	Unknown	8	≤0.5	Yes, VME	–	Yes, VME
73	<i>E. coli</i>	R	Unknown	8	>4	No	+	No
74	<i>E. coli</i>	R	Unknown	4	4	No	+	No
75	<i>E. coli</i>	R	Unknown	16	>4	No	+	No
76	<i>K. pneumoniae</i>	R	PmrA G53C	64	>4	No	+	No
77–78	<i>K. pneumoniae</i> (n = 2)	R	PmrA G53S	16 or 32	>4	No	+	No
79–80	<i>K. pneumoniae</i> (n = 2)	R	PmrB T157P	16 or 32	>4	No	+	No
81	<i>K. pneumoniae</i>	R	PhoP D191Y	128	≤0.5	Yes, VME	+	No
82	<i>K. pneumoniae</i>	R	PhoQ R16C	128	>4	No	+	No
83	<i>K. pneumoniae</i>	R	MgrB N42Y et K43I	64	>4	No	+	No
84	<i>K. pneumoniae</i>	R	MgrB I45T	64	>4	No	+	No
85 to 87	<i>K. pneumoniae</i> (n = 3)	R	MgrB truncated	64 or 128	>4	No	+	No
88	<i>K. pneumoniae</i>	R	Deletion of 11 nucleotides into <i>mgrB</i> gene	>128	>4	No	+	No
89	<i>K. pneumoniae</i>	R	blaCTX-M-15/ISEcp1 into <i>mgrB</i>	64	>4	No	+	No
90	<i>K. pneumoniae</i>	R	IS5 into <i>mgrB</i> gene	64	>4	No	+	No
91	<i>K. pneumoniae</i>	R	IS102 into <i>mgrB</i> gene	>128	>4	No	+	No
92	<i>K. pneumoniae</i>	R	ISKpn14 into <i>mgrB</i> gene	32	>4	No	+	No
93	<i>K. pneumoniae</i>	R	ISKpn13 into <i>mgrB</i> gene	128	>4	No	+	No
94	<i>K. pneumoniae</i>	R	ISKpn26 into <i>mgrB</i> gene	64	>4	No	+	No
95	<i>K. pneumoniae</i>	R	IS903 into <i>mgrB</i> gene	128	>4	No	+	No
96	<i>K. pneumoniae</i>	R	IS903b into <i>mgrB</i> gene	64	>4	No	+	No
97	<i>K. pneumoniae</i>	R	IS5 into <i>mgrB</i> gene	128	>4	No	+	No
98	<i>K. pneumoniae</i>	R	IS10R into <i>mgrB</i> promotor	128	>4	No	+	No
99	<i>K. pneumoniae</i>	R	ISKpn14 into <i>mgrB</i> promotor	32	>4	No	+	No
100	<i>K. pneumoniae</i>	R	CrrB N141Y	>128	>4	No	+	No
101	<i>K. pneumoniae</i>	R	CrrB P151L	>128	>4	No	+	No
102	<i>K. pneumoniae</i>	R	CrrB G183V	>128	>4	No	+	No
103	<i>K. pneumoniae</i>	R	Plasmid mediated <i>mcr-1</i> gene	16	4	No	+	No
104	<i>K. pneumoniae</i>	R	Unknown	16	>4	No	+	No
105	<i>K. pneumoniae</i>	R	Unknown	64	>4	No	+	No
106	<i>K. pneumoniae</i>	R	Unknown	32	>4	No	+	No
107	<i>K. pneumoniae</i>	R	Unknown	>128	>4	No	+	No
108	<i>K. pneumoniae</i>	R	Unknown	64	>4	No	+	No
109	<i>K. pneumoniae</i>	R	Unknown	64	>4	No	+	No
110	<i>K. pneumoniae</i>	R	Unknown	32	>4	No	+	No
111	<i>E. cloacae</i>	R	Unknown	32	>4	No	+	No
112	<i>E. cloacae</i>	R	Unknown	>128	>4	No	+	No
113	<i>E. cloacae</i>	R	Unknown	32	>4	No	+	No
114	<i>E. cloacae</i>	R	Unknown	>128	1	Yes, VME	+	No
115	<i>E. cloacae</i>	R	Unknown	64	≤0.5	Yes, VME	+	No
116	<i>E. cloacae</i>	R	Unknown	>128	≤0.5	Yes, VME	+	No
117	<i>E. cloacae</i>	R	Unknown	16	≤0.5	Yes, VME	+	No
118	<i>E. cloacae</i>	R	Unknown	>128	≤0.5	Yes, VME	+	No
119	<i>E. cloacae</i>	R	Unknown	>128	≤0.5	Yes, VME	+	No

Table 1 (continued)

Isolate	Species (no. of isolates)	Phenotype	Mechanism of resistance to colistin <sup>a</sup>	BMD	Phoenix	Discrepancies	Rapid Polymyxin NP test	
				MIC colistin (mg/L)	MIC colistin (mg/L)		Result	Discrepancies
120	<i>E. asburiae</i>	R	Unknown	>128	≤0.5	Yes, VME	+	No
121	<i>Salmonella enterica</i>	R	Plasmid mediated <i>mcr-1</i> gene	16	>4	No	+	No
122	<i>S. enterica</i>	R	Unknown	4	2	Yes, VME	+	No
123	<i>S. enterica</i>	R	Unknown	4	>4	No	+	No

BMD, broth microdilution; ME, major error (false resistance compared to the results obtained by broth microdilution reference method); MIC, minimum inhibitory concentration; NA, not applicable; R, resistant; S, susceptible; VME, very major error (false susceptibility compared to the results obtained by broth microdilution reference method).

<sup>a</sup> Unknown: no mutation in genes known to be involved in colistin resistance (*pmrA*, *pmrB*, *phoP*, *phoQ*, *mgrB* and *crrB* genes).

perform in routine laboratories because it requires qualified staff, is time-consuming and requires manual preparation of antibiotic solutions [5].

Automated dilution methods such as those performed by the BD Phoenix system could be an alternative for the screening of colistin resistance for laboratories that cannot perform manual BMD. However, to our knowledge, the performance of this automated testing for colistin susceptibility—especially its accuracy for the detection of isolates exhibiting a plasmid-mediated colistin resistance—has never been evaluated. Recently a rapid colourimetric test, the Rapid Polymyxin NP test, has been developed for detecting polymyxin resistance in *Enterobacteriaceae* within 2 hours [6].

The objective of this study was to evaluate the performance of the BD Phoenix automated system to detect plasmid-mediated and chromosomally encoded colistin resistance using a collection of clinical enterobacterial isolates. We also aimed to compare their performances to those of the Rapid Polymyxin NP test and the BMD reference method.

## Materials and methods

### Bacteria strains

This study was carried out using 123 nonduplicated clinical isolates of various enterobacterial species. The collection included 40 colistin-susceptible and 83 colistin-resistant isolates. Of the 83 colistin-resistant isolates, 16 belonged to a genus known to be naturally resistant to colistin (*Morganella*, *Proteus*, *Providencia*, *Serratia* and *Hafnia*), and 67 isolates belonged to the *Escherichia*, *Klebsiella*, *Enterobacter* or *Salmonella* genera with acquired resistance mechanisms to colistin. Identification of the isolates at the species level was performed using the Microflex benchtop matrix-assisted desorption ionization–time of flight mass spectrometer (Bruker, Champs-sur-Marne, France). Isolates were grown on Luria Bertani (GibcoBRL, Cergy Pontoise, France) or Mueller-Hinton (MH; bioMérieux, Marcy-l'Etoile, France) agar plates at 35 ± 2°C for 18 hours. The colistin-susceptible *Escherichia coli* ATCC 25922 strain was included in all experiments as a quality control.

### Susceptibility testing

#### Reference antimicrobial susceptibility testing

The BMD method was performed according to the EUCAST/CLSI joint guidelines (<http://www.eucast.org/>). Briefly, BMD panels were prepared extemporaneously in 96-well sterile polystyrene microplates (Sarstedt, Nümbrecht, Germany). Dilutions of colistin (Sigma-Aldrich, St Louis, MO, USA) ranging from 0.125 to 128 mg/L were made in cation-adjusted MH broth (Bio-Rad, Marnes-la-Coquette, France), without addition of polysorbate 80 (Tween 80) and with a final concentration of 5 × 10<sup>5</sup> CFU/mL of bacteria in each well. This procedure was performed in triplicate in separate experiments, and the minimum inhibitory concentrations (MICs)

were read after 16 to 20 hours of incubation at 35 ± 2°C in ambient air. Results were interpreted according to the EUCAST breakpoints [7], i.e. isolates with colistin MICs of ≤2 mg/L were categorized as susceptible although those with MICs of >2 mg/L were resistant.

#### BD Phoenix automated system

Colistin susceptibility testing was assessed using the BD Phoenix automated system (BD Phoenix 100; BD Diagnostic Systems, Le Pont de Claix, France), which performs an automated BMD method. The panel selected to perform this evaluation was the Gram-negative panel NMIC-93 using the BMD method for colistin concentrations ranging from 0.5 to 4 mg/L in order to cover the EUCAST breakpoints [7]. Bacterial suspension and panel inoculation were performed according to the manufacturer's guidelines. Panels were incubated for up to 16 hours at 35 ± 2°C under ambient air, and results were interpreted with BD EpiCenter software.

#### Rapid Polymyxin NP test

The Rapid Polymyxin NP test is based on the detection of the glucose metabolism related to bacterial growth in presence of a fixed concentration of colistin (3.75 mg/L) in cation-adjusted MH broth medium [6]. Formation of acid metabolites consecutive to the glucose metabolism is evidenced by a colour change (orange to yellow) of the pH indicator (red phenol). The test is positive (colistin resistance) if a strain grows in presence of colistin, whereas it is negative (colistin susceptibility) if a strain does not grow in presence of colistin. Results of the Rapid Polymyxin NP test were read at 2 hours of incubation at 35 ± 2°C in ambient air.

#### Molecular characterization of colistin resistance

Molecular mechanisms responsible for plasmid-mediated (*mcr-1* and *mcr-2* genes) and chromosomally encoded (*pmrA*, *pmrB*, *phoP*, *phoQ*, *mgrB* and *crrB* alterations) colistin resistance were determined as described previously [1,2,8–12].

#### Data analysis

The results obtained with the BD Phoenix system and the Rapid Polymyxin NP test were compared to those obtained with the reference BMD method. Discrepancies were determined for each method in order to assess their performance to detect colistin resistance. For strains for which discrepant susceptibility results were obtained, the isolates were retested with the three methods. Unsolved discrepancies were then maintained in the database for performance evaluation. Errors were ranked as follows: a very major error (VME) was defined when isolates were categorized as susceptible using the BD Phoenix system or the Rapid Polymyxin NP test but resistant by the BMD method (false-susceptible result), and a major error (ME) was defined when isolates were found to be resistant using the BD Phoenix system or the Rapid Polymyxin NP test but were found to be susceptible by using the BMD method

(false-resistant result). The number of resistant isolates and the number of susceptible isolates were used as denominators for VME and ME calculations, respectively. Acceptance criteria that provide requirements and specifications to evaluate performances of antimicrobial susceptibility test devices were those defined by the International Organization for Standardization standards (VME and ME must be  $\leq 3\%$ ) [13].

## Results

The features of the 123 enterobacterial isolates included in this study to evaluate the performance of the BD Phoenix system and Rapid Polymyxin NP test for determining colistin susceptibility are presented in Table 1.

Forty isolates defined as colistin susceptible according to the results of the BMD method (MICs of colistin ranging 0.12–2  $\mu\text{g}/\text{mL}$ ) were found susceptible by the BD Phoenix system (Table 1). A single susceptible *Klebsiella pneumoniae* isolate with a MIC of colistin of 2 mg/L was found to be resistant using the Rapid Polymyxin NP test. The MIC value of colistin for this same isolate as determined by the BD Phoenix system was underestimated (MIC  $\leq 0.5$  mg/L), but the isolate was well categorized as susceptible.

Of the 83 colistin-resistant enterobacterial isolates (MICs of colistin ranging from 4 to  $>128$  mg/L), the BD Phoenix system failed to detect colistin resistance for seven *Enterobacter* spp. isolates, a single *K. pneumoniae*, a single *Salmonella enterica* and a single *E. coli* isolate, whereas the Rapid Polymyxin NP test only failed to detect a single colistin-resistant *E. coli* isolate (Table 1). Identical results were obtained when those strains were repeatedly tested with the BD Phoenix system and the Rapid Polymyxin NP test, indicating good reproducibility of methods.

It is worth noting that 13 non-clonally-related colistin-resistant *E. coli*, one *K. pneumoniae* and one *S. enterica* isolate possessing the plasmid-mediated *mcr-1* gene were tested (MICs of colistin ranged 4–64 mg/L using the BMD method), and all were identified as resistant with the BD Phoenix system and the Rapid Polymyxin NP test. Similarly, the *E. coli* isolate possessing the plasmid-mediated *mcr-2* gene (MIC 4 mg/L) was detected by the two methods.

## Discussion

Of the 40 colistin-susceptible enterobacterial isolates, no ME (i.e. false resistance) was found with the BD Phoenix system, and only a single susceptible *K. pneumoniae* isolate with an MIC of colistin at 2 mg/L (and therefore just below the EUCAST breakpoint value of  $>2$  mg/L) was falsely identified as colistin resistant with the Rapid Polymyxin NP test, for an ME rate of 2.5%.

Of the 83 colistin-resistant enterobacterial isolates, the BD Phoenix system and the Rapid Polymyxin NP test showed excellent performances to detect the 13 isolates with plasmid-mediated colistin resistance, regardless of the level of resistance. However, ten VME (i.e. false susceptibility) were found with the BD Phoenix system, whereas a single VME was found with the Rapid Polymyxin NP test (Table 1). A high VME rate of 12% was thus found with the BD Phoenix system, whereas a low VME rate of 1.2% was found with the Rapid Polymyxin NP test. The single colistin-resistant *E. coli* isolate that was not detected with the BD Phoenix system and the Rapid Polymyxin NP test presented a low level of resistance (MIC of colistin at 8 mg/L). Its mechanism of colistin resistance remains unknown; neither chromosomally encoded mutations in genes known to be involved in lipopolysaccharide modifications—i.e. *mgrB*, *pmrAB* and *phoPQ* genes—nor plasmid-mediated *mcr-1* and *mcr-2* genes were detected.

The *S. enterica* isolate identified as susceptible (MIC 2 mg/L) with the BD Phoenix system presented a low level of colistin resistance

(MIC 4 mg/L), and its mechanism of resistance remains unknown (neither chromosomal mutations nor plasmid-mediated resistance).

The *K. pneumoniae*–resistant isolate and the seven *Enterobacter* spp.–resistant isolates not detected with the BD Phoenix system exhibited MIC values of colistin ranging from 16 to  $>128$  mg/L and were identified as colistin resistant with the Rapid Polymyxin NP test. During the determination of MICs by the BMD method, skipped wells (i.e. wells that exhibit no growth, although growth does occur at higher concentrations) were observed for 88% of those isolates (the *K. pneumoniae* isolate and six *Enterobacter* spp. isolates). This observation suggests that the failure of the BD Phoenix system to detect colistin resistance in those isolates could be related to a heteroresistance phenotype (defined by the presence of two subpopulations exhibiting different susceptibilities to colistin) [14]. The skipped wells observed during the MIC determination of those isolates by the BMD method are mainly for dilutions comprised between 0.125 and 4 mg/L. The BD Phoenix panel used in this study contained dilutions of colistin ranging from 0.5 to 4 mg/L. It is therefore likely that the failure of detection of heteroresistance for those isolates was linked to the absence of testing at higher colistin concentrations. The low sensitivity to detect colistin heteroresistance has already been described for another automated system, i.e. the bioMérieux VITEK system [15].

A limitation of our study is the absence of testing of non-fermenting Gram-negative rods in our collection.

## Conclusions

This study shows that the BD Phoenix system and the Rapid Polymyxin NP test are reliable tools for detection of plasmid-mediated colistin resistance (*mcr-1* and *mcr-2* genes), which is currently a major concern. However, the BD Phoenix system is not reliable for detection of colistin heteroresistance in enterobacterial isolates. Thus, we recommend the determination of MICs by the BMD method when susceptible results are obtained and if clinical use is required. In contrast, the Rapid Polymyxin NP test showed good agreement with the BMD method, and results were obtained rapidly (within 2 hours). However, the BMD method should be performed if determination of MIC values is necessary.

## Acknowledgements

We thank colleagues who provided polymyxin-resistant isolates: A. Brink, J. W. Decousser, J. Fernandez-Dominguez, J. Y. Madec, M. V. Villegas, M. Yilmaz and S. Kumar-Malhotra.

## Transparency declaration

Supported in part by Hospital Pellegrin, Bordeaux University Hospital (France) and the University of Fribourg (Switzerland). An international patent form has been filed on behalf of the University of Fribourg corresponding to the Rapid Polymyxin NP test.

## References

- [1] Liu YY, Wang Y, Walsh TR, Yi LX, Zhang R, Spencer J, et al. Emergence of plasmid-mediated colistin resistance mechanism MCR-1 in animals and human beings in China: a microbiological and molecular biological study. *Lancet Infect Dis* 2015;16:161–8.
- [2] Xavier BB, Lammens C, Ruhel R, Kumar-Singh S, Butaye P, Goossens H, et al. Identification of a novel plasmid-mediated colistin-resistance gene, *mcr-2*, in *Escherichia coli*, Belgium, June 2016. *Euro Surveill* 2016;21.
- [3] Skov RL, Monnet DL. Plasmid-mediated colistin resistance (*mcr-1* gene): three months later, the story unfolds. *Euro Surveill* 2016;21.
- [4] Poirel L, Jayol A, Nordmann P. Polymyxins: antibacterial activity, susceptibility testing, and resistance mechanisms encoded by plasmids or chromosomes. *Clin Microbiol Rev* 2017;30:557–96.

- [5] Jorgensen JH, Ferraro MJ. Antimicrobial susceptibility testing: a review of general principles and contemporary practices. *Clin Infect Dis* 2009;49: 1749–55.
- [6] Nordmann P, Jayol A, Poirel L. Rapid detection of polymyxin resistance in *Enterobacteriaceae*. *Emerg Infect Dis* 2016;22:1038–43.
- [7] European Committee on Antimicrobial Susceptibility Testing. Breakpoint tables for interpretation of MICs and zone diameters, version 1.0. Basel: EUCAST; 2016.
- [8] Jayol A, Poirel L, Villegas MV, Nordmann P. Modulation of *mgrB* gene expression as a source of colistin resistance in *Klebsiella oxytoca*. *Int J Antimicrob Agents* 2015;46:108–10.
- [9] Poirel L, Jayol A, Bontron S, Villegas MV, Ozdamar M, Turkoglu S, et al. The *mgrB* gene as a key target for acquired resistance to colistin in *Klebsiella pneumoniae*. *J Antimicrob Chemother* 2014;70:75–80.
- [10] Sun S, Negrea A, Rhen M, Andersson DI. Genetic analysis of colistin resistance in *Salmonella enterica* serovar *Typhimurium*. *Antimicrob Agents Chemother* 2009;53:2298–305.
- [11] Quesada A, Porrero MC, Tellez S, Palomo G, Garcia M, Dominguez L. Polymorphism of genes encoding PmrAB in colistin-resistant strains of *Escherichia coli* and *Salmonella enterica* isolated from poultry and swine. *J Antimicrob Chemother* 2015;70:71–4.
- [12] Cheng YH, Lin TL, Lin YT, Wang JT. Amino acid substitutions of CrrB responsible for resistance to colistin through CrrC in *Klebsiella pneumoniae*. *Antimicrob Agents Chemother* 2016;60:3709–16.
- [13] International Standard Organization. Clinical laboratory testing and in vitro diagnostic test systems. Susceptibility testing of infectious agents and evaluation of performance of antimicrobial susceptibility test devices. Part 2: evaluation of performance of antimicrobial susceptibility test devices. International Standard ISO 20776-2:2007. Geneva: ISO; 2007.
- [14] Landman D, Salamera J, Quale J. Irreproducible and uninterpretable polymyxin B MICs for *Enterobacter cloacae* and *Enterobacter aerogenes*. *J Clin Microbiol* 2013;51:4106–11.
- [15] Lo-Ten-Foe JR, de Smet AM, Diederer BM, Kluytmans JA, van Keulen PH. Comparative evaluation of the VITEK 2, disk diffusion, Etest, broth microdilution, and agar dilution susceptibility testing methods for colistin in clinical isolates, including heteroresistant *Enterobacter cloacae* and *Acinetobacter baumannii* strains. *Antimicrob Agents Chemother* 2007;51: 3726–30.

## **2. Evaluation des systèmes UMIC®, MicroScan® et Sensititre®**

### **Contexte :**

Suite à la publication de l'EUCAST du 22 mars 2016, les panels commerciaux de dilutions en milieu liquide comme les systèmes UMIC® (Biocentric), MicroScan® (Beckman Coulter) et Sensititre® (ThermoFisher Diagnostics) semblaient également être une alternative intéressante pour tester la sensibilité des souches à la colistine. Cependant, leurs performances pour la détection de la résistance à la colistine étaient peu connues voire complètement inconnues.

### **Objectif de l'étude :**

L'objectif de cette étude était donc de tester les performances des systèmes UMIC®, MicroScan® et Sensititre® pour la détection de la résistance à la colistine sur un panel de souches de BGN caractérisées au niveau moléculaire.

### **Matériel et méthodes :**

Nous avons testé 185 souches de BGN d'espèces variées, incluant 133 souches résistantes (résistances naturelles ou acquises, chromosomiques ou plasmidiques) et 52 souches sensibles à la colistine et nous avons comparé les résultats obtenus à ceux de la méthode de référence (dilutions en milieu liquide manuelles).

### **Principaux résultats :**

#### *Performances du système UMIC®*

Le système UMIC® a donné le taux le plus important de résultats de fausses sensibilités (11,3 %), comparé aux systèmes Sensititre® et MicroScan® (3 % et 0,8 %, respectivement). En effet, ce système n'a pas détecté la résistance de cinq souches (trois *E. coli*, une *K. pneumoniae* et une *S. enterica*) qui exprimaient des résistances plasmidiques (*mcr-1*, *mcr-2* ou *mcr-4-like*). Il n'a pas non plus détecté des résistances à la colistine de bas niveaux (CMI égales à 4 ou 8 mg/L selon la méthode de référence) chez deux souches de *Hafnia alvei*, une souche de *E. coli* et trois souches de *S. enterica* (CMI = 1 ou 2 mg/L selon le système UMIC®). Des résultats de fausses sensibilités ont également été obtenus avec ce système (CMI comprises entre 0,12 et 2 mg/L selon le système UMIC®) pour quatre souches de *S. maltophilia* présentant des résistances de haut niveau (CMI comprises entre 8 et 32 mg/L selon la méthode de référence).



### *Performances du système Microscan®*

Le système MicroScan® a détecté correctement toutes les souches de BGN résistantes à la colistine, à l'exception d'une souche de *A. baumannii* avec une CMI de la colistine égale à 128 mg/L (CMI selon le système MicroScan®  $\leq 2$  mg/L). Cependant, parmi les 20 souches de BGN non fermentants sensibles à la colistine, 13 souches ont été faussement détectées comme résistantes à la colistine. Une souche de *E. cloacae* a également été faussement détectée résistante.

### *Performances du système Sensititre®*

Le système Sensititre® a détecté correctement les 23 souches d'entérobactéries exprimant une résistance plasmidique à la colistine. Seules quatre souches présentant une hétérorésistance (deux souches de *Enterobacter* spp. et deux souches de *S. enterica*) ont faussement été identifiées comme sensibles à la colistine (CMI selon le système Sensititre® = 2 mg/L). A l'exception d'une souche de *P. aeruginosa*, les CMIs déterminées avec le système Sensititre® pour toutes les souches résistantes à la colistine étaient égales ou différaient seulement d'une dilution comparées aux CMIs obtenues avec la méthode de référence de dilutions en milieu liquide.

### **Conclusion :**

Le système UMIC® est un système unitaire facile à utiliser mais il ne détecte pas 11,3 % des souches résistantes à la colistine, et notamment les souches présentant des résistances plasmidiques (*mcr*). Etant donné les risques de diffusion de ces résistances plasmidiques, ces échecs de détection ne sont pas acceptables. Les performances du système MicroScan® sont excellentes pour tester la sensibilité à la colistine chez les entérobactéries mais les résistances détectées avec ce système chez les BGN non fermentants doivent être confirmées par une autre méthode (taux élevé de fausses résistances (26,9 %) chez les BGN non fermentants). Le système Sensititre® est le système commercial qui a donné les meilleurs résultats avec une catégorisation correcte des souches dans 97,8 % des cas. C'est donc le système qui semble le plus adapté pour la détermination des CMIs de la colistine pour les laboratoires ne pouvant pas réaliser des dilutions en milieu liquide par la méthode manuelle. Le système Sensititre® représente donc une alternative à la détermination des CMIs en milieu liquide par la méthode manuelle.

**Ce travail a été publié dans *Journal of Antimicrobial Chemotherapy* en mai 2018 (IF = 4.919).**



## Evaluation of three broth microdilution systems to determine colistin susceptibility of Gram-negative bacilli

Aurélie Jayol<sup>1–4</sup>, Patrice Nordmann<sup>1–3,5</sup>, Catherine André<sup>6</sup>, Laurent Poirel<sup>1–3\*</sup> and Véronique Dubois<sup>4,6</sup>

<sup>1</sup>Emerging Antibiotic Resistance Unit, Medical and Molecular Microbiology, Department of Medicine, University of Fribourg, Fribourg, Switzerland; <sup>2</sup>INSERM European Unit (LEA-IAME Paris, France), University of Fribourg, Fribourg, Switzerland; <sup>3</sup>National Reference Center for Emerging Antibiotic Resistance (NARA), University of Fribourg, Fribourg, Switzerland; <sup>4</sup>Laboratory of Bacteriology, University Hospital of Bordeaux, Bordeaux, France; <sup>5</sup>University of Lausanne and University Hospital Center, Lausanne, Switzerland; <sup>6</sup>CNRS UMR5234, University of Bordeaux, Bordeaux, France

\*Corresponding author. Medical and Molecular Microbiology, Department of Medicine, University of Fribourg, Chemin du Musée 18, CH-1700 Fribourg, Switzerland. Tel: +41-26-300-9582; E-mail: laurent.poirel@unifr.ch

Received 9 November 2017; returned 29 November 2017; revised 4 December 2017; accepted 2 January 2018

**Background:** The broth microdilution (BMD) method is currently the recommended technique to determine susceptibility to colistin.

**Objectives:** We evaluated the accuracy of three commercialized BMD panels [Sensititre (ThermoFisher Diagnostics), UMIC (Biocentric) and MicroScan (Beckman Coulter)] to determine colistin susceptibility.

**Methods:** A collection of 185 isolates of Gram-negative bacilli (133 colistin resistant and 52 colistin susceptible) was tested. Manual BMD according to EUCAST guidelines was used as the reference method, and EUCAST 2017 breakpoints were used for susceptibility categorization.

**Results:** The UMIC system gave the highest rate of very major errors (11.3%) compared with the Sensititre and MicroScan systems (3% and 0.8%, respectively). A high rate of major errors (26.9%) was found with the MicroScan system due to an overestimation of the MICs for the non-fermenting Gram-negative bacilli, whereas no major errors were found with the Sensititre and UMIC systems.

**Conclusions:** The UMIC system was easy to use, but failed to detect >10% of colistin-resistant isolates. The MicroScan system showed excellent results for enterobacterial isolates, but non-susceptible results for non-fermenters should be confirmed by another method and the range of MICs tested was narrow. The Sensititre system was the most reliable marketed BMD panel with a categorical agreement of 97.8%.

### Introduction

Occurrence of MDR Gram-negative bacilli is a growing concern and has led to a renewed interest in the use of polymyxins (colistin, polymyxin B) as last-resort antibiotics.<sup>1</sup> However, colistin susceptibility testing is currently challenging, with the disc-diffusion method and the Etest systems giving high rates of false-susceptibility results (up to 30%).<sup>2</sup> Since March 2016, the joint CLSI-EUCAST Polymyxin Breakpoints Working Group has recommended the broth microdilution (BMD) method as the reference method to determine susceptibility to colistin ([www.eucast.org](http://www.eucast.org)).<sup>3</sup> However, this method is often not implementable in routine practice due to a laborious manual preparation. Marketed BMD panels such as Sensititre (ThermoFisher Diagnostics, Dardilly, France), UMIC (Biocentric, Bandol, France) and MicroScan (Beckman Coulter, Villepinte, France) systems may be considered as interesting alternatives, but the performances of

these systems for detecting colistin resistance have not been carefully evaluated.

To date, only two studies have investigated the performance of the MicroScan system for colistin susceptibility testing. One study included *Acinetobacter* spp. isolates only and showed a categorical agreement (CA) of 87.3%,<sup>4</sup> while the second included *Klebsiella pneumoniae* isolates only and showed a susceptibility of 88.1%.<sup>5</sup> A single study has evaluated the Sensititre method, and a 96% CA with the reference BMD method was found, with no false-susceptibility results reported.<sup>6</sup> The performance of the UMIC system for determining colistin susceptibility has never been assessed.

The objective of this study was therefore to evaluate and compare the performances of the Sensititre system, the UMIC system and the MicroScan system for determining colistin susceptibility using a collection of 185 isolates of Gram-negative bacilli.

## Materials and methods

### Bacterial isolates

A collection of 185 Gram-negative isolates was tested. Fifty-two isolates were susceptible to colistin, 19 isolates belonged to a genus naturally resistant to colistin (*Proteus*, *Providencia*, *Morganella*, *Serratia* and *Hafnia*) and 114 isolates belonged to various species (*Klebsiella* spp., *Escherichia coli*, *Enterobacter* spp., *Salmonella enterica*, *Pseudomonas aeruginosa*, *Stenotrophomonas maltophilia* and *Acinetobacter baumannii*) and presented acquired resistance to colistin. The colistin-resistant isolates were collected worldwide from clinical samples. Identification was performed using the Microflex bench-top MALDI-TOF mass spectrometer (Bruker, Champs-sur-Marne, France). None of the strains was clonally related.

### Molecular genotyping for colistin resistance

Colistin-resistant isolates were screened for the plasmid-mediated colistin resistance genes *mcr-1*, *mcr-2*, *mcr-3* and *mcr-4* as described previously.<sup>7-11</sup> Chromosomally encoded mutations in genes potentially involved in colistin resistance (*pmrA*, *pmrB*, *phoP*, *phoQ*, *mgrB* and *crpB* genes) were also searched for as described previously.<sup>12-18</sup>

### Colistin susceptibility testing

#### Preparation of the BMD panels

The features of the various commercialized panels used in this study are summarized in Table 1. Each isolate was tested with the four techniques in the same experiment and from the same starting bacterial suspension with a turbidity equivalent to that of a 0.5 McFarland standard. The panels were prepared as recommended by each manufacturer. The BMD reference method was performed according to the EUCAST guidelines (www.eucast.org) in 96-well non-treated polystyrene microplates (ref. 82.1582.001; Sarstedt, Nümbrecht, Germany). Dilutions of colistin sulfate (ref. C4461; Sigma-Aldrich, St Louis, MO, USA) ranging from 0.12 to 128 mg/L were made extemporaneously in CAMHB (ref. YT3462; ThermoFisher Diagnostics), without addition of polysorbate 80 (Tween 80), and a final concentration of  $5 \times 10^5$  cfu/mL of bacteria was added to each well. All the BMD panels were read visually after 18–20 h of incubation at  $35 \pm 2^\circ\text{C}$ . The colistin-susceptible

ATCC 25922 *E. coli* and ATCC 27853 *P. aeruginosa* strains were included in all the experiments as quality controls.

### Analysis of the results

It should be noted that all inspections were visual and not automated. Categorization of the isolates was performed on the basis of the EUCAST susceptible and resistant breakpoints ( $\leq 2$  and  $> 2$  mg/L, respectively). For *S. maltophilia* isolates, the same breakpoint of 2 mg/L was arbitrarily chosen, given the lack of EUCAST breakpoints.

Results obtained with the three commercialized BMD panels were compared with those obtained with the BMD reference method. Discrepancies were determined for each method in order to assess how accurately they determined susceptibility to colistin. Isolates for which discrepant susceptibility results were observed were retested twice with the four methods. Unsolved discrepancies were then maintained in the database for performance evaluation. Errors were ranked as follows: very major errors (VMEs), for isolates categorized as susceptible using the marketed panel, but resistant by the BMD reference method (false-susceptibility result); and major errors (MEs), for isolates categorized as resistant using the marketed panel, but susceptible by the BMD reference method (false-resistant result). The number of resistant and susceptible isolates were used as denominators for VME and ME calculations, respectively. CA was defined as the percentage of isolates classified into the same category by the commercialized panel compared with the BMD reference method. Acceptance criteria that provide the requirements and specifications to evaluate performances of antimicrobial susceptibility test devices were those defined by the ISO standards (VMEs and MEs must be  $\leq 3\%$  and CA must be  $\geq 90\%$ ).<sup>19</sup>

## Results

The 133 colistin-resistant Gram-negative isolates analysed in this study presented various levels of resistance (MICs ranging from 4 to  $> 128$  mg/L by using the BMD reference method) (Table 2).

The tested isolates exhibited various genotypes conferring colistin resistance, i.e. related to various chromosomal mutations, and/or acquisition of plasmid-mediated genes (Table 2). Thirty-five *K. pneumoniae* isolates presented mutations in *pmrAB*, *phoPQ*,

**Table 1.** Features of the BMD panels

	Sensititre	UMIC	MicroScan
Manufacturer	ThermoFisher Diagnostics	Biocentric	Beckman Coulter
Reference of the panel	FRCOL (custom plate)	UM-COL-040	NM44
Range of colistin concentrations tested (mg/L)	0.12–128	0.06–64	2–4
Description of the panel	96-well microplate to test only colistin <sup>a</sup>	12-well panel to test only colistin	96-well microplate: 2 wells to test colistin, the others to test additional antibiotics <sup>a</sup>
Number of strains tested by panel	8	1	1
Medium	CAMHB with TES (ref. YT3462)	CAMHB	water with PLURONIC
Inoculation	manual or semi-automated with the AIM <sup>TM</sup> automated inoculation delivery system	manual	semi-automated with the RENOK inoculator system
Incubation time (h)	18–24	18–24	16–20
Reading	visual, semi-automated with Vizion <sup>®</sup> or automated with Optiread <sup>®</sup>	visual	visual, semi-automated with autoSCAN-4 or automated with the WalkAway plus system

<sup>a</sup>Other panels testing colistin are also available.

**Table 2.** MIC results obtained with the four BMD methods

Species (number of isolates)	Genotype	Colistin MIC (mg/L)			
		reference BMD	Sensititre	UMIC	MicroScan
Isolates naturally resistant to colistin					
<i>Proteus mirabilis</i> (n = 1), <i>Proteus vulgaris</i> (n = 1), <i>Providencia stuartii</i> (n = 1), <i>Morgenalla morgani</i> (n = 1), <i>Serratia marcescens</i> (n = 1)	NA	>128	>128	>64	>4
<i>H. alvei</i> (n = 2)	NA	8	4–8	<b>2</b>	4 to >4
<i>H. alvei</i> (n = 1)	NA	8	8	4–8	>4
<i>H. alvei</i>	NA	16	16	16	>4
<i>Hafnia paralvei</i>	NA	4	4	4	4
<i>H. paralvei</i> (n = 9)	NA	8	4–8	4–8	>4
Isolates with acquired colistin resistance					
<i>K. pneumoniae</i>	plasmid-mediated <i>mcr-1</i> gene	8	4	<b>2</b>	>4
<i>K. pneumoniae</i>	PmrA G53C	32	16	32	>4
<i>K. pneumoniae</i>	PmrA G53S	16	16	16	>4
<i>K. pneumoniae</i>	PmrA G53S	32	16	16	>4
<i>K. pneumoniae</i>	PmrB T157P	8	8	8	>4
<i>K. pneumoniae</i>	PmrB T157P	16	8	16	>4
<i>K. pneumoniae</i>	PhoP D191Y	64	32	64	>4
<i>K. pneumoniae</i>	PhoQ R16C	128	64	64	>4
<i>K. pneumoniae</i>	MgrB C28 (MgrB truncated)	128	64	64	>4
<i>K. pneumoniae</i> (n = 2)	MgrB Q30 (MgrB truncated)	64	32	64	>4
<i>K. pneumoniae</i>	MgrB W47 (MgrB truncated)	32	16	16	>4
<i>K. pneumoniae</i>	MgrB W20R	16	16	16	>4
<i>K. pneumoniae</i>	MgrB M27K	16	16	32	>4
<i>K. pneumoniae</i>	MgrB C39Y	4	4	8	>4
<i>K. pneumoniae</i>	MgrB N42Y and K43I	4	4	8	>4
<i>K. pneumoniae</i>	MgrB I45T	32	32	16	>4
<i>K. pneumoniae</i>	MgrB P46S	16	8	32	>4
<i>K. pneumoniae</i>	MgrB ISEcp1/blaCTX-M-15	32	16	32	>4
<i>K. pneumoniae</i>	IS10R in <i>mgrB</i> promotor	64	64	64	>4
<i>K. pneumoniae</i>	ISKpn14 in <i>mgrB</i> promotor	32	32	16	>4
<i>K. pneumoniae</i>	IS102-like in <i>mgrB</i> gene	128	64	>64	>4
<i>K. pneumoniae</i>	IS5-like in <i>mgrB</i> gene	32	16	32	>4
<i>K. pneumoniae</i>	IS5-like in <i>mgrB</i> gene	32	16	64	>4
<i>K. pneumoniae</i>	ISKpn13 in <i>mgrB</i> gene	64	32	>64	>4
<i>K. pneumoniae</i>	ISKpn14 in <i>mgrB</i> gene	8	8	16	>4
<i>K. pneumoniae</i>	IS903b-like in <i>mgrB</i> gene	64	32	64	>4
<i>K. pneumoniae</i>	<i>mgrB</i> Δnt23 (frameshift)	8	8	8	>4
<i>K. pneumoniae</i>	<i>mgrB</i> Δnt74 (frameshift)	32	32	64	>4
<i>K. pneumoniae</i>	<i>mgrB</i> Δnt100 (frameshift)	32	16	32	>4
<i>K. pneumoniae</i>	<i>mgrB</i> Δnt23/33 (frameshift)	128	64	>64	>4
<i>K. pneumoniae</i> (n = 2)	Δ <i>mgrB</i>	32	16	32	>4
<i>K. pneumoniae</i>	CrrB N141Y	128	64	>64	>4
<i>K. pneumoniae</i>	CrrB P151L	>128	>128	>64	>4
<i>K. pneumoniae</i>	CrrB G183V	32	32	>64	>4
<i>K. pneumoniae</i>	—	4	4	8	>4
<i>K. pneumoniae</i>	—	8	4	4	>4
<i>K. pneumoniae</i>	—	16	16	16	>4
<i>K. pneumoniae</i>	—	64	32	32	>4
<i>K. pneumoniae</i>	—	128	64	>64	>4
<i>Klebsiella oxytoca</i>	ISKpn26-like in <i>mgrB</i> promotor	32	16	32	>4

Continued

**Table 2.** *Continued*

Species (number of isolates)	Genotype	Colistin MIC (mg/L)			
		reference BMD	Sensititre	UMIC	MicroScan
<i>E. coli</i>	plasmid-mediated <i>mcr-1</i> gene	4	4	<b>2</b>	>4
<i>E. coli</i>	plasmid-mediated <i>mcr-1</i> gene	8	4	<b>2</b>	>4
<i>E. coli</i> (n = 13)	plasmid-mediated <i>mcr-1</i> gene	8	4–8	4–8	>4
<i>E. coli</i> (n = 2)	plasmid-mediated <i>mcr-1</i> gene	16	8–16	8	>4
<i>E. coli</i>	plasmid-mediated <i>mcr-1</i> gene	64	64	32	>4
<i>E. coli</i>	plasmid-mediated <i>mcr-2</i> gene	4	4	<b>2</b>	>4
<i>E. coli</i>	PmrA G15R	8	8	8	>4
<i>E. coli</i>	PmrB R11L	4	4	<b>2</b>	>4
<i>E. coli</i>	PmrB L13P	16	8	8	>4
<i>E. coli</i>	PmrB G19E	4	4	4	4
<i>E. coli</i>	PmrB G22E	8	8	4	>4
<i>E. coli</i>	PmrB T114P	4	4	4	>4
<i>E. coli</i>	PmrB R138H	8	4	8	>4
<i>E. coli</i>	PmrB R138H, G305R	8	4	8	>4
<i>E. coli</i>	PmrB D152V	8	8	4	>4
<i>E. coli</i>	PmrB D315N	16	16	8	>4
<i>E. coli</i>	PmrB T114P+plasmid-mediated <i>mcr-1</i> gene	16	16	16	>4
<i>E. coli</i> (n = 4)	—	8	4	4	>4
<i>E. coli</i>	—	16	16	8	>4
<i>E. cloacae</i>	—	4	<b>2</b>	4	4
<i>E. cloacae</i>	—	8	8	16	>4
<i>E. cloacae</i> (n = 2)	—	16	8	16	>4
<i>E. cloacae</i>	—	16	16	32	>4
<i>E. cloacae</i>	—	64	32	>64	4
<i>E. cloacae</i> (n = 2)	—	>128	>128	>64	>4
<i>E. cloacae</i>	—	>128	>128	>64	>4
<i>E. aerogenes</i>	—	4	<b>2</b>	4	>4
<i>E. aerogenes</i>	—	4	4	4	4
<i>S. enterica</i> <sup>a</sup>	ArcA T111P, EnvZ Del13nt	4	<b>2</b>	<b>2</b>	4
<i>S. enterica</i> <sup>a</sup>	<i>pmrD</i> gene amplification, ArcA V9L, MurD S161T	4	<b>2</b>	<b>2</b>	>4
<i>S. enterica</i>	—	4	4	<b>1</b>	>4
<i>S. enterica</i>	plasmid-mediated <i>mcr-4-like</i> gene	4	4	<b>2</b>	>4
<i>S. enterica</i>	—	4	4	4	>4
<i>S. enterica</i>	plasmid-mediated <i>mcr-1</i> gene	16	8	8	>4
<i>S. enterica</i> <sup>a</sup>	<i>pmrD</i> gene amplification, ArcA L50Q, MurD D418Y, FliM L54Q	64	32	32	>4
<i>P. aeruginosa</i>	—	16	8	16	>4
<i>P. aeruginosa</i>	—	64	16	>64	>4
<i>P. aeruginosa</i>	—	128	64	>64	>4
<i>S. maltophilia</i>	—	4	4	8	>4
<i>S. maltophilia</i>	—	8	4	<b>1</b>	>4
<i>S. maltophilia</i>	—	8	16	<b>2</b>	>4
<i>S. maltophilia</i>	—	16	16	<b>0.12</b>	>4
<i>S. maltophilia</i>	—	32	16	<b>0.5</b>	>4
<i>S. maltophilia</i>	—	32	16	64	>4
<i>S. maltophilia</i>	—	64	32	4	>4
<i>S. maltophilia</i>	—	128	64	>64	>4
<i>A. baumannii</i>	PmrA E8D, PmrB R263P	128	64	64	≤ <b>2</b>
<i>A. baumannii</i>	PmrA E54G, PmrB E140V	>128	>128	>64	>4

*Continued*

**Table 2.** Continued

Species (number of isolates)	Genotype	Colistin MIC (mg/L)			
		reference BMD	Sensititre	UMIC	MicroScan
<i>A. baumannii</i>	PmrB I163S	32	32	32	>4
<i>A. baumannii</i>	PmrB P170L	16	16	32	>4
<i>A. baumannii</i>	PmrB G260D	>128	>128	>64	>4
<i>A. baumannii</i>	PmrB Q265P	>128	>128	>64	>4
<i>A. baumannii</i>	—	16	16	16	>4
<i>A. baumannii</i>	—	>128	>128	>64	>4
Colistin-susceptible isolates					
<i>K. pneumoniae</i> (n = 5)	NA	≤0.12–0.25	0.5	0.25–0.5	≤2
<i>K. pneumoniae</i>	NA	1	1	0.5	≤2
<i>K. oxytoca</i>	NA	≤0.12	0.25–0.5	0.12–0.25	≤2
<i>E. coli</i> (n = 5)	NA	≤0.12	0.25–0.5	0.12–0.5	≤2
<i>E. coli</i> (n = 6)	NA	0.25	0.25–0.5	0.25–0.5	≤2
<i>E. coli</i> (n = 2)	NA	0.5	0.5	0.25–0.5	≤2
<i>E. cloacae</i> (n = 4)	NA	≤0.12	0.25–0.5	0.12–0.5	≤2
<i>E. cloacae</i>	NA	0.25	0.5	0.25	<b>4</b>
<i>E. cloacae</i>	NA	0.25	0.5	0.5	≤2
<i>E. cloacae</i>	NA	0.5	0.5	0.5	≤2
<i>E. aerogenes</i> (n = 2)	NA	≤0.12	0.5	0.12–0.25	≤2
<i>S. enterica</i>	NA	0.5	1	0.5	≤2
<i>S. enterica</i>	NA	2	2	2	≤2
<i>Citrobacter koseri</i>	NA	0.25	0.5	0.5	≤2
<i>P. aeruginosa</i> (n = 2)	NA	0.25	0.5	0.12–0.25	≤2
<i>P. aeruginosa</i> (n = 8)	NA	1	1–2	1	<b>4</b>
<i>P. aeruginosa</i> (n = 2)	NA	1	2	1	≤2
<i>P. aeruginosa</i>	NA	2	2	1	≤2
<i>S. maltophilia</i>	NA	0.5	0.5	0.5	≤2
<i>S. maltophilia</i>	NA	1	1	1	> <b>4</b>
<i>S. maltophilia</i>	NA	1	2	0.12	<b>4</b>
<i>A. baumannii</i>	NA	0.5	1	2	<b>4</b>
<i>A. baumannii</i> (n = 2)	NA	1	1–2	0.5	<b>4</b>
<i>A. baumannii</i>	NA	1	1	0.5	≤2

NA, not applicable.

The discordant results compared with the reference method are in bold.

<sup>a</sup>These *S. enterica* strains were genotyped by Hjort et al.<sup>22</sup>

*mgrB* or *crrB* genes, and 11 *E. coli* and 6 *A. baumannii* isolates exhibited mutations in *pmrAB* genes. Twenty-three enterobacterial isolates recovered worldwide carried plasmid-mediated colistin resistance *mcr-1*, *mcr-2* or *mcr-4-like* genes.

### Comparison of the UMIC system with the BMD reference method

The UMIC system did not detect two colistin-resistant *Hafnia alvei* isolates that presented low MIC values of colistin (8 mg/L). It failed also to detect five isolates (three *E. coli*, a single *K. pneumoniae* and a single *Salmonella enterica*) possessing plasmid-mediated colistin resistance genes (*mcr-1*, *mcr-2* or *mcr-4-like*) and exhibiting a low level of resistance (MICs from 4 to 8 mg/L). Several enterobacterial isolates (a single *E. coli* and three *S. enterica* isolates) also exhibiting low MICs of colistin (MIC of 4 mg/L), but lacking the plasmid-mediated *mcr-1*, *mcr-2*, *mcr-3* and *mcr-4* genes, were

also falsely detected as susceptible by the UMIC system. For all these isolates, the UMIC system gave MICs of 1 or 2 mg/L. Finally, four *S. maltophilia* isolates with higher colistin MICs (ranging from 8 to 32 mg/L) were not detected as resistant by the UMIC system, which gave lower MICs ranging from 0.12 to 2 mg/L. For one *S. maltophilia* isolate, the CA was correct, but the colistin MIC found with the UMIC system was 4 mg/L, while the MIC was actually 64 mg/L by manual BMD.

### Comparison of the Sensititre system with the BMD reference method

This comparison showed a high rate of agreement. The Sensititre system detected correctly the 19 isolates naturally resistant to colistin and all the 23 enterobacterial isolates harbouring plasmid-mediated colistin resistance. Except for a single *P. aeruginosa* isolate, the MICs determined by the Sensititre system for all the resistant isolates were

equal or only differed by one dilution when compared with those determined by the BMD reference method. However, two *Enterobacter* spp. and two *S. enterica* isolates presenting low MICs (4 mg/L) were falsely detected as susceptible by the Sensititre system, which gave MICs of 2 mg/L.

**Comparison of the MicroScan system with the BMD reference method**

The MicroScan system detected all the colistin-resistant isolates, except a single *A. baumannii* isolate showing an MIC of colistin of 128 mg/L, whereas the MicroScan system gave an MIC of ≤2 mg/L. However, 13 out of the 20 colistin-susceptible non-fermenters (13 *P. aeruginosa*, 3 *S. maltophilia* and 4 *A. baumannii*) were found resistant to colistin with the MicroScan system. Moreover, a single *Enterobacter cloacae* isolate was also found falsely resistant with an MIC of 4 mg/L.

**Discussion**

In this study, we evaluated three marketed BMD panels (Sensititre, UMIC and MicroScan) to determine MICs of colistin for a collection of 185 isolates of Gram-negative bacilli.

The UMIC system is a novel BMD panel for colistin susceptibility testing. Its advantages are the absence of any need for specific equipment and the form of the panel allowing colistin susceptibility to be tested for a single strain. However, the UMIC system failed to detect 15 isolates among the 133 colistin-resistant isolates, giving a high rate of VMEs (11.3%) (Table 3). It failed to detect colistin resistance in two *H. alvei* isolates (Table 2). In fact, we recently showed that the *H. alvei* species exhibits intrinsic resistance to colistin, though the resistance was of low level (MICs ranging from 4 to 16 mg/L).<sup>20</sup> UMIC failed also to detect nine other enterobacterial isolates with low-level resistance to colistin (MICs ranging from 4 to 8 mg/L), whereas the MICs of colistin reported with this system

were close to the breakpoints (MICs of 1 or 2 mg/L, respectively) (Table 2). Five of those isolates possessed a plasmid-mediated colistin resistance determinant (*mcr-1*, *mcr-2* or *mcr-4-like*). This mis-detection could underestimate the carriage of plasmid-mediated colistin resistance isolates, and thus participate in the spread of this resistance trait by delaying the rapid implementation of adequate hygiene measures. For those enterobacterial isolates with MICs of 1 or 2 mg/L, a more sensitive method should be used for confirmation, such as the BMD reference method or the newly developed rapid test (Rapid Polymyxin NP test).<sup>21</sup> The UMIC system also widely underestimated the MICs for isolates belonging to the *S. maltophilia* species, and failed to detect high-level resistance (MICs from 8 to 32 mg/L).

The performance of the Sensititre system was much better, but this system failed to detect four colistin-resistant isolates (one *E. cloacae* isolate, one *Enterobacter aerogenes* isolate and two *S. enterica* isolates) (Table 2), giving an acceptable VME rate of 3% (Table 3). The two tested *Salmonella* isolates not detected as colistin resistant were characterized as heteroresistant by Hjort *et al.*<sup>22</sup> This lack of detection could therefore be due to the presence of colistin-resistant subpopulations. Guérin *et al.*<sup>23</sup> also showed that some subpopulations of *E. cloacae* isolates may exhibit heteroresistance to colistin, which could explain a misdetection of that resistance for one of the isolates in our study. Of the 133 colistin-resistant isolates, the Sensititre system detected 129, underlining the accuracy of this system for detecting colistin resistance with a high CA of 97.8%. However, the main disadvantage is that this system is not adapted to test only one strain (at least eight strains need to be tested per panel) (Table 1).

The performance of the MicroScan system for detection of colistin resistance in Gram-negative bacilli was excellent regardless of the nature of the resistance mechanism. Only a single colistin-resistant *A. baumannii* isolate was not detected (Table 2), giving the lowest VME rate of 0.8% (Table 3). The main inconvenience of this panel was the narrow range of colistin concentrations tested, the absence of a panel to test colistin only, and the high rate of

**Table 3.** Performances of the BMD panels

		Sensititre		UMIC		MicroScan <sup>a</sup>	
						all the isolates	all isolates excluding <i>E. cloacae</i> , <i>Salmonella</i> , <i>Acinetobacter</i> and <i>Stenotrophomonas</i> isolates <sup>a</sup>
VME rate	Enterobacteriaceae	3.5% (4/114)	Enterobacteriaceae	9.6% (11/114)	Enterobacteriaceae	0% (0/114)	0% (0/105)
	NFGNB	0% (0/19)	NFGNB	21.1% (4/19)	NFGNB	5.3% (1/19)	0% (0/3)
	all species	3.0% (4/133)	all species	11.3% (15/133)	all species	0.8% (1/133)	0% (0/98)
ME rate	Enterobacteriaceae	0% (0/32)	Enterobacteriaceae	0% (0/32)	Enterobacteriaceae	3.1% (1/32)	0% (0/23)
	NFGNB	0% (0/20)	NFGNB	0% (0/20)	NFGNB	65.0% (13/20)	61.5% (8/13)
	all species	0% (0/52)	all species	0% (0/52)	all species	26.9% (14/52)	22.2% (8/36)
CA rate	Enterobacteriaceae	97.3% (142/146)	Enterobacteriaceae	92.5% (135/146)	Enterobacteriaceae	99.3% (145/146)	100% (128/128)
	NFGNB	100% (39/39)	NFGNB	89.7% (35/39)	NFGNB	64.1% (25/39)	50% (8/16)
	all species	97.8% (181/185)	all species	91.9% (170/185)	all species	91.9% (170/185)	94.4% (136/144)

NFGNB, non-fermenting Gram-negative bacteria.

<sup>a</sup>The procedural manual of the MicroScan system indicates that results for *E. cloacae*, *Salmonella* and non-Enterobacteriaceae (except *Pseudomonas*) should not be reported for colistin.



false-resistance results found for non-fermenters (65%) (Table 3). This finding supports a previous report showing a high rate of false-resistance results in *Acinetobacter* species.<sup>4</sup> The global rate of MEs for the MicroScan system was thus 26.9%, whereas Sensititre and UMIC systems did not give MEs (Table 3). However, the procedural manual of the MicroScan panel indicates that results for *E. cloacae*, *Salmonella* and non-fermenting Gram-negative bacilli except *Pseudomonas* spp. should not be reported, and hence the MICs and categorization results are not provided for those species. By excluding the results for those species, the ME rate was lower (22.2% instead of 26.9%) (Table 3), but still not acceptable (>3%) because of a high rate of false resistance found for *P. aeruginosa* isolates (8/13). Therefore, non-susceptibility results for non-fermenters including *P. aeruginosa* should be confirmed by the BMD reference method.

## Conclusions

This study showed that variable results of colistin MICs can occur depending on the BMD panels used. It revealed that the UMIC system is not reliable for detection of colistin resistance, especially for isolates with a low level of colistin resistance and for *S. maltophilia* isolates. The performance of the MicroScan system was excellent, but this system is not suited for testing the colistin susceptibility of non-fermenters because of a high rate of false resistance. The Sensititre system showed excellent concordance with the BMD reference method and was reliable for testing colistin susceptibility for all the species of Gram-negative bacilli tested.

## Acknowledgements

We thank colleagues who provided polymyxin-resistant isolates, namely Drs D. I. Andersson, A. Brink, J. W. Decousser, J. Fernandez-Dominguez, J. Y. Madec, M. V. Villegas, M. Yilmaz, H. Nicoloff and S. Kumar-Malhotra.

We are very grateful to ThermoFisher Diagnostics and Beckman Coulter for providing the Sensititre and MicroScan panels.

## Funding

This work was financed by grants from the University of Bordeaux (France) and the University of Fribourg (Switzerland) and from the Swiss National Reference Center for Emerging Antibiotic Resistance (NARA).

## Transparency declarations

None to declare.

## References

- Falagas ME, Rafailidis PI, Ioannidou E et al. Colistin therapy for microbiologically documented multidrug-resistant Gram-negative bacterial infections: a retrospective cohort study of 258 patients. *Int J Antimicrob Agents* 2010; **35**: 194–9.
- Humphries RM. Susceptibility testing of the polymyxins: where are we now? *Pharmacotherapy* 2014; **35**: 22–7.
- EUCAST. *Breakpoint Tables for Interpretation of MICs and Zone Diameters, Version 6.0, 2016*.
- Lee SY, Shin JH, Lee K et al. Comparison of the Vitek 2, MicroScan, and Etest methods with the agar dilution method in assessing colistin susceptibility of bloodstream isolates of *Acinetobacter* species from a Korean university hospital. *J Clin Microbiol* 2013; **51**: 1924.
- Perez LR. Evaluation of polymyxin susceptibility profile among KPC-producing *Klebsiella pneumoniae* using Etest and MicroScan WalkAway automated system. *APMIS* 2015; **123**: 951–4.
- Hindler JA, Humphries RM. Colistin MIC variability by method for contemporary clinical isolates of multidrug-resistant Gram-negative bacilli. *J Clin Microbiol* 2013; **51**: 1678–84.
- Liu YY, Wang Y, Walsh TR et al. Emergence of plasmid-mediated colistin resistance mechanism MCR-1 in animals and human beings in China: a microbiological and molecular biological study. *Lancet Infect Dis* 2015; **16**: 161–8.
- Xavier BB, Lammens C, Ruhel R et al. Identification of a novel plasmid-mediated colistin-resistance gene, *mcr-2*, in *Escherichia coli*, Belgium, June 2016. *Euro Surveill* 2016; **21**: pii=30280.
- Poirel L, Jayol A, Nordmann P. Polymyxins: antibacterial activity, susceptibility testing, and resistance mechanisms encoded by plasmids or chromosomes. *Clin Microbiol Rev* 2017; **30**: 557–96.
- Carattoli A, Villa L, Feudi C et al. Novel plasmid-mediated colistin resistance *mcr-4* gene in *Salmonella* and *Escherichia coli*, Italy 2013, Spain and Belgium, 2015 to 2016. *Euro Surveill* 2017; **22**: pii=30589.
- Yin W, Li H, Shen Y et al. Novel plasmid-mediated colistin resistance gene *mcr-3* in *Escherichia coli*. *mBio* 2017; **8**: e00543-17.
- Poirel L, Jayol A, Bontron S et al. The *mgrB* gene as a key target for acquired resistance to colistin in *Klebsiella pneumoniae*. *J Antimicrob Chemother* 2014; **70**: 75–80.
- Jayol A, Poirel L, Villegas MV et al. Modulation of *mgrB* gene expression as a source of colistin resistance in *Klebsiella oxytoca*. *Int J Antimicrob Agents* 2015; **46**: 108–10.
- Cheng YH, Lin TL, Lin YT et al. Amino acid substitutions of CrrB responsible for resistance to colistin through CrrC in *Klebsiella pneumoniae*. *Antimicrob Agents Chemother* 2016; **60**: 3709–16.
- Lee JY, Ko KS. Mutations and expression of PmrAB and PhoPQ related with colistin resistance in *Pseudomonas aeruginosa* clinical isolates. *Diagn Microbiol Infect Dis* 2014; **78**: 271–6.
- Sun S, Negrea A, Rhen M et al. Genetic analysis of colistin resistance in *Salmonella enterica* serovar Typhimurium. *Antimicrob Agents Chemother* 2009; **53**: 2298–305.
- Quesada A, Porrero MC, Tellez S et al. Polymorphism of genes encoding PmrAB in colistin-resistant strains of *Escherichia coli* and *Salmonella enterica* isolated from poultry and swine. *J Antimicrob Chemother* 2015; **70**: 71–4.
- Beceiro A, Llobet E, Aranda J et al. Phosphoethanolamine modification of lipid A in colistin-resistant variants of *Acinetobacter baumannii* mediated by the PmrAB two-component regulatory system. *Antimicrob Agents Chemother* 2011; **55**: 3370–9.
- International Organization for Standardization (ISO). *Clinical Laboratory Testing and In Vitro Diagnostic Test Systems—Susceptibility Testing of Infectious Agents and Evaluation of Performance of Antimicrobial Susceptibility Test Devices—Part 2: Evaluation of Performance of Antimicrobial Susceptibility Test Devices*. International Standard ISO 20776-2:2007. Geneva, Switzerland: ISO, 2007.
- Jayol A, Saly M, Nordmann P et al. *Hafnia*, an enterobacterial genus naturally resistant to colistin revealed by three susceptibility testing methods. *J Antimicrob Chemother* 2017; **72**: 2507–11.
- Nordmann P, Jayol A, Poirel L. Rapid detection of polymyxin resistance in *Enterobacteriaceae*. *Emerg Infect Dis* 2016; **22**: 1038–43.
- Hjort K, Nicoloff H, Andersson DI. Unstable tandem gene amplification generates heteroresistance (variation in resistance within a population) to colistin in *Salmonella enterica*. *Mol Microbiol* 2016; **102**: 274–89.
- Guérin F, Isnard C, Sinel C et al. Cluster-dependent colistin heteroresistance in *Enterobacter cloacae* complex. *J Antimicrob Chemother* 2016; **71**: 3058–61.

## **C. Epidémiologie de la résistance à la colistine chez les BGN**

### **1. Détermination du taux de résistance à la colistine chez les entérobactéries productrices de carbapénèmases en France**

#### **Contexte :**

Lors de l'initiation de ce travail, la prévalence de la résistance à la colistine chez les entérobactéries multirésistantes aux antibiotiques était inconnue en France. Par ailleurs, des études réalisées dans d'autres pays d'Europe révélaient des prévalences très variables (9), mais les méthodes utilisées dans ces études pour déterminer la résistance n'étaient pas toujours optimales (sous-estimation du taux de résistance avec la méthode des disques ou les bandelettes Etest®). D'autre part, des épidémies avec des souches de *K. pneumoniae* productrices de carbapénèmases et résistantes à la colistine ayant été décrites dans plusieurs pays d'Europe et en Amérique du Nord (9), nous souhaitions déterminer si de telles épidémies avaient eu lieu en France.

#### **Objectif de l'étude :**

L'objectif de cette étude était donc de déterminer rétrospectivement le taux de résistance à la colistine chez les souches d'entérobactéries productrices de carbapénèmases reçues au Centre National de Référence (CNR) des résistances aux antibiotiques en France en 2014 (directeur : Pr P. Nordmann) et de déterminer si ces souches étaient reliées clonalement.

#### **Matériel et méthodes :**

Nous avons testé 972 souches d'entérobactéries productrices de carbapénèmases. Ces souches incluaient 577 *Klebsiella* spp., 236 *E. coli*, 108 *Enterobacter* spp., 50 *Citrobacter* spp. et une souche de *Salmonella* spp. Les CMI de la colistine ont été déterminées par dilutions en milieu liquide. Les mécanismes de résistance à la colistine ont été recherchés par PCR et séquençage. L'étude de la clonalité a été réalisée par PFGE et la détermination des séquences-types a été déterminée par MLST.

#### **Principaux résultats :**

Sur 561 souches de *K. pneumoniae*, 35 étaient résistantes à la colistine. Parmi elles, 15 souches avaient été isolées dans un même hôpital dans le Nord de la France. Ces 15 souches étaient clonales et de ST11 et étaient productrices de la carbapénémase OXA-48 et de la bêta-lactamase à spectre étendu (BLSE) CTX-M-15. Elles présentaient des CMI de la colistine variables (de 4 à 64 mg/L) : le gène *pmrA* des 5 souches avec les CMI les plus élevées (32 ou 64 mg/L) était muté (substitution G53S de la protéine PmrA), tandis que les 10 souches avec les CMI plus basses (4 ou 8 mg/L) possédaient un gène *pmrA* sauvage. Ceci suggérait l'implication de la substitution PmrA G53S dans l'acquisition d'un haut niveau de résistance à la



colistine chez ces cinq souches. Les 20 autres souches de *K. pneumoniae* avaient été isolées dans différentes régions et produisaient des carbapénèmases variées (OXA-48, KPC-2, NDM-1, OXA-48+NDM-1). Elles présentaient des CMI de la colistine de 16 à > 128 mg/L et le séquençage du gène *mgrB* a permis d'identifier des altérations variées responsables de l'acquisition de la résistance à la colistine (mutations, insertions ou duplications de séquences, délétions).

Sur 91 souches de *E. cloacae*, 7 étaient résistantes à la colistine et produisaient différents types de carbapénèmases (OXA-48, VIM-1, IMP-1, OXA-48+VIM-1).

Aucune des souches de *E. coli* et de *Citrobacter* spp. n'étaient résistantes à la colistine.

### **Conclusion :**

Nous avons trouvé un taux relativement élevé de résistance à la colistine en France en 2014 chez les *K. pneumoniae* et les *E. cloacae* (6,2 % et 7,7 %, respectivement) producteurs de carbapénèmases. Nous avons identifié de façon rétrospective une épidémie de *K. pneumoniae* productrices de la carbapénémase OXA-48 et résistantes à la colistine impliquant 15 cas dans le Nord de la France.

**Ce travail a été publié dans *Eurosurveillance* en septembre 2016 (IF = 5.983).**

# National survey of colistin resistance among carbapenemase-producing *Enterobacteriaceae* and outbreak caused by colistin-resistant OXA-48-producing *Klebsiella pneumoniae*, France, 2014

A Jayol<sup>1</sup>, L Poirel<sup>1</sup>, L Dortet<sup>2,3,4</sup>, P Nordmann<sup>1,2,5</sup>

1. Emerging Antibiotic Resistance Unit, Medical and Molecular Microbiology, Department of Medicine, University of Fribourg, Fribourg, Switzerland
2. Associated National Reference Centre for Antibiotic Resistance, Le Kremlin-Bicêtre, France
3. Faculty of Medicine, South-Paris University, Le Kremlin-Bicêtre, France
4. Bacteriology-Hygiene unit, Hospital Bicêtre, Assistance Publique /Hôpitaux de Paris, Le Kremlin-Bicêtre, France
5. University of Lausanne and University Hospital Center, Lausanne, Switzerland

Correspondence: Laurent Poirel (laurent.poirel@unifr.ch)

## Citation style for this article:

Jayol A, Poirel L, Dortet L, Nordmann P. National survey of colistin resistance among carbapenemase-producing *Enterobacteriaceae* and outbreak caused by colistin-resistant OXA-48-producing *Klebsiella pneumoniae*, France, 2014. *Euro Surveill.* 2016;21(37):pii=30339. DOI: <http://dx.doi.org/10.2807/1560-7917.ES.2016.21.37.30339>

Article submitted on 30 October 2015 / accepted on 04 April 2016 / published on 15 September 2016

From January 2014 to December 2014, 972 consecutive non- replicate carbapenemase-producing *Enterobacteriaceae* isolates from colonised or infected patients were collected at the Associated French National Reference Centre as part of the French national survey on antimicrobial resistance. It included 577 *Klebsiella* spp. (59%), 236 *Escherichia coli* (24%), 108 *Enterobacter* spp. (11%), 50 *Citrobacter* spp. (5%), and a single *Salmonella* spp. isolate (0.1%). Of 561 *K. pneumoniae* isolates, 35 were found to be resistant to colistin (6.2%). PFGE analysis revealed a clonal outbreak involving 15 *K. pneumoniae* isolates belonging to sequence type ST11, recovered in a single hospital in the Picardie region in northern France. Those clonally related isolates showed variable levels of resistance to colistin, ranging from 4 to 64 mg/L. They harboured the *bla*<sub>OXA-48</sub> carbapenemase gene and the *bla*<sub>CTX-M-15</sub> extended-spectrum beta-lactamase gene. Among the 91 *Enterobacter cloacae* isolates, seven were resistant to colistin and produced different types of carbapenemases. Surprisingly, none of the *E. coli* and *Citrobacter* spp. isolates showed resistance to colistin. This national survey including carbapenemase-producing isolates recovered in 2014 reported a high rate of colistin resistance in *K. pneumoniae* and *E. cloacae* (6.2% and 7.7%, respectively) in France.

## Introduction

Carbapenemase-producing *Enterobacteriaceae* (CPE) resistant to colistin are increasingly reported. They represent an additional link in the development of pan-drug resistance. However, the epidemiology of colistin resistance among enterobacterial isolates is

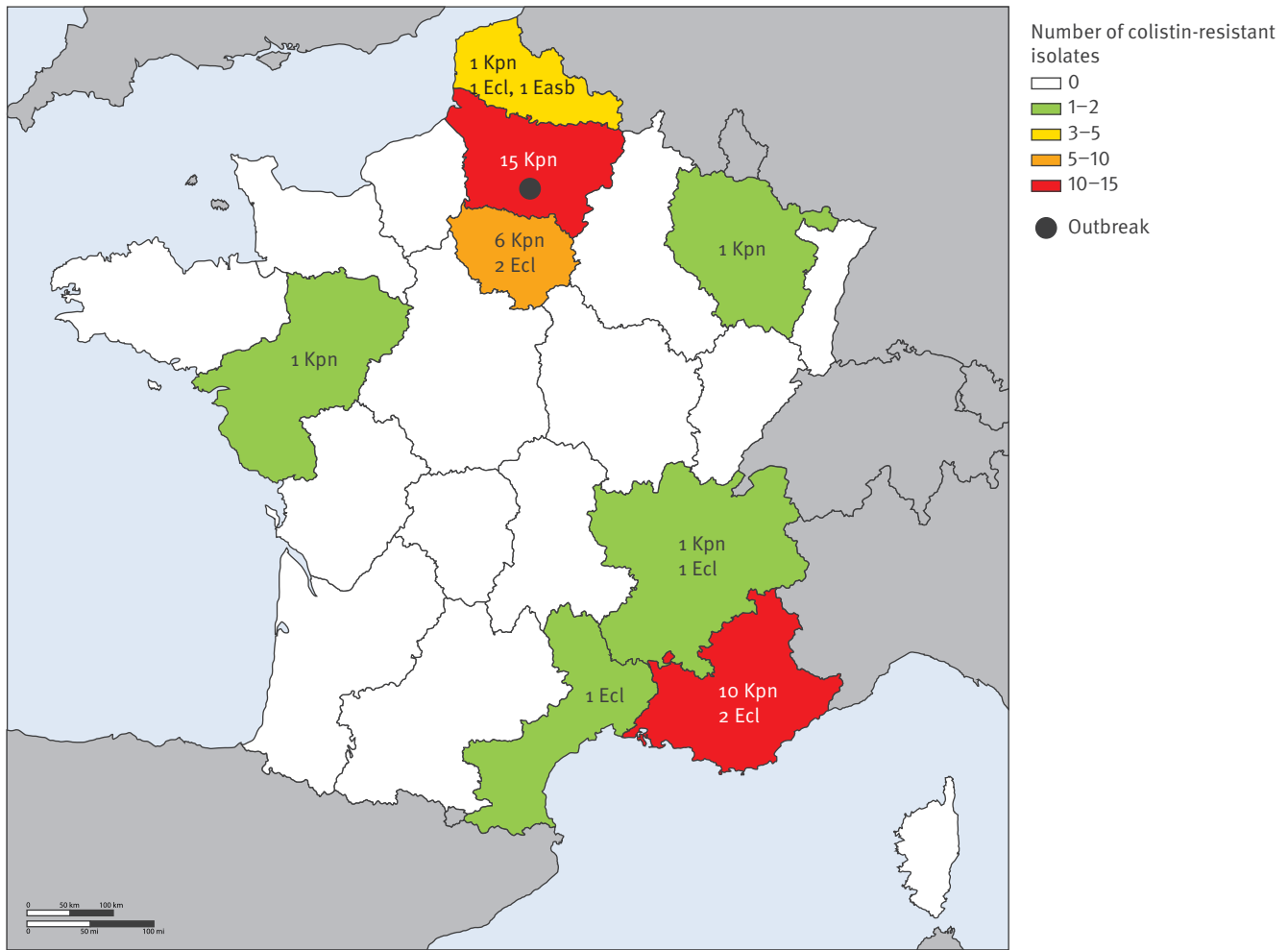
currently almost unknown in most parts of the world. In Italy, an increase in carbapenemase-producing *Enterobacteriaceae* has been noted in the past years, but the situation remains unknown in France [1]. The lack of information about the prevalence of colistin resistance among multidrug-resistant enterobacterial isolates derives from several reasons: (i) so far, there has been limited interest in that field, (ii) methods used for determination of colistin susceptibility are not adequate, and (iii) the lack of well-defined breakpoints does not allow precise determination of prevalence. However, the recent identification of a plasmid-borne polymyxin resistance determinant (MCR-1) raised a very serious concern in that resistance to colistin might widely disseminate [2].

The aim of this study was to evaluate retrospectively the prevalence of colistin resistance among a collection of CPE strains recovered in France during a period of one year and to analyse the phenotypic, genotypic features and clonality of the colistin-resistant isolates.

## Methods

### Carbapenemase-producing *Enterobacteriaceae* isolates

From January to December 2014, 972 consecutive non-duplicate isolates of carbapenemase-producing *Enterobacteriaceae* were isolated in private laboratories and hospitals in France either by screening for colonisation or by analysing clinical samples in the context of infections. They were recovered from rectal swabs or stools (n=625), urine samples (n=250), respiratory

**FIGURE 1**Geographic distribution of colistin-resistant *Enterobacteriaceae* isolates, France, January–December 2014 (n = 43)

Easb: *Enterobacter asburiae*; Ecl: *Enterobacter cloacae*; Kpn: *Klebsiella pneumoniae*.

tract samples (n=35), blood samples (n=22), wounds (n=24), catheter (n=7), vaginal swabs (n=3) and other specimens (n=6). Those isolates were sent to the Associated French National Reference Centre for characterisation of resistance mechanisms to carbapenems as part of the French antibiotic resistance survey. The 972 carbapenemase-producing *Enterobacteriaceae* isolates included 577 isolates of *Klebsiella* spp. (59%), 236 isolates of *Escherichia coli* (24%), 108 isolates of *Enterobacter* spp. (11%), 50 isolates of *Citrobacter* spp. (5%), and a single isolate of *Salmonella* spp. (0.1%). Species that are naturally resistant to colistin (*Proteus* spp., *Morganella morganii*, *Providencia* spp., and *Serratia* spp.) had been excluded before the initiation of this study. Only a single isolate per patient was included in the study. All isolates were identified using the Microflex bench-top MALDI-TOF mass spectrometer (Bruker, Champs-sur-Marne, France).

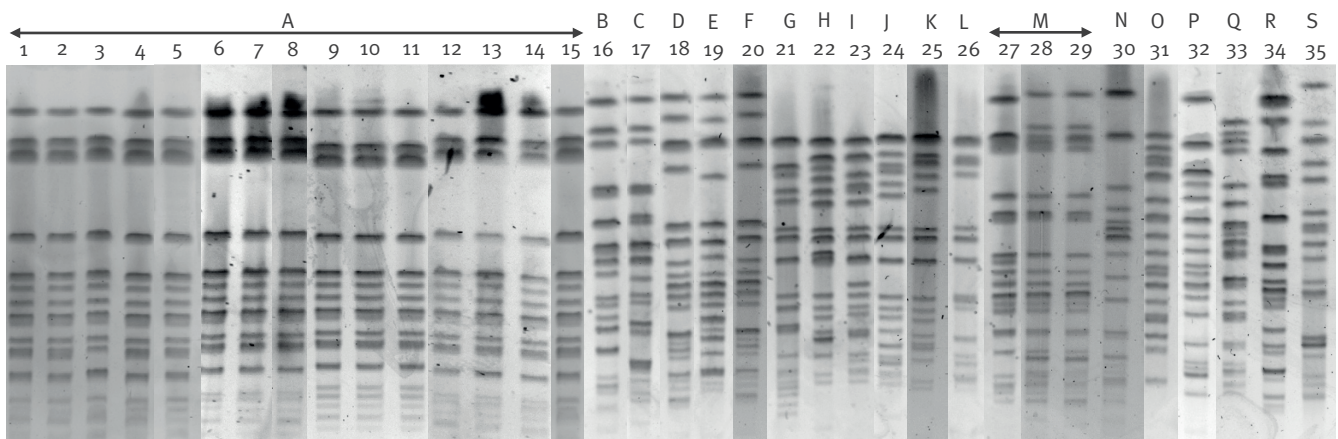
### Antimicrobial susceptibility testing

Minimum inhibitory concentrations (MIC) of colistin (CS) were determined using broth microdilution method according to the guidelines of the Clinical Laboratory Standards Institute (CLSI) [3]. As recommended, *E. coli* ATCC 25922 was used as quality control strain.

For the colistin-resistant isolates, susceptibility to other classes of antibiotics was also tested. Susceptibility to imipenem, ertapenem, and tigecycline was tested by broth microdilution method according to CLSI guidelines, whereas susceptibility to the other antibiotics was tested by the standardised agar disk diffusion method according to the guidelines of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) [4]. The antibiotics tested using disk diffusion method were: amoxicillin (AMX), amoxicillin/clavulanic acid (AMC), cefotaxime (CTX), ceftazidime (CAZ), cefepime (FEP), temocillin (TEM), ciprofloxacin (CIP), gentamicin (GM), amikacin

**FIGURE 2**

PFGE patterns of *Xba*I-digested chromosomal DNA of colistin-resistant *Klebsiella pneumoniae* isolates, France, January–December 2014 (n = 35)



The numbers correspond to the isolates from Table 2 and the letters indicate the PFGE type.

(AK), trimethoprim-sulfamethoxazole (SXT) and fosfomycin (FOS).

The MIC results for colistin and the disk diffusion diameters were interpreted according to susceptibility breakpoints of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) [4].

### Molecular characterisation

The *mgrB* genes of *K. pneumoniae* and *Enterobacter* spp. isolates were amplified using specific primers (Table 1), knowing that the MgrB protein is a negative regulator of the PhoPQ two-component system and that alterations in the *mgrB* gene are commonly involved in acquisition of colistin resistance in *K. pneumoniae* [5-7]. The plasmid-mediated *mcr-1* gene encoding colistin resistance was sought as described previously [2]. Detection of extended-spectrum beta-lactamases (ESBL) and carbapenemases genes was performed with specific primers as described previously [8]. Both strands of the amplification products obtained were sequenced with an ABI 3100 sequencer (Applied Biosystems, Foster City, US). The nucleotide and deduced protein sequences were analysed at the National Centre for Biotechnology Information website ([www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)) by the Basic Local Alignment Search Tool (BLAST) programme.

### Genotyping

Genotyping was performed to evaluate the clonal relationship of the colistin-resistant *K. pneumoniae* and *E. cloacae* isolates by pulsed-field gel electrophoresis (PFGE) with *Xba*I-digested genomic DNA and interpreted according to Tenover criteria [9]. Multilocus sequence typing (MLST) for *K. pneumoniae* was performed using the simplified protocol at the Institut Pasteur website (<http://bigsd.pasteur.fr/klebsiella/klebsiella.html>) [10].

## Results

### *Klebsiella pneumoniae*

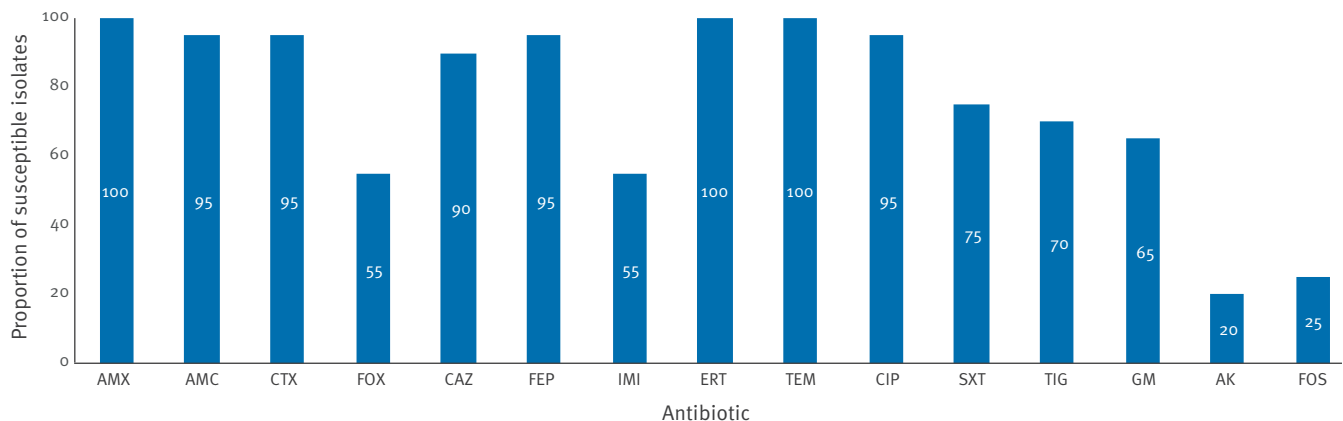
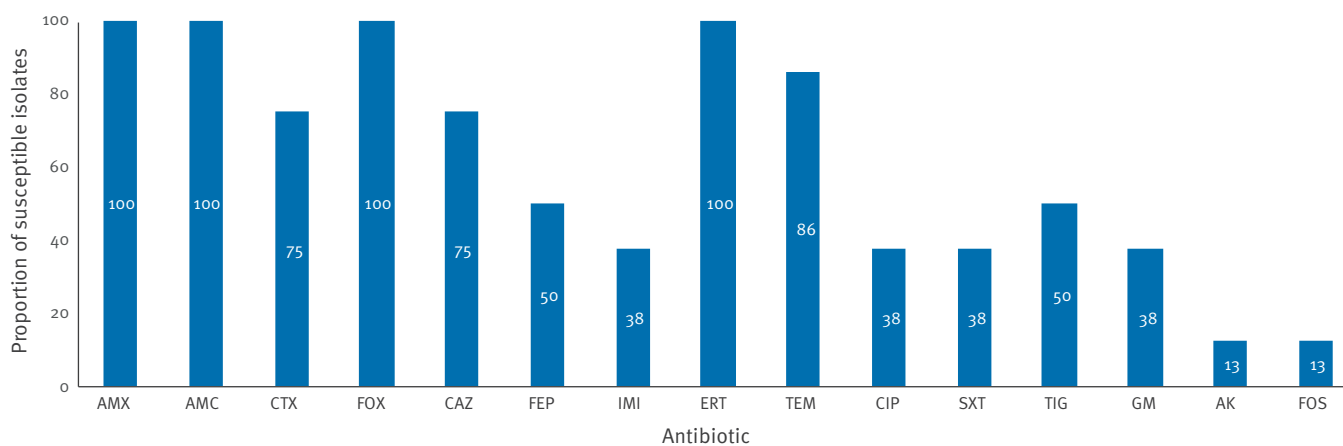
Of 561 *K. pneumoniae* isolates, 35 were found to be resistant to colistin (6%). Fifteen of the 35 colistin-resistant *K. pneumoniae* isolates were recovered from a single hospital in the Picardie region, northern France (Figure 1). We could not obtain the exact dates of their isolations due to the retrospective nature of the study. These isolates had mostly been recovered from rectal swab specimens, but also from a catheter, a urinary sample, a wound exudate and a respiratory specimen (isolates 1 to 15, Table 2). PFGE analysis revealed that the 15 isolates were clonally related (Figure 2, Table 2). The clone was of the ST11 type, and was susceptible only to ceftazidime, amikacin and fosfomycin (Table 2). A single isolate among these 15 was susceptible to tigecycline. The 15 isolates harboured both the *bla*OXA-48 carbapenemase gene, and the *bla*CTX-M-15 extended-spectrum beta-lactamase (ESBL) gene, and the MICs for colistin ranged from 4 to 64 mg/L (Table 2).

The other 20 colistin-resistant *K. pneumoniae* strains were mostly recovered from the regions Ile-de-France (n=6) and Provence-Alpes-Côte d'Azur (n=10) (Figure 1). These strains presented high MIC values for colistin ranging from 16 to >128 mg/L (isolates 16 to 35, Table 2). They produced either the carbapenemases OXA-48 (15/20), KPC-2 (3/20), NDM-1 (1/20), or both OXA-48 and NDM-1 together (1/20) (Table 2). Overall, 14 of the 20 isolates produced the ESBL CTX-M-15. PFGE analysis identified 18 clonal patterns among the 20 isolates (n=3 for clone M) (Figure 2, Table 2), and MLST assigned the isolates to eight sequence types (STs) (Table 2).

Sequencing of the *mgrB* gene of those *K. pneumoniae* isolates revealed various *mgrB* alterations and none

**FIGURE 3**

Antimicrobial non-susceptibilities among colistin-resistant isolates, France, January–December 2014

**A. *Klebsiella pneumoniae* (except the strains responsible for the outbreak in Picardie) (n=35)****B. *Enterobacter* spp. (n=8)**

AK: amikacin; AMC: amoxicillin/clavulanic acid; AMX: amoxicillin; CAZ: ceftazidime; CIP: ciprofloxacin; CTX: cefotaxime; ERT: ertapenem; FEP: cefepime; FOS: fosfomicin; FOX: ceftiofur; GM: gentamicin; IMI: imipenem; SXT: trimethoprim-sulfamethoxazole; TEM: temocillin; TIG: tigecycline.

of the strains harboured the plasmid-encoded *mcr-1* gene.

Antimicrobial susceptibility data for the colistin-resistant *K. pneumoniae* isolates not involved in the outbreak revealed that most isolates (19/20) were non-susceptible to third- and fourth-generation cephalosporins (Figure 3A). They were also frequently resistant to ciprofloxacin (19/20), trimethoprim-sulfamethoxazole (15/20) and tigecycline (14/20). They were less often resistant to gentamicin and ceftiofur (13/20 and 11/20, respectively). Amikacin and fosfomicin remained the most active agents against colistin-resistant *K. pneumoniae* (16/20 and 15/20 were susceptible, respectively) (Figure 3A).

***Enterobacter* spp.**

Among the 91 *Enterobacter cloacae* isolates, seven were resistant to colistin (7.7%). They showed high MIC

values for colistin (ranging from 16 to >128 mg/L) (isolates 36 to 42, Table 2). They produced the carbapenemases OXA-48 (4/7), VIM-1 (1/7), IMP-1 (1/7), or both OXA-48 and VIM-1 together (1/7) (Table 2). In total, three of seven strains were CTX-M producers, with two isolates producing CTX-M-15 and a single isolate producing CTX-M-2. The colistin-resistant *E. cloacae* isolates were recovered in different geographical regions in France (Figure 1, Table 2), and results of the PFGE analysis revealed that they were not clonally related (data not shown).

The single carbapenem-resistant *E. asburiae* strain was resistant to colistin. It had an MIC of colistin above 128 mg/L and produced the VIM-1 carbapenemase (isolate 43, Table 2).



**TABLE 1**

Oligonucleotides used as primers in this study, France, January–December 2014

Oligonucleotides	Sequence (5'-3')	Reference
<i>Kpn mgrB</i> ext F	TTA AGA AGG CCG TGC TAT CC	[7]
<i>Kpn mgrB</i> ext R	AAG GCG TTC ATT CTA CCA CC	[7]
<i>Kpn mgrB</i> int F	CGG TGG GTT TTA CTG ATA GTC	This study
<i>Kpn mgrB</i> int R	GAA CAT CCT GGT CGC ACA TT	This study
<i>Ent mgrB</i> ext F	CGG TTT ACT CTA TGA AAC AAG TGC	This study
<i>Ent mgrB</i> ext R	GCG AAG GAA GGA AAT CAC CT	This study

All *Enterobacter* spp. isolates had a wild-type *mgrB* gene, leaving unexplained the colistin resistance mechanism (*E. cloacae* and *E. asburiae*) (Table 2).

Of the eight colistin-resistant *Enterobacter* spp. isolates, four were non-susceptible to cefepime and tigecycline, and three were non-susceptible to ciprofloxacin, trimethoprim-sulfamethoxazole and gentamicin (Figure 3B). Amikacin and fosfomycin were the most active agents against colistin-resistant *E. cloacae* (all seven isolates were susceptible) (Figure 3B).

### Other species

None of the *E. coli* (n=236) and *Citrobacter* spp. (n=50) isolates were resistant to colistin.

### Discussion

We describe here a clonal outbreak involving 15 *K. pneumoniae* isolates recovered from a single hospital in the Picardie region in northern France. This outbreak was caused by a colistin-resistant OXA-48 and CTX-M-15-producing *K. pneumoniae* of ST11 type that was susceptible only to ceftazidime, amikacin and fosfomycin. Surprisingly, those clonally related isolates had variable MIC values for colistin ranging from 4 to 64 mg/L. An ST11 clone co-producing OXA-48 and CTX-M-15 was responsible for a large outbreak involving 44 patients in a hospital in Madrid, Spain, from 2009 to 2014 but only 3.4% of the isolates were resistant to colistin [11].

Several outbreaks of colistin-resistant KPC-producing *K. pneumoniae* (mainly attributed to the international epidemic clone type ST258) have been reported across Europe, in Greece [12,13], Hungary [14], Italy [15-17] and the Netherlands [18]. A single outbreak of colistin-resistant VIM-1-producing *K. pneumoniae* has also been described in Spain [19].

We report also 20 colistin-resistant *K. pneumoniae* strains recovered from the regions Ile-de-France and Provence-Alpes-Côte d'Azur. These strains belonged to 10 sequence types (n=2 ST147, n=3 ST258, n=6 ST101, n=3 ST307) and PFGE analysis identified 18 patterns among the 20 isolates. All three KPC-producing *K. pneumoniae* isolates belonged to ST258, the most

common clone for KPC-producing isolates [20]. The OXA-48-producing *K. pneumoniae* isolates belonged to nine sequence types with six strains that were ST101, the most common clone identified among OXA-48-positive *K. pneumoniae* [21].

Sequencing of the *mgrB* gene revealed *mgrB* alterations which are likely to be responsible for colistin resistance as described previously [5-7]. Interestingly, the three strains belonging to the single clone M recovered in the Provence-Alpes Côte-d'Azur region had different mechanisms of *mgrB* inactivation (Table 2). The occurrence of such different mechanisms of colistin resistance among clonally related isolates indicates that it is not the product of clonal dissemination of a single colistin-resistant *K. pneumoniae* strain, but rather clonal dissemination of a carbapenemase-producing isolate, which has acquired colistin resistance thereafter.

The rates of colistin resistance among the carbapenemase-producing isolates were 7.7% for *Enterobacter* spp. and 3.6% for *K. pneumoniae* isolates (excluding the isolates responsible for the outbreak in the Picardie region). The resistance rate observed among the carbapenemase-producing *K. pneumoniae* isolates was much lower than the high rates reported in the neighbouring countries of southern Europe such as Spain (20%) [19] and Italy (43%) [1].

None of the 236 carbapenemase-producing *E. coli* isolates were colistin-resistant or carried the *mcr-1* gene. This is surprising considering that a recent report of the French antimicrobial resistance Resapath surveillance network identified the plasmid-borne *mcr-1* gene in 21% of ESBL-producing *E. coli* isolates recovered from faeces of veal calves in France between 2005 and mid-2014 [22]. The plasmid-borne *mcr-1* colistin resistance gene has also been found in many neighbouring countries of France, for example among ESBL-producing *Enterobacteriaceae* isolates recovered from river water and imported vegetable samples in Switzerland [23], in *E. coli* isolates recovered from calves and piglets in Belgium [24], in swine and human wound infections in Germany [25], and in food and human bloodstream infections in Denmark [26]. The *mcr-1* gene was also detected in *Salmonella enterica* from food samples in Portugal [27] and France [28]. An *E. coli* isolate co-harboring the *blaVIM-1* carbapenemase gene and the *mcr-1* gene was described in Switzerland [29] and an isolate co-producing NDM-9 and MCR-1 was reported from China [30]. We believe that the plasmid carrying the *mcr-1* gene might be currently more prevalent among ESBL-producing isolates than among carbapenemase-producing isolates in human samples, which would explain why we did not identify this gene in our collection of carbapenemase-producing isolates.

Amikacin and fosfomycin were most effective against the colistin and carbapenem-resistant *K. pneumoniae* (susceptibility rates of 80% and 75%, respectively) and

**TABLE 2A**

 Characteristics of the colistin-resistant *Klebsiella pneumoniae* and *Enterobacter* spp. clinical isolates, France, January–December 2014 (n = 43)

Isolate	Site of isolation	Origin	MIC CS <sup>a</sup>	<i>mgrB</i> genotype	Carbapenemase	Associated beta-lactamase	Co-resistances <sup>b</sup>	ST	PFGE
1	?	Picardie	32	<i>mgrB</i> WT	OXA-48	CTX-M-15	CIP GM SXT	11	A
2	Catheter	Picardie	8	<i>mgrB</i> WT	OXA-48	CTX-M-15	CIP GM SXT TIG	11	A
3	Rectal swab	Picardie	4	<i>mgrB</i> WT	OXA-48	CTX-M-15	CIP GM SXT TIG	11	A
4	Rectal swab	Picardie	4	<i>mgrB</i> WT	OXA-48	CTX-M-15	CIP GM SXT TIG	11	A
5	Rectal swab	Picardie	64	<i>mgrB</i> WT	OXA-48	CTX-M-15	CIP GM SXT TIG	11	A
6	Urine	Picardie	32	<i>mgrB</i> WT	OXA-48	CTX-M-15	CIP GM SXT TIG	11	A
7	Rectal swab	Picardie	64	<i>mgrB</i> WT	OXA-48	CTX-M-15	CIP GM SXT TIG	11	A
8	Wound	Picardie	4	<i>mgrB</i> WT	OXA-48	CTX-M-15	CIP GM SXT TIG	11	A
9	Respiratory	Picardie	4	<i>mgrB</i> WT	OXA-48	CTX-M-15	CIP GM SXT TIG	11	A
10	Rectal swab	Picardie	4	<i>mgrB</i> WT	OXA-48	CTX-M-15	CIP GM SXT TIG	11	A
11	Rectal swab	Picardie	8	<i>mgrB</i> WT	OXA-48	CTX-M-15	CIP GM SXT TIG	11	A
12	Rectal swab	Picardie	64	<i>mgrB</i> WT	OXA-48	CTX-M-15	CIP GM SXT TIG	11	A
13	Rectal swab	Picardie	8	<i>mgrB</i> WT	OXA-48	CTX-M-15	CIP GM SXT TIG	11	A
14	Rectal swab	Picardie	4	<i>mgrB</i> WT	OXA-48	CTX-M-15	CIP GM SXT TIG	11	A
15	Rectal swab	Picardie	4	<i>mgrB</i> WT	OXA-48	CTX-M-15	CIP GM SXT TIG	11	A
16	Rectal swab	Nord-Pas-de-Calais	128	IS <sub>1R</sub> in promoter region (between nt -45 and -46)	OXA-48	CTX-M-15	CIP GM SXT TIG	147	B
17	Rectal swab	Ile-de-France	128	<i>mgrB</i> WT	NDM	CTX-M-15	CIP GM SXT TIG	147	C
18	Rectal swab	PACA	>128	ISK <sub>pn26</sub> -like in coding region (between nt +74 and +75)	KPC	-	CIP AK SXT TIG	258	D
19	Rectal swab	Rhône-Alpes	128	MgrB truncated (27 amino acids)	KPC	-	CIP AK SXT TIG	258	E
20	Blood	PACA	16	Full gene deletion <sup>c</sup>	KPC	-	CIP AK SXT TIG	258	F
21	Rectal swab	Lorraine	64	Single nucleotide deletion (nt 74)	OXA-48	CTX-M-15	CIP GM	101	G
22	Rectal swab	Ile-de-France	32	Single nucleotide deletion (nt 23)	OXA-48	CTX-M-15	CIP GM	101	H
23	Rectal swab	Ile-de-France	64	IS <sub>1R</sub> in promoter region (between nt -36 and -37)	OXA-48 + NDM	CTX-M-15	CIP GM	101	I
24	Urine	PACA	64	IS <sub>1R</sub> in promoter region (between nt -45 and -46)	OXA-48	CTX-M-15	CIP GM SXT TIG	101	J
25	Abcess	Ile-de-France	32	MgrB M27K	OXA-48	CTX-M-15	CIP GM	101	K
26	Urine	Pays de la Loire	128	Duplication of 19 nucleotides	OXA-48	CTX-M-15	CIP SXT FOS	101	L
27	Urine	PACA	64	IS <sub>1R</sub> in coding region (between nt +21 and +22)	OXA-48	CTX-M-15	CIP GM SXT TIG	307	M
28	Rectal swab	PACA	64	<i>mgrB</i> WT	OXA-48	CTX-M-15	CIP GM SXT TIG FOS	307	M
29	Rectal swab	PACA	64	IS <sub>5</sub> -like in coding region (between nt +74 and +75)	OXA-48	CTX-M-15	CIP GM SXT TIG	307	M
30	Urine	PACA	>128	Full gene deletion <sup>b</sup>	OXA-48	-	CIP GM AK SXT TIG FOS	611	N
31	Rectal swab	Ile-de-France	32	ISK <sub>pn14</sub> -like in promoter region (between nt -45 and -46)	OXA-48	-	CIP GM SXT TIG	23	O

 Isolates 1-35: *Klebsiella pneumoniae*; 36–42: *Enterobacter cloacae*; 43: *Enterobacter asburiae*.

AK: amikacin; CIP: ciprofloxacin; CS: colistin; FOS: fosfomicin; GM: gentamicin; MIC: minimum inhibitory concentration; NA: not applicable; nt: nucleotide; PACA: Provence-Alpes-Côte-d'Azur; PFGE: pulsed-field gel electrophoresis; ST: sequence type; SXT: trimethoprim-sulfamethoxazole; TIG: tigecycline; WT: wildtype.

<sup>a</sup> MIC of colistin determined by broth microdilution method.

<sup>b</sup> Resistant or intermediate susceptibility to antibiotic.

<sup>c</sup> Full gene deletion: no PCR product was detected with external or internal primers.

**TABLE 2B**

Characteristics of the colistin-resistant *Klebsiella pneumoniae* and *Enterobacter* spp. clinical isolates, France, January–December 2014 (n = 43)

Isolate	Site of isolation	Origin	MIC CS <sup>a</sup>	<i>mgrB</i> genotype	Carbapenemase	Associated beta-lactamase	Co-resistances <sup>b</sup>	ST	PFGE
32	Rectal swab	PACA	32	IS102-like in coding region (between nt +36 and +37)	OXA-48	CTX-M-15	CIP GM SXT TIG	20	P
33	Respiratory	Ile-de-France	32	IS1R in promoter region (between nt -61 and -62)	OXA-48	CTX-M-15	CIP SXT TIG FOS		Q
34	Blood	PACA	>128	<i>mgrB</i> WT	OXA-48	CTX-M-15	CIP SXT TIG	39	R
35	Rectal swab	PACA	32	MgrB truncated (32 amino acids)	OXA-48	-	FOS	13	S
36	Rectal swab	Nord-Pas-de-Calais	64	<i>mgrB</i> WT	OXA-48 + VIM	CTX-M-15	CIP GM SXT	NA	T
37	Stools	Languedoc-Roussillon	64	<i>mgrB</i> WT	OXA-48	CTX-M-15	GM AK	NA	U
38	Respiratory	Ile-de-France	32	<i>mgrB</i> WT	VIM	-	SXT TIG	NA	V
39	Rectal swab	PACA	>128	<i>mgrB</i> WT	OXA-48	-	CIP GM SXT TIG	NA	W
40	Rectal swab	Ile-de-France	16	<i>mgrB</i> WT	OXA-48	-	FOS	NA	X
41	Rectal swab	PACA	>128	<i>mgrB</i> WT	IMP	CTX-M-2	No	NA	Y
42	Respiratory	Rhône-Alpes	>128	<i>mgrB</i> WT	OXA-48	-	TIG	NA	Z
43	Urine	Nord-Pas-de-Calais	>128	<i>mgrB</i> WT	VIM-1	-	CIP TIG	NA	α

Isolates 1-35: *Klebsiella pneumoniae*; 36-42: *Enterobacter cloacae*; 43: *Enterobacter asburiae*.

AK: amikacin; CIP: ciprofloxacin; CS: colistin; FOS: fosfomicin; GM: gentamicin; MIC: minimum inhibitory concentration; NA: not applicable; nt: nucleotide; PACA: Provence-Alpes-Côte-d'Azur; PFGE: pulsed-field gel electrophoresis; ST: sequence type; SXT: trimethoprim-sulfamethoxazole; TIG: tigecycline; WT: wildtype.

<sup>a</sup> MIC of colistin determined by broth microdilution method.

<sup>b</sup> Resistant or intermediate susceptibility to antibiotic.

*E. cloacae* isolates (susceptibility rates of 87%). The rate of tigecycline non-susceptibility was high (70% for *K. pneumoniae* and 50% for *Enterobacter* spp.), probably because of a strong selective pressure by this last-line antibiotic.

## Conclusion

This national survey on carbapenemase-producing isolates recovered in 2014 discovered a high rate of colistin resistance in *K. pneumoniae* and *E. cloacae* (6.2% and 7.7%, respectively) in France. These resistance rates remain much lower than those observed in other European countries such as Greece, Italy and Spain. No plasmid-encoded *mcr-1* gene was identified here. Therefore it seems that it is still possible to control the spread of those multidrug-resistant isolates based on accurate identification of colistin resistance and isolation of plasmid-encoded MCR-1 producers. Amikacin and fosfomicin remained the antibiotic agents most effective against those isolates which were resistant to polymyxins and produced a carbapenemase.

## Acknowledgements

This work was supported by the University of Fribourg and partially funded by the Institut de Veille Sanitaire (InVS).

## Conflict of interest

None declared.

## Authors' contributions

AJ, LP, and PN contributed to the design of the study. AJ performed the experiments. AJ, LP, and PN analysed the data. AJ, LP, LD, and PN contributed to the writing of the manuscript.

## References

1. Monaco M, Giani T, Raffone M, Arena F, Garcia-Fernandez A, Pollini S, et al. Colistin resistance superimposed to endemic carbapenem-resistant *Klebsiella pneumoniae*: a rapidly evolving problem in Italy, November 2013 to April 2014. *Euro Surveill.* 2014;19(42). DOI: 10.2807/1560-7917.ES2014.19.42.20939 PMID: 25358041
2. Liu YY, Wang Y, Walsh TR, Yi LX, Zhang R, Spencer J, et al. Emergence of plasmid-mediated colistin resistance mechanism MCR-1 in animals and human beings in China: a microbiological and molecular biological study. *Lancet Infect Dis.* 2016;16(2):161-8. DOI: 10.1016/S1473-3099(15)00424-7 PMID: 26603172
3. Clinical and Laboratory Standards Institute (CLSI). Methods for dilution of antimicrobial susceptibility tests for bacteria that grow aerobically. Approved standard, 9th ed. CLSI document M07-A10. Wayne: CLSI; 2015.
4. European Committee on Antimicrobial Susceptibility Testing (EUCAST). Breakpoints tables for interpretation of MICs and zone diameters, Version 5.0. Växjö: EUCAST. 2015.
5. Cannatelli A, Giani T, D'Andrea MM, Di Pilato V, Arena F, Conte V, et al. MgrB inactivation is a common mechanism of colistin



- resistance in KPC-producing *Klebsiella pneumoniae* of clinical origin. *Antimicrob Agents Chemother.* 2014;58(10):5696-703. DOI: 10.1128/AAC.03110-14 PMID: 25022583
6. Cheng YH, Lin TL, Pan YJ, Wang YP, Lin YT, Wang JT. Colistin resistance mechanisms in *Klebsiella pneumoniae* strains from Taiwan. *Antimicrob Agents Chemother.* 2015;59(5):2909-13. DOI: 10.1128/AAC.04763-14 PMID: 25691646
  7. Poirel L, Jayol A, Bontron S, Villegas MV, Ozdamar M, Türkoglu S, et al. The *mgrB* gene as a key target for acquired resistance to colistin in *Klebsiella pneumoniae*. *J Antimicrob Chemother.* 2015;70(1):75-80. DOI: 10.1093/jac/dku323 PMID: 25190723
  8. Nordmann P, Naas T, Poirel L. Global spread of carbapenemase-producing Enterobacteriaceae. *Emerg Infect Dis.* 2011;17(10):1791-8. DOI: 10.3201/eid1710.110655 PMID: 22000347
  9. Tenover FC, Arbeit RD, Goering RV, Mickelsen PA, Murray BE, Persing DH, et al. Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. *J Clin Microbiol.* 1995;33(9):2233-9. PMID: 7494007
  10. Diancourt L, Passet V, Verhoef J, Grimont PA, Brisse S. Multilocus sequence typing of *Klebsiella pneumoniae* nosocomial isolates. *J Clin Microbiol.* 2005;43(8):4178-82.
  11. Brañas P, Villa J, Viedma E, Mingorance J, Orellana MA, Chaves F. Molecular epidemiology of carbapenemase-producing *Klebsiella pneumoniae* in a hospital in Madrid: Successful establishment of an OXA-48 ST11 clone. *Int J Antimicrob Agents.* 2015;46(1):111-6. DOI: 10.1016/j.ijantimicag.2015.02.019 PMID: 25914088
  12. Antoniadou A, Kontopidou F, Poulakou G, Koratzanis E, Galani I, Papadomichelakis E, et al. Colistin-resistant isolates of *Klebsiella pneumoniae* emerging in intensive care unit patients: first report of a multiclonal cluster. *J Antimicrob Chemother.* 2007;59(4):786-90. DOI: 10.1093/jac/dkl562 PMID: 17307769
  13. Kontopoulou K, Protonotariou E, Vasilakos K, Kriti M, Koteli A, Antoniadou E, et al. Hospital outbreak caused by *Klebsiella pneumoniae* producing KPC-2 beta-lactamase resistant to colistin. *J Hosp Infect.* 2010;76(1):70-3. DOI: 10.1016/j.jhin.2010.03.021 PMID: 20705205
  14. Tóth A, Damjanova I, Puskás E, Jánvári L, Farkas M, Dobák A, et al. Emergence of a colistin-resistant KPC-2-producing *Klebsiella pneumoniae* ST258 clone in Hungary. *Eur J Clin Microbiol Infect Dis.* 2010;29(7):765-9. DOI: 10.1007/s10096-010-0921-3 PMID: 20401676
  15. Giani T, Arena F, Vaggelli G, Conte V, Chiarelli A, Henrici De Angelis L, et al. Large nosocomial outbreak of colistin-resistant, carbapenemase-producing *Klebsiella pneumoniae* traced to clonal expansion of an *mgrB* deletion mutant. *J Clin Microbiol.* 2015;53(10):3341-4. DOI: 10.1128/JCM.01017-15 PMID: 26202124
  16. Mammina C, Bonura C, Di Bernardo F, Aleo A, Fasciana T, Sodano C, et al. Ongoing spread of colistin-resistant *Klebsiella pneumoniae* in different wards of an acute general hospital, Italy, June to December 2011. *Euro Surveill.* 2012;17(33):17. PMID: 22913977
  17. Mezzatesta ML, Gona F, Caio C, Petrolito V, Sciortino D, Sciacca A, et al. Outbreak of KPC-3-producing, and colistin-resistant, *Klebsiella pneumoniae* infections in two Sicilian hospitals. *Clin Microbiol Infect.* 2011;17(9):1444-7. DOI: 10.1111/j.1469-0691.2011.03572.x PMID: 21668577
  18. Weterings V, Zhou K, Rossen JW, van Stenis D, Thewessen E, Kluytmans J, et al. An outbreak of colistin-resistant *Klebsiella pneumoniae* carbapenemase-producing *Klebsiella pneumoniae* in the Netherlands (July to December 2013), with inter-institutional spread. *Eur J Clin Microbiol Infect Dis.* 2015;34(8):1647-55. DOI: 10.1007/s10096-015-2401-2 PMID: 26067658
  19. Pena I, Picazo JJ, Rodríguez-Avial C, Rodríguez-Avial I. Carbapenemase-producing Enterobacteriaceae in a tertiary hospital in Madrid, Spain: high percentage of colistin resistance among VIM-1-producing *Klebsiella pneumoniae* ST11 isolates. *Int J Antimicrob Agents.* 2014;43(5):460-4. DOI: 10.1016/j.ijantimicag.2014.01.021 PMID: 24657043
  20. Nordmann P. Carbapenemase-producing Enterobacteriaceae: overview of a major public health challenge. *Med Mal Infect.* 2014;44(2):51-6. DOI: 10.1016/j.medmal.2013.11.007 PMID: 24360201
  21. Potron A, Poirel L, Rondinaud E, Nordmann P. Intercontinental spread of OXA-48 beta-lactamase-producing Enterobacteriaceae over a 11-year period, 2001 to 2011. *Euro Surveill.* 2013;18(31). DOI: 10.2807/1560-7917.ES2013.18.31.20549 PMID: 23929228
  22. Haenni M, Poirel L, Kieffer N, Châtre P, Saras E, Métayer V, et al. Co-occurrence of extended spectrum beta lactamase and MCR-1 encoding genes on plasmids. *Lancet Infect Dis.* 2016;16(3):281-2. DOI: 10.1016/S1473-3099(16)00007-4 PMID: 26774244
  23. Zurfuh K, Poirel L, Nordmann P, Nüesch-Inderbilen M, Hächler H, Stephan R. Occurrence of the plasmid-borne *mcr-1* colistin resistance gene in ESBL-producing Enterobacteriaceae in river water and imported vegetable samples in Switzerland. *Antimicrob Agents Chemother.* 2016; 60(4):2594-5.
  24. Malhotra-Kumar S, Xavier BB, Das AJ, Lammens C, Butaye P, Goossens H. Colistin resistance gene *mcr-1* harboured on a multidrug resistant plasmid. *Lancet Infect Dis.* 2016;16(3):283-4. DOI: 10.1016/S1473-3099(16)00012-8 PMID: 26774247
  25. Falgenhauer L, Waezsada SE, Yao Y, Imirzalioglu C, Käsbohrer A, Roesler U, et al. Colistin resistance gene *mcr-1* in extended-spectrum beta-lactamase-producing and carbapenemase-producing Gram-negative bacteria in Germany. *Lancet Infect Dis.* 2016;16(3):282-3. DOI: 10.1016/S1473-3099(16)00009-8 PMID: 26774242
  26. Hasman H, Hammerum AM, Hansen F, Hendriksen RS, Olesen B, Agersø Y, et al. Detection of *mcr-1* encoding plasmid-mediated colistin-resistant *Escherichia coli* isolates from human bloodstream infection and imported chicken meat, Denmark 2015. *Euro Surveill.* 2015;20(49). DOI: 10.2807/1560-7917.ES.2015.20.49.30085 PMID: 26676364
  27. Tse H, Yuen KY. Dissemination of the *mcr-1* colistin resistance gene. *Lancet Infect Dis.* 2016;16(2):145-6. DOI: 10.1016/S1473-3099(15)00532-0 PMID: 26711362
  28. Webb HE, Granier SA, Marault M, Millemann Y, den Bakker HC, Nightingale KK, et al. Dissemination of the *mcr-1* colistin resistance gene. *Lancet Infect Dis.* 2016;16(2):144-5. DOI: 10.1016/S1473-3099(15)00538-1 PMID: 26711363
  29. Poirel L, Kieffer N, Liassine N, Thanh D, Nordmann P. Plasmid-mediated carbapenem and colistin resistance in a clinical isolate of *Escherichia coli*. *Lancet Infect Dis.* 2016;16(3):281. DOI: 10.1016/S1473-3099(16)00006-2 PMID: 26774246
  30. Yao X, Doi Y, Zeng L, Lv L, Liu JH. Carbapenem-resistant and colistin-resistant *Escherichia coli* co-producing NDM-9 and MCR-1. *Lancet Infect Dis.* 2016;16(3):288-9. DOI: 10.1016/S1473-3099(16)00057-8 PMID: 26842777

## License and copyright

This is an open-access article distributed under the terms of the Creative Commons Attribution (CC BY 4.0) Licence. You may share and adapt the material, but must give appropriate credit to the source, provide a link to the licence, and indicate if changes were made.

This article is copyright of the authors, 2016.

## **2. Détermination de la prévalence du portage fécal de BGN résistants à la colistine dans l'Ouest de la France**

### **Contexte :**

Suite à la découverte du gène plasmidique de résistance à la colistine, *mcr-1*, à la fin de l'année 2015 en Chine (60), de nombreuses études ont montré une diffusion de ce gène à l'échelle mondiale chez l'Homme et l'animal (114). Parmi elles, une étude réalisée en France a révélé une prévalence importante de ce gène plasmidique parmi des souches de *E. coli* isolées chez le veau (115). Cette découverte faisait craindre une diffusion du gène *mcr-1* de l'animal à l'Homme en France.

### **Objectif de l'étude :**

L'objectif de cette étude prospective était de déterminer la prévalence du portage fécal de souches de BGN résistantes à la colistine chez les patients pris en charge au CHU de Bordeaux, France. Par ailleurs, un second objectif était de déterminer la prévalence du portage fécal de souches d'entérobactéries productrices du gène *mcr-1* chez ces patients.

### **Matériel et méthodes :**

De février à mai 2016, les écouvillons rectaux de 653 patients (patients communautaires admis aux urgences ou patients hospitalisés) ont étéensemencés sur la gélose SuperPolymyxin. L'identification d'espèce des bactéries ayant cultivé sur ce milieu a été effectuée par spectrométrie de masse MALDI-TOF (Brüker) et les CMI de la colistine ont été déterminées par dilutions en milieu liquide. Les gènes plasmidiques de résistance à la colistine, *mcr-1* et *mcr-2*, ont été recherchés par PCR.

### **Principaux résultats :**

Parmi les 653 patients testés, 152 étaient porteurs de souches présentant une résistance naturelle à la colistine incluant 75 *Proteus* spp., 22 *Morganella* spp., 12 *Providencia* spp., 6 *Serratia* spp., 35 *Hafnia* spp., tandis que seulement 10 patients étaient porteurs de souches présentant une résistance acquise à la colistine (3 *E. coli*, 2 *E. cloacae*, 3 *K. pneumoniae*, 1 *Raoultella ornithinolytica*, 1 *S. maltophilia*). Aucune de ces souches ne produisait les gènes plasmidiques *mcr-1* ou *mcr-2*.

### **Conclusion :**

Cette étude prospective indique une forte prévalence (23 %) du portage rectal de BGN présentant une résistance intrinsèque à la colistine, mais une faible prévalence (1,4 %) du portage rectal de BGN présentant une résistance acquise à la colistine chez les patients admis ou hospitalisés au CHU de Bordeaux.

**Ce travail a été publié dans *Journal of Medical Microbiology* en juin 2017 (IF = 2.269).**

# Prevalence of faecal carriage of colistin-resistant Gram-negative rods in a university hospital in western France, 2016

Marion Saly,<sup>1,2</sup> Aurelie Jayol,<sup>1,2,3,4,5,\*</sup> Laurent Poirer,<sup>3,4,5</sup> Francis Megraud,<sup>1</sup> Patrice Nordmann<sup>3,4,5,6</sup> and Veronique Dubois<sup>1,2</sup>

## Abstract

Plasmid-mediated and chromosomally-encoded colistin resistance is increasingly being reported worldwide. We aimed to determine the prevalence of faecal carriage of colistin-resistant Gram-negative rod isolates in a university hospital in western France. From February to May 2016, rectal swabs from 653 patients hospitalized in various clinical settings were recovered and subsequently screened for colistin resistance using the SuperPolymyxin medium. Antimicrobial susceptibilities were determined according to EUCAST guidelines. Genetic detection of plasmid-mediated colistin resistance was performed by PCR. The faecal carriage with intrinsic colistin-resistant isolates was high (23 %), while the faecal carriage with Gram-negative rods showing acquired resistance was low (1.4 %). No isolate carried the plasmid-mediated *mcr-1/mcr-2* genes. It was noteworthy that none of the patients carrying isolates with acquired colistin resistance had previously received a colistin-based treatment, while these isolates were not multidrug resistant.

Plasmid-mediated *mcr-1* [1] and *mcr-2* genes [2] conferring colistin resistance in *Enterobacteriaceae* have been discovered recently in animals worldwide. Simultaneously, human infections and faecal carriage with *Enterobacteriaceae* isolates carrying the *mcr-1* gene were reported extensively during 2015 [3]. In France, a high prevalence of the plasmid-borne *mcr-1* gene has been reported among extended-spectrum  $\beta$ -lactamase (ESBL)-producing *Escherichia coli* isolates collected from the faeces of diarrheic veal calves [4]. This finding may suggest that the dissemination of *mcr-1* from animals to humans may have already occurred quite extensively.

We therefore conducted a prospective study to evaluate the prevalence of faecal carriage of acquired and intrinsic colistin-resistant Gram-negative rods among patients admitted to or hospitalized at the Pellegrin University Hospital of Bordeaux (1350 beds), which is the largest public hospital in south-west France. From February to May 2016, rectal swabs from 653 patients hospitalized in various clinical settings (emergency, intensive care unit, surgery, medical units) were screened. Colistin-resistant isolates were screened using the SuperPolymyxin medium, which contains colistin sulfate at a concentration of 3.5 mg l<sup>-1</sup>, allowing the isolation of any colistin-resistant Gram-negative rods [5]. Colonies growing on this medium were identified

using a MALDI-TOF mass spectrometer (Bruker, Champs-sur-Marne, France). Isolates belonging to species that are intrinsically resistant to colistin were not further investigated. Minimum inhibitory concentrations (MICs) of colistin were determined for the other isolates using the reference broth microdilution (BMD) method. The results were interpreted according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines, 2016 [6]. All colistin-resistant isolates were subsequently screened for the *mcr-1* and *mcr-2* genes by PCR, as described previously [1, 2].

Out of the 653 rectal swabs, 150 gave isolates that are known to be intrinsically resistant species, namely *Proteus* sp. (*n*=75), *Morganella* sp. (*n*=22), *Providencia* sp. (*n*=12), *Serratia* sp. (*n*=6) and *Hafnia* sp. (*n*=35) [the latter genus was recently described as being naturally resistant to colistin (A. Jayol, personal communication)].

Nine isolates with an acquired colistin trait were recovered, including three *Escherichia coli*, two *Klebsiella pneumoniae*, one *Raoultella ornithinolytica*, two *Enterobacter cloacae* and one *Stenotrophomonas maltophilia* (Table 1). The MIC values of colistin ranged from 4 to 128 mg l<sup>-1</sup> for those isolates that exhibited various profiles of resistance to the other

Received 30 January 2017; Accepted 7 April 2017

**Author affiliations:** <sup>1</sup>Laboratory of Bacteriology, University of Bordeaux, France; <sup>2</sup>CNRS UMR5234 MFP, University of Bordeaux, France; <sup>3</sup>Molecular Microbiology, Department of Medicine, University of Fribourg, Fribourg, Switzerland; <sup>4</sup>French INSERM European Unit, University of Fribourg (LEA-IAME), Fribourg, Switzerland; <sup>5</sup>National Reference Center for Emerging Antibiotic Resistance, University of Fribourg, Fribourg, Switzerland; <sup>6</sup>University of Lausanne and University Hospital Center, Lausanne, Switzerland.

\*Correspondence: Aurelie Jayol, aurelie.jayol@hotmail.fr

**Keywords:** polymyxin; epidemiology; *mcr-1* gene; rectal swabs; SuperPolymyxin.

**Abbreviations:** BMD, broth microdilution; ESBL, extended-spectrum  $\beta$ -lactamase; MIC, minimum inhibitory concentration.

**Table 1.** Colistin-resistant *Enterobacteriaceae* isolates

Species	MIC of CS (mg l <sup>-1</sup> )	Additional antibiotic resistances	ESBL	Unit
<i>E. coli</i>	4	AMX, TIC, SXT	No	Emergency
<i>E. coli</i>	4	AMX, TIC, FQ, GM	No	Surgery ICU
<i>E. coli</i>	8	AMX, C1G	No	Neurosurgery ICU
<i>K. pneumoniae</i>	64	FQ, SXT	No	Medical ICU
<i>K. pneumoniae</i>	32	AMX, TIC, C3G, C4G, FQ, FSF, FT, GM, SXT	Yes	Surgery
<i>R. ornithinolytica</i>	16	No	No	Gastroenterology
<i>E. cloacae</i>	64	AMX, TIC, C3G, C4G, FT, GM, SXT	Yes	Medical ICU
<i>E. cloacae</i>	8	FT	No	Emergency
<i>S. maltophilia</i>	128	-	No	Medical ICU

AMX, amoxicillin; C1G, first-generation cephalosporin; C3G, third-generation cephalosporin; C4G, fourth-generation cephalosporin; CS, colistin; ESBL, extended-spectrum beta-lactamase; FQ, fluoroquinolone; FSF, fosfomycin; FT, nitrofurantoin; GM, gentamicin; ICU, intensive care unit; SXT, cotrimoxazole; TIC, ticarcillin.

antibiotics (Table 1). Only two isolates (a single *K. pneumoniae* and a single *E. cloacae*) were multidrug resistant and produced an extended-spectrum  $\beta$ -lactamase. All those isolates were negative for the *mcr-1* and *mcr-2* genes. The two colistin-resistant *E. cloacae* isolates presented 'skip wells' with the BMD method, suggesting a heteroresistant phenotype. These isolates could belong to a cluster-dependent colistin-heteroresistant complex, as described recently [7].

Out of the nine patients carrying an acquired colistin-resistance trait, five were hospitalized in an intensive care unit, and two were from the community. None of the patients had previously received a colistin-based treatment, further highlighting the occurrence of colistin-resistant isolates without obvious corresponding antibiotic selection, as previously notified by Olaitan *et al* [8].

This prospective study indicates a high prevalence (23 %) of faecal carriage with intrinsic colistin-resistant Gram-negative rods, but a low prevalence (1.4 %) with isolates showing acquired resistance. No isolate carried the plasmid-mediated *mcr-1* and *mcr-2* genes, suggesting that there is currently low diffusion of these resistance determinants among human clinical samples. Similar observations were recently made during hospital- and community-based surveys in Switzerland [9]. However, single *E. coli* and *K. pneumoniae* isolates with a plasmid-borne *mcr-1* gene were isolated in 2016 from clinical infections in our hospital (A. Jayol, personal communication). Both isolates were ESBL producers and the *E. coli* one was recovered from a patient without a recent history of travel. Regular screening of plasmid-mediated colistin resistance, which is now quite easy to implement owing to the availability of a selective medium (SuperPolymyxin), may now be important to monitor and therefore possibly detect at an early stage the emergence of

colistin-resistant isolates, with the ultimate goal of preventing their further spread in France.

#### Funding information

This work was supported by internal funding from the University of Bordeaux, France, and the University of Fribourg, Switzerland.

#### Conflicts of interest

The authors declare that there are no conflicts of interest.

#### References

- Liu YY, Wang Y, Walsh TR, Yi LX, Zhang R *et al*. Emergence of plasmid-mediated colistin resistance mechanism MCR-1 in animals and human beings in China: a microbiological and molecular biological study. *Lancet Infect Dis* 2016;16:161–168.
- Xavier BB, Lammens C, Ruhel R, Kumar-Singh S, Butaye P *et al*. Identification of a novel plasmid-mediated colistin-resistance gene, *mcr-2*, in *Escherichia coli*, Belgium, June 2016. *Euro Surveill* 2016; 21.
- Skov RL, Monnet DL. Plasmid-mediated colistin resistance (*mcr-1* gene): three months later, the story unfolds. *Euro Surveill* 2016;21.
- Haenni M, Poirer L, Kieffer N, Châtre P, Saras E *et al*. Co-occurrence of extended spectrum  $\beta$  lactamase and MCR-1 encoding genes on plasmids. *Lancet Infect Dis* 2016;16:281–282.
- Nordmann P, Jayol A, Poirer L. A universal culture medium for screening polymyxin-resistant gram-negative isolates. *J Clin Microbiol* 2016;54:1395–1399.
- EUCAST. *Breakpoints tables for interpretation of MICs and zone diameters, Version 1.0*. EUCAST. 2016.
- Guérin F, Isnard C, Sinel C, Morand P, Dhalluin A *et al*. Cluster-dependent colistin hetero-resistance in *Enterobacter cloacae* complex. *J Antimicrob Chemother* 2016;71:3058–3061.
- Olaitan AO, Morand S, Rolain JM. Emergence of colistin-resistant bacteria in humans without colistin usage: a new worry and cause for vigilance. *Int J Antimicrob Agents* 2016;47:1–3.
- Liassine N, Assouvie L, Descombes MC, Tendon VD, Kieffer N *et al*. Very low prevalence of MCR-1/MCR-2 plasmid-mediated colistin resistance in urinary tract *Enterobacteriaceae* in Switzerland. *Int J Infect Dis* 2016;51:4–5.

## **D. Mécanismes chromosomiques de résistance à la colistine**

### **1. Diversité des mécanismes chromosomiques de résistance chez *K. pneumoniae***

#### **1.1. Identification d'une mutation dans la protéine PmrB responsable de l'acquisition d'une résistance à la colistine**

##### **Contexte :**

Lorsque ce travail a débuté, des études réalisées chez *P. aeruginosa* et *A. baumannii* avaient révélé l'implication de mutations dans les gènes des systèmes à deux composants PmrA/PmrB et PhoP/PhoQ, dans l'acquisition de la résistance à la colistine (116–118), mais les mécanismes chromosomiques de résistance à la colistine chez *K. pneumoniae* étaient encore inconnus.

##### **Objectif de l'étude :**

L'objectif de cette étude était donc de rechercher les mécanismes responsables de l'acquisition de résistances à la colistine dans une collection de souches de *K. pneumoniae*.

##### **Matériel et méthodes :**

Trente-cinq souches de *K. pneumoniae* résistantes à la colistine isolées en Afrique du Sud, Colombie, et Turquie ont été incluses dans cette étude. Les CMI de la colistine ont été déterminées par dilutions en milieu liquide. L'opéron *pmrAB* a été séquencé pour rechercher des mutations. Une analyse *in silico* à l'aide du logiciel EMBOSS 6.3.1 a été réalisée pour prédire l'impact des mutations observées sur la modification de la structure secondaire de la protéine PmrB. Des expériences de complémentation ont été réalisées par clonage du gène sauvage *pmrB* dans un plasmide vecteur et électroporation du plasmide recombinant dans les souches résistantes à la colistine. De plus, l'expression des différents gènes *pmr* impliqués dans la modification du lipopolysaccharide a été évaluée par RT-PCR. Enfin, l'étude de la clonalité des souches présentant les mêmes mutations au sein du gène *pmrB* a été réalisée par PFGE et par détermination des séquence-type par MLST.

##### **Principaux résultats :**

Une substitution T157P a été identifiée dans la protéine PmrB de six souches de *K. pneumoniae* résistantes à la colistine (CMI de la colistine égales à 16 mg/L). D'après l'analyse *in silico*, cette mutation semblait être à l'origine d'une modification de la structure secondaire de la protéine (interruption de l'hélice alpha). L'étude de clonalité a révélé que ces six souches appartenaient à quatre clones différents. Les expériences de complémentation de ces souches avec le gène *pmrB* sauvage ont conduit à une restauration de la sensibilité à la colistine (CMI = 1-2 mg/L) et ont ainsi confirmé l'implication de la mutation présente

au sein du gène *pmrB* dans l'acquisition de la résistance à la colistine. Enfin, des expériences de RT-PCR ont révélé que cette mutation PmrB T157P entraînait la surexpression des opérons *pmrCAB* et *pmrHFIJKLM* responsable de l'ajout de groupements cationiques à la surface du lipopolysaccharide et par conséquent de l'acquisition de la résistance à la colistine.

### **Conclusion :**

Nous avons identifié pour la première fois un mécanisme de résistance à la colistine chez *K. pneumoniae*. Nous avons identifié une substitution T157P dans la protéine PmrB responsable de l'acquisition de la résistance à la colistine dans cette espèce. Cette substitution a été identifiée chez six souches appartenant à quatre clones différents et isolées sur trois continents différents, ce qui suggère que la position 157 dans la protéine PmrB constitue un site privilégiée de mutation (« hot spot ») pour l'acquisition de la résistance à la colistine chez *K. pneumoniae*.

**Ce travail a été publié dans *Antimicrobial Agents and Chemotherapy* le 9 juin 2014 (IF = 4.415).**

# Resistance to Colistin Associated with a Single Amino Acid Change in Protein PmrB among *Klebsiella pneumoniae* Isolates of Worldwide Origin

Aurélie Jayol,<sup>a</sup> Laurent Poirel,<sup>a,b</sup> Adrian Brink,<sup>d</sup> Maria-Virginia Villegas,<sup>e</sup> Mesut Yilmaz,<sup>f</sup> Patrice Nordmann<sup>a,b,c</sup>

INSERM U914, South-Paris Medical School, K-Bicêtre, France<sup>a</sup>; Medical and Molecular Microbiology Unit, Department of Medicine, Faculty of Science, University of Fribourg, Fribourg, Switzerland<sup>b</sup>; Hôpital Fribourgeois–Hôpital Cantonal de Fribourg, Fribourg, Switzerland<sup>c</sup>; Department of Clinical Microbiology, Ampath National Laboratory Services, Milpark Hospital, Johannesburg, South Africa<sup>d</sup>; International Center for Medical Research and Training, CIDEIM, Cali, Colombia<sup>e</sup>; Medical Microbiology Department, School of Medicine, Istanbul Medipol University, Istanbul, Turkey<sup>f</sup>

A series of colistin-resistant *Klebsiella pneumoniae* isolates recovered from different countries was investigated in order to evaluate the involvement of the PmrA/PmrB two-component system in this resistance. Six isolates possessed a mutated PmrB protein, which is encoded by the *pmrB* gene, part of the *pmrCAB* operon involved in lipopolysaccharide modification. The same amino acid substitution (Thr157Pro) in PmrB was identified in the six isolates. The six isolates belonged to four distinct clonal groups, recovered in South Africa (sequence type 14 [ST14]), Turkey (ST101), and Colombia (ST258 and ST15). Three out of the four clones produced a carbapenemase, OXA-181, OXA-48, or KPC-3, while a single isolate did not produce any carbapenemase. Expression assays revealed an overexpression of the *pmrA* (70-fold), *pmrB* (70-fold), *pmrC* (170-fold), and *pmrK* (40-fold) genes in the *pmrB*-mutated isolate compared to expression of the *pmrB* wild-type isogenic *K. pneumoniae* isolate, confirming that the PmrB substitution was responsible for increased expression levels of those genes. Complementation assays leading to the expression of a wild-type PmrB protein restored the susceptibility to colistin in all isolates, confirming that the substitution in PmrB was responsible for the resistance phenotype. This study identified a key amino acid located in the PmrB protein as being responsible for the overexpression of *pmrCAB* and *pmrHFIJKLM* operons, leading to resistance to colistin.

*Klebsiella pneumoniae* is a Gram-negative pathogen often associated with nosocomial infections, including pneumonia, bacteremia, urinary tract infection, and sometimes even life-threatening septic shock (1). Infection caused by multidrug-resistant (MDR) *K. pneumoniae* is now a worldwide issue, considering the wide dissemination of MDR clones. In particular, the recent worldwide emergence of carbapenemase-producing isolates has often resulted in very limited therapeutic options. As a consequence, polymyxin antibiotics are now considered the last resort in the armamentarium for treatment of infections caused by MDR Gram-negative pathogens.

Polymyxins are derivatives of the *Bacillus polymyxa* subspecies *colistinus* that are active only against Gram-negative bacteria. Structurally, they are decapeptides bound to a fatty acid chain (2). The clinically available forms, polymyxin B and colistin (also known as polymyxin E), are administered intravenously or by inhalation. Like other antimicrobial peptides (APs), colistin interacts with the lipid A moiety of the Gram-negative bacterial lipopolysaccharide (LPS). The polycationic peptide ring competes for and substitutes the calcium and magnesium bridges stabilizing the LPS, promoting membrane permeability and thus disrupting the integrity of the outer membrane of Gram-negative bacteria, thus leading to bacterial death (2).

Nevertheless, resistance to polymyxins has been reported in *K. pneumoniae*, but little is known about the molecular mechanisms sustaining this resistance trait. The two-component regulatory systems (TCS) PmrA/PmrB and PhoP/PhoQ have been identified as regulatory systems involved in the resistance to polymyxin B (3). The insertional inactivation of the PhoQ/PhoP *mgrB*-encoding regulator has also been recently associated with colistin resistance (4, 5).

The main mechanisms leading to resistance to polymyxins are modifications of the bacterial outer membrane, mainly through the covalent addition of both phosphoethanolamine (pEtN) and 4-deoxyaminoarabinose (Lara4N) to the LPS (6–8). The *pmrCAB* operon encodes the PmrC phosphoethanolamine phosphotransferase, the PmrA response regulator (also named BasR), and the PmrB sensor kinase (also named BasS) (6). In *Escherichia coli* and *Salmonella enterica* species, PmrB acts as a sensor cytoplasmic membrane-bound kinase activated by high concentrations of iron (Fe<sup>3+</sup>) and an acidic pH (pH 5.5). Upon activation, it activates PmrA by phosphorylation (2). *In vivo*, PmrAB is involved in sensing the environment and is required for intramacrophage survival and virulence (6). Mutations in the *pmrA* or *pmrB* genes usually result in constitutive activation of PmrA, which upregulates in turn three loci, namely, *pmrC* encoding an aminotransferase involved in the decoration of the LPS with pEtN, *pmrE* (previously identified as *pagA* or *ugd*) encoding a UDP-glucose dehydrogenase, which is the first enzyme in the Ara4N biosynthetic pathway, and the *pmrHFIJKLM* operon (also called *pmrF*, *pbg*, or *arn* operon) encoding enzymes responsible for the synthesis and

Received 13 January 2014 Returned for modification 25 February 2014

Accepted 30 May 2014

Published ahead of print 9 June 2014

Address correspondence to Patrice Nordmann, patrice.nordmann@unifr.ch.

Supplemental material for this article may be found at <http://dx.doi.org/10.1128/AAC.00084-14>.

Copyright © 2014, American Society for Microbiology. All Rights Reserved.

doi:10.1128/AAC.00084-14



transfer of the Lara4N to lipid A (6). Overall, this constitutive activation of PmrA leads to a more positively charged LPS, thus reducing the affinity of positively charged polymyxins.

Using a collection of *K. pneumoniae* isolates recovered worldwide, in this study we aimed to investigate whether the mechanism leading to resistance to colistin might be related to the PmrAB two-component system.

## MATERIALS AND METHODS

**Bacterial strains and growth conditions.** Thirty-five colistin-resistant *K. pneumoniae* clinical isolates were included in this study. They had been collected in France, Turkey, Colombia, and South Africa. Isolates were identified by using the API20E system (bioMérieux, Marcy l'Etoile, France). *E. coli* TOP10 (Invitrogen, Illkirch, France) was used as the host strain for cloning experiments with selection based on kanamycin (50 µg/ml). Colistin-resistant *K. pneumoniae* clinical isolates were also used for transformation assays and selection based on zeocin (a formulation of phleomycin D1, a glycopeptide antibiotic produced by *Streptomyces verticillus*) (100 µg/ml).

**Antimicrobial susceptibility assays.** The antibiotic susceptibility testing was performed using Etest strips (AB bioMérieux, La Balme-les-Grottes, France) on Mueller-Hinton agar plates (Bio-Rad, Marnes-la-Coquette, France) with 0.5 McFarland standard inoculum. MICs were also determined by broth microdilution in cation-adjusted Mueller-Hinton broth (MHB-CA) according to CLSI guidelines (9–11). Polymyxin B and colistin (Sigma-Aldrich, Saint-Quentin Fallavier, France) were tested with Tween 80 (a surfactant preventing binding of colistin to drug panels) over a range of dilution from 0.125 to 64 µg/ml (9, 12). Briefly, MHB-CA with a final concentration of 0.002% Tween 80 and  $5 \times 10^5$  CFU/ml in each well was used. Following the EUCAST breakpoints (<http://www.eucast.org/>), isolates with a colistin MIC of  $\leq 2$  µg/ml were categorized as susceptible, although those with MICs of  $> 2$  µg/ml were resistant.

**PCR amplification and sequencing.** The chromosomal DNA was isolated using the commercially available QIAquick kit (Qiagen, Courtaboeuf, France), according to the manufacturer's instructions. The *pmrA*, *pmrB*, *phoP*, *phoQ*, and *mgrB* genes possibly involved in resistance to colistin were amplified using specific oligonucleotides (Table 1). The PCR amplification for detection of the narrow- and expanded-spectrum  $\beta$ -lactamase genes *bla*<sub>CTX-M</sub>, *bla*<sub>SHV</sub>, *bla*<sub>TEM</sub>, *bla*<sub>OXA</sub>, *bla*<sub>OXA-48-like</sub>, and *bla*<sub>KPC</sub> was carried out as described previously (13, 14). The amplified DNA fragments were purified with the QIAquick PCR purification kit (Qiagen). Both strands of the amplification products obtained were sequenced with an ABI 3100 sequencer (Applied Biosystems, Foster City, CA). The nucleotide and deduced protein sequences were analyzed at the National Center for Biotechnology Information website ([www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)) by the Basic Local Alignment Search Tool (BLAST) program.

**Analysis of the primary and secondary structures of the PmrB protein.** The primary structure of the PmrB protein was analyzed using the Ensembl Bacteria database (<http://bacteria.ensembl.org/index.html>). The predicted secondary structures of the two PmrB proteins (wild-type and mutated) were obtained using the GOR method with EMBOSS 6.3.1 software, available on the Mobylye@Pasteur portal (<http://mobylye.pasteur.fr/cgi-bin/portal.py>).

**Complementation assays.** The *pmrB* gene from the *K. pneumoniae* reference strain ATCC 53153 (colistin MIC of 0.125 µg/ml) was amplified by PCR using 2× Phusion HF master mix (Finnzymes; Life Technologies, Illkirch, France) and primers *pmrB* ext F and *pmrB* ext R. The partial and therefore noncoding *mdh* sequence (supposed to encode a malate dehydrogenase [15]) was PCR amplified with primers *mdh* ext F and *mdh* ext R (Table 1). Using the Zero Blunt TOPO PCR cloning kit (Invitrogen), the amplified fragments were cloned into the high-copy-number plasmid pCR-BluntII-TOPO encoding resistance to kanamycin and zeocin. The resulting plasmids pTOPO-*pmrB* and pTOPO-*mdh* were separately transformed into electrocompetent *E. coli* TOP10 strains by electroporation. Transformants were selected by overnight incubation at 37°C on Mueller-

TABLE 1 Oligonucleotides used in this study

Primer	Sequence (5' to 3')	Gene	Reference or source
<i>pmrA</i> ext F	CAT TTC CGC GCA CTG TCT GC	<i>pmrA</i>	This study
<i>pmrA</i> ext R	CAG CTT TCA GTT GCA AAC AG	<i>pmrA</i>	This study
<i>pmrB</i> ext F	ACC TAC GCG AAA AGA TTG GC	<i>pmrB</i>	This study
<i>pmrB</i> ext R	GAT GAG GAT AGC GCC CAT GC	<i>pmrB</i>	This study
<i>phoP</i> ext F	GAG CTT CAG ACT ACT ATC GA	<i>phoP</i>	This study
<i>phoP</i> ext R	GGG AAG ATA TGC CGC AAC AG	<i>phoP</i>	This study
<i>phoQ</i> ext F	ATA CCC ACA GGA CGT CAT CA	<i>phoQ</i>	This study
<i>phoQ</i> ext R	CAG GTG TCT GAC AGG GAT TA	<i>phoQ</i>	This study
<i>mgrB</i> ext F	TTA AGA AGG CCG TGC TAT CC	<i>mgrB</i>	4
<i>mgrB</i> ext R	AAG GCG TTC ATT CTA CCA CC	<i>mgrB</i>	4
<i>mdh</i> ext F	CCC AAC TCG CTT CAG GTT CAG	<i>mdh</i>	15
<i>mdh</i> ext R	CCG TTT TTC CCC AGC AGC AG	<i>mdh</i>	15
<i>pmrC</i> int F	GCG TGA TGA ATA TCC TCA CCA	<i>pmrC</i>	This study
<i>pmrC</i> int R	CAC GCC AAA GTT CCA GAT GA	<i>pmrC</i>	This study
<i>pmrA</i> int F	GAT GAA GAC GGG CTG CAT TT	<i>pmrA</i>	This study
<i>pmrA</i> int R	ACC GCT AAT GCG ATC CTC AA	<i>pmrA</i>	This study
<i>pmrB</i> int F	TGC CAG CTG ATA AGC GTC TT	<i>pmrB</i>	This study
<i>pmrB</i> int R	TTC TGG TTG TTG TGC CCT TC	<i>pmrB</i>	This study
<i>pmrD</i> int F	GAT CGC AGA GAT TGA AGC CT	<i>pmrD</i>	This study
<i>pmrD</i> int R	GCG TTG CGG ATC TTC AAA GT	<i>pmrD</i>	This study
<i>pmrE</i> int F	GCA TAC CGT AAT GCC GAC TA	<i>pmrE</i>	This study
<i>pmrE</i> int R	GGG TTG ATC TCT GTG ACA TC	<i>pmrE</i>	This study
<i>pmrK</i> int F	AGT ATC GGT CAG TGG CTG TT	<i>pmrK</i>	This study
<i>pmrK</i> int R	CCG CTT ATC ACG AAA GAT CC	<i>pmrK</i>	This study
<i>rpsL</i> int F	CCG TGG CGG TCG TGT TAA AGA	<i>rpsL</i>	4
<i>rpsL</i> int R	GCC GTA CTT GGA GCG AGC CTG	<i>rpsL</i>	4

Hinton agar supplemented with kanamycin (50 µg/ml). Plasmids were then isolated using the QIAprep spin miniprep column kit (Qiagen) and transformed into colistin-resistant *K. pneumoniae* clinical isolates. Transformants were selected by overnight incubation at 37°C on Mueller-Hinton agar supplemented with zeocin (100 µg/ml), and the presence of the cloned gene was checked by PCR. The MICs of transformants for colistin and polymyxin were determined as described above.

**Strain genotyping by PFGE and MLST.** The genetic relatedness of the isolates was assessed by pulsed-field gel electrophoresis (PFGE) analysis with XbaI-digested genomic DNA as described previously (16). Multilocus sequence typing (MLST) was carried out with seven standard house-keeping loci (*rpoB*, *gapA*, *mdh*, *pgi*, *phoE*, *infB*, and *tonB*) according to Diancourt et al. (15). Sequence types were analyzed by using the Institut Pasteur database (<http://www.pasteur.fr/recherche/genopole/PF8/mlst/Kpneumoniae.html>).

**Growth curves.** In order to evaluate the putative impact of modified LPS, growth curves were determined. Briefly, 200 ml Luria broth was inoculated independently with  $10^8$  CFU of each strain, and cultures were grown for 24 h at 37°C under gentle shaking. Absorbance at a wavelength of 600 nm was measured during 24 h. The colony counting was performed by serial dilution and final plating on solid medium. Experiments were repeated three times. Zeocin (100 µg/ml) was added to the growth medium in order to select the recombinant plasmids when *K. pneumoniae* strain AF1b complemented with pTOPO-*pmrB* or pTOPO-*mdh* was used.

**Transcriptional analysis by quantitative real-time PCR.** Quantitative real-time PCR (qRT-PCR) was used to measure the expression of the *pmrC*, *pmrA*, *pmrB*, *pmrD*, *pmrE*, and *pmrK* genes, using the primers listed in Table 1. RNA preparations were obtained using the RNeasy minikit (Qiagen), according to the manufacturer's instructions. qRT-PCR was carried out using a Rotor-Gene Q instrument (Qiagen). Expression of the *rpsL* gene encoding a ribosomal protein was evaluated with the primers *rpsL* int F and *rpsL* int R (Table 1), and this expression rate was used as a signal reporter (4). QuantiFast SYBR green (Qiagen) was used as an in-



**TABLE 2** MICs, molecular features, and genotyping analysis of *K. pneumoniae* isolates carrying the *pmrB* gene mutation and of isolate AF1a, the isogenic colistin-susceptible counterpart of AF1b

Isolate	Country of isolation	MIC ( $\mu\text{g/ml}$ ) for <sup>a</sup> :					Colistin profile <sup>b</sup>	PmrB amino acid change	Carbapenemase	ESBL <sup>c</sup>	Additional $\beta$ -lactamases	MLST <sup>d</sup>	PFGE <sup>e</sup> pulsotype
		ERT	IPM	MEM	DOR	CST							
AF1a	South Africa	>32	>32	>32	>32	0.125	S	WT <sup>f</sup>	OXA-181	CTX-M-15	TEM-1, SHV-1	ST14	1
AF1b	South Africa	>32	>32	>32	>32	3	R	T157P	OXA-181	CTX-M-15	TEM-1, SHV-1	ST14	1
C3	Colombia	>32	>32	>32	>32	4	R	T157P	KPC-3	None	TEM-1, SHV-1	ST258	2
C4	Colombia	>32	>32	>32	>32	4	R	T157P	KPC-3	None	TEM-1, SHV-1	ST258	2
C19	Colombia	0.25	0.19	0.094	0.064	4	R	T157P	None	None	TEM-1, SHV-1	ST15	3
T2	Turkey	16	>32	>32	24	6	R	T157P	OXA-48	CTX-M-15	TEM-1, SHV-1, OXA-1	ST101	4
T3	Turkey	16	>32	>32	24	6	R	T157P	OXA-48	CTX-M-15	TEM-1, SHV-1, OXA-1	ST101	4

<sup>a</sup> ERT, ertapenem; IPM, imipenem; MEM, meropenem; DOR, doripenem; CST, colistin.

<sup>b</sup> R, resistant; S, susceptible.

<sup>c</sup> ESBL, extended-spectrum  $\beta$ -lactamase.

<sup>d</sup> MLST, multilocus sequence type; ST, sequence type.

<sup>e</sup> PFGE, pulsed-field gel electrophoresis.

<sup>f</sup> WT, wild type.

ternal standard (4). Data were compared to those obtained with the *rpsL* gene using the threshold cycle ( $\Delta\Delta C_T$ ) method (relative), and the obtained values were then normalized against the values obtained for the susceptible isolate. Experiments were repeated three times.

**Nucleotide sequence accession number.** The nucleotide and protein sequences of the mutated PmrB protein were registered in GenBank under accession no. [KJ626267](https://www.ncbi.nlm.nih.gov/nuclseq/KJ626267).

## RESULTS

**A point mutation in the *pmrB* gene of colistin-resistant *K. pneumoniae* isolates.** Sequence analysis of the *pmrB* genes of all the colistin-resistant clinical isolates from our collection revealed the same single-nucleotide substitution (A to C) in six isolates (AF1b, C3, C4, C19, T2, and T3) compared to those in the *pmrB* gene sequences from *K. pneumoniae* available in GenBank (Table 2). No amino acid substitutions were identified in the PmrA, PhoP, PhoQ, and MgrB proteins in those isolates compared with sequences obtained from wild-type isolates (data not shown). This mutated PmrB protein differed from the wild type by a single threonine to proline amino acid substitution at position 157. Since a colistin-susceptible isolate, AF1a (colistin MIC of 0.125  $\mu\text{g/ml}$ ), recovered from the same patient from whom the colistin-resistant isolate AF1b had been recovered was available (17), its *pmrB* gene was also sequenced. A Thr residue was identified at position 157 (as for the other wild-type strains), thus reinforcing the hypothesis that Thr157Pro might play a key role in acquired resistance to colistin (Table 2).

**Impact of amino acid 157 substitution on the structure of the PmrB protein.** By analyzing the primary structure of the PmrB protein, amino acid 157 was identified in the PmrB domain, which is involved in the dimerization of the protein. We might speculate that the replacement of a polar by a nonpolar amino acid in this domain may have a significant impact in the dimerization process of PmrB and therefore induce constitutive activation of PmrA.

The predicted secondary structures of the two PmrB proteins (wild type and mutated) revealed that the Thr157Pro amino acid substitution significantly modified the secondary structure of the mutated protein, with an interruption of the alpha-helix (see Fig. S1 in the supplemental material).

**Thr157Pro substitution in protein PmrB identified among clonally unrelated colistin-resistant *K. pneumoniae* isolates.** The genetic relationship of the six colistin-resistant isolates exhibiting the same Thr157Pro substitution, together with the colistin-

susceptible isolate AF1a, was evaluated. PFGE analysis confirmed that isolates AF1a and AF1b recovered from a single patient were clonally related (17) (data not shown). In addition, isolates C3 and C4, recovered from two patients from the same hospital, were also clonally related, and isolates T2 and T3, again from two patients from the same hospital, were clonally related (data not shown). Overall, four colistin-resistant clones were identified (Table 2). Further data obtained by MLST were in accordance with the PFGE results (Table 2).

**Identification of additional resistance mechanisms to broad-spectrum  $\beta$ -lactams.** The clonally related isolates AF1a (colistin-susceptible) and AF1b (colistin-resistant) recovered from South Africa were found to produce the OXA-181 (OXA-48-like) carbapenemase (Table 2). The clonally related C3 and C4 isolates from Colombia produced carbapenemase KPC-3. Isolate C19 from Colombia, clonally unrelated compared to other isolates in this study, did not produce any carbapenemase. Finally, clonally related isolates T2 and T3 recovered from Turkey produced carbapenemase OXA-48.

**Complementation experiments.** To determine whether resistance to colistin might be related to the PmrB mutation, complementation experiments were performed for each clonally unrelated strain (AF1b, C3, C19, and T2). The wild-type *pmrB* gene was cloned into the high-copy-number plasmid pCR-BluntII-TOPO, which was then transformed into the colistin-resistant clinical isolates. The results of complementation showed a complete reversion to susceptibility to colistin when we transformed each of those resistant isolates with plasmid pTOPO-*pmrB* (Table 3). As expected, transformation with plasmid pTOPO-*mdh* used as a negative control did not restore the susceptibility to colistin.

Additionally, three other clonally unrelated and colistin-resistant *K. pneumoniae* for which no substitution was identified in PmrB were transformed with plasmid pTOPO-*pmrB* encoding the wild-type PmrB. MICs of colistin remained unchanged, thus showing that restoration of the susceptibility to colistin by complementation with a wild-type PmrB protein was not due to PmrB overproduction. This was also a direct proof of the involvement of the protein PmrB in the resistance pattern to colistin.

**Growth rates of colistin-susceptible and colistin-resistant strains.** No difference was observed between the growth of isogenic *K. pneumoniae* AF1a (colistin-susceptible) and AF1b (colistin-resistant) isolates, thus suggesting that the PmrB substitution and

**TABLE 3** MIC results for colistin and polymyxin B determined by broth microdilution before and after complementation with plasmids pTOPO-*mdh* (negative control) and pTOPO-*pmrB* for the four clonally unrelated and colistin-resistant *K. pneumoniae* isolates

Isolate	MIC ( $\mu\text{g/ml}$ ) of colistin:			MIC ( $\mu\text{g/ml}$ ) of polymyxin B:		
	Before complementation	After complementation with pTOPO- <i>mdh</i>	After complementation with pTOPO- <i>pmrB</i>	Before complementation	After complementation with pTOPO- <i>mdh</i>	After complementation with pTOPO- <i>pmrB</i>
AF1b	16	16	1	8	8	1
C3	16	16	2	8	8	1
C19	16	16	1	8	8	2
T2	16	16	1	8	8	2

the subsequent modification of the LPS did not modify the growth properties of resistant isolates (data not shown). This observation was further confirmed by the lack of difference between the growth curves obtained for *K. pneumoniae* AF1b complemented with wild-type PmrB protein (plasmid pTOPO-*pmrB*) (colistin-susceptible) or not complemented with PmrB but with plasmid pTOPO (pTOPO-*mdh*) (colistin-resistant) (data not shown).

**Thr157Pro substitution in PmrB mutation associated with overexpression of *pmrCAB* and *pmrHFIJKLM* operons.** Expression levels of the *pmr* genes were measured in order to evaluate the impact of the *pmrB* mutation. RT-PCR assays were performed with isolates AF1a and AF1b, since they corresponded to likely isogenic strains. Upregulation of the expression levels of the *pmrC*, *pmrA*, *pmrB*, and *pmrK* genes was observed for AF1b compared to that for the isogenic colistin-susceptible isolate AF1a. The relative mean increases were estimated to be 170-fold for *pmrC*, 70-fold for *pmrA*, 70-fold for *pmrB*, and 40-fold for *pmrK*, whereas no significant differences in expression levels were observed with the *pmrD* and *pmrE* genes (increases of 1.9- and 1.4-fold, respectively) (Fig. 1).

## DISCUSSION

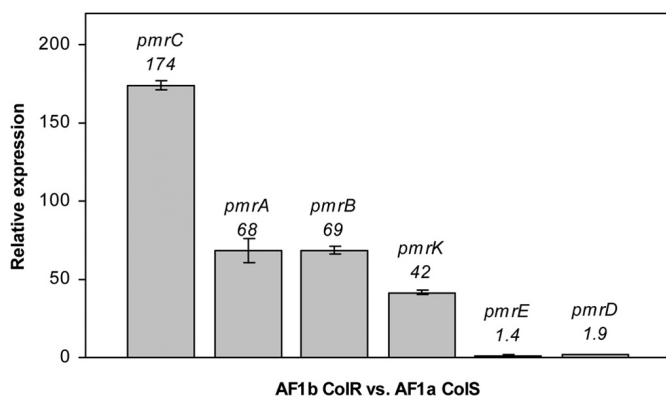
Since data on the molecular bases of colistin resistance in *K. pneumoniae* are scarce, we analyzed a collection of colistin-resistant isolates of worldwide origin. Interestingly, we found six isolates harboring an unique and identical amino acid substitution in the protein PmrB. Complementation assays performed with a wild-type PmrB protein generated a complete reversion of the colistin-resistant trait. This result suggested that the PmrB substitution identified (Thr157Pro) was involved in the resistance of the clin-

ical isolates. Of note, while this work was in progress, Choi and Ko (18) identified the same mutation in a colistin-resistant *K. pneumoniae* isolate and suggested that it might be involved in colistin resistance, but no further demonstration was provided to confirm this hypothesis. The involvement of the protein PmrB in resistance to colistin correlates results obtained with colistin-resistant *Pseudomonas aeruginosa* isolates (19) or colistin-resistant *Acinetobacter baumannii* isolates (20, 21).

Furthermore, since we had two very likely isogenic isolates (one susceptible and one resistant) recovered from the same patient, this hypothesis was confirmed by the identification of this same Thr to Pro substitution at position 157 in the colistin-resistant isolate. Of note, the colistin-resistant isolate had been recovered after a selective digestive tract decontamination process based on oral colistin administration (17). Considering that a single amino acid substitution in a single protein (here PmrB) may generate resistance to colistin, we suggest that colistin-based oral decontamination has to be used with caution.

The identification of four clonally unrelated *K. pneumoniae* clinical isolates harboring the same Thr157Pro substitution in PmrB is surprising considering that they have been recovered worldwide and do not correspond to the spread of a single clone. It strongly suggests that position 157 in protein PmrB is a key position for colistin resistance. It also suggests that the Pro residue might be a key residue for resistance to colistin in *K. pneumoniae*, in contrast to recent observations made in *P. aeruginosa* or *A. baumannii* isolates from which many different amino acid substitutions occurring in PmrB have been shown to be involved in colistin resistance (18–20). Further studies, using site-directed mutagenesis in PmrB, will be performed to evaluate our hypotheses, targeting different positions, including amino acids at position 157.

Analysis of the *pmrC*, *pmrA*, and *pmrB* transcription levels by qRT-PCR in isolate AF1b carrying a *pmrB* mutation compared to those in the susceptible and isogenic AF1a indicated that this mutation leads to an overexpression of the entire *pmrCAB* operon. This result suggests that the modified PmrB induces the constitutive activation of PmrA, which in turn autoregulates (activates) the *pmrCAB* promoter, as previously demonstrated in *Salmonella* spp. (6). Expression of the *pmrC* gene, being the first gene in the *pmrCAB* operon, has been shown to lead to phosphoethanolamine addition to lipid A in *Salmonella* spp. Moreover, analysis of the level of transcription of the *pmrK* gene, which belongs to the *pmrHFIJKLM* operon, confirmed that this operon was also activated by PmrA as described previously (8). The products of this operon are responsible for 4-deoxyaminoarabinose addition and therefore modification of the LPS target. Addition of both pEtN and Ara4N to the LPS creates a more positively charged LPS molecule and thus reduces the affinity of positively charged polymyxins (6–8).



**FIG 1** Relative expression levels of the *pmrC*, *pmrA*, *pmrB*, *pmrK*, *pmrE*, and *pmrD* genes in the AF1b colistin-resistant strain (ColR) compared with those in the AF1a colistin-susceptible strain (ColS). Values are the means and the standard deviations from three independent experiments.

The expression of the *pmrE* gene was not significantly modified here in the studied colistin-resistant *K. pneumoniae* isolates. Therefore, in contrast to what has been observed for *S. enterica* and *E. coli* (22), PmrA may upregulate *pmrE* expression in *K. pneumoniae*. Also, the expression of the *pmrD* gene was not significantly modified, which is different from what has been observed in *S. enterica* (23), for which the transcription of the *pmrD* gene was repressed by the PmrA protein. Those findings are in accordance with data obtained by Cheng et al. (3) showing that the *pmrD* expression is independent of PmrA/PmrB in *Klebsiella*.

In conclusion, we showed here that a specific point mutation in the PmrB protein is responsible for an upregulation of the *pmrCAB* and *pmrHFIJKLM* operons that confers resistance to colistin in *K. pneumoniae*. The identification of six clinical isolates of worldwide origin belonging to four distinct clones with this same substitution in PmrB suggests that this constitutes a clinically relevant mechanism of resistance in *K. pneumoniae*. In addition, it confirms that the PmrAB two-component system plays a major regulatory role in polymyxin B resistance in that species.

## ACKNOWLEDGMENTS

This work was funded by the INSERM, France, by the University of Fribourg, Switzerland, and by grants from the European Community (R-GNOSIS, FP7/HEALTH-F3-2011-282512, and MagicBullet, FP7/HEALTH-F3-2001-278232).

## REFERENCES

- Podschun R, Ullmann U. 1998. *Klebsiella* spp. as nosocomial pathogens: epidemiology, taxonomy, typing methods, and pathogenicity factors. Clin. Microbiol. Rev. 11:589–603.
- Falagas ME, Rafailidis PI, Matthaïou DK. 2010. Resistance to polymyxins: mechanisms, frequency and treatment options. Drug Resist. Updat. 13:132–138. <http://dx.doi.org/10.1016/j.drug.2010.05.002>.
- Cheng HY, Chen YF, Peng HL. 2010. Molecular characterization of the PhoPQ-PmrD-PmrAB mediated pathway regulating polymyxin B resistance in *Klebsiella pneumoniae* CG43. J. Biomed. Sci. 17:60. <http://dx.doi.org/10.1186/1423-0127-17-60>.
- Cannatelli A, D'Andrea MM, Giani T, Di Pilato V, Arena F, Ambretti S, Gaibani P, Rossolini GM. 2013. *In vivo* emergence of colistin resistance in *Klebsiella pneumoniae* producing KPC-type carbapenemase mediated by insertional inactivation of the PhoQ/PhoP *mgrB* regulator. Antimicrob. Agents Chemother. 57:5521–5526. <http://dx.doi.org/10.1128/AAC.01480-13>.
- López-Camacho E, Gómez-Gil R, Tobes R, Manrique M, Lorenzo M, Galván B, Salvarelli E, Moatassim Y, Salanueva JJ, Pareja E, Codoñer FM, Alvarez-Tejado M, Garcillán-Barcia MP, De la Cruz F, Mingorance J. 2014. Genomic analysis of the emergence and evolution of multidrug resistance during a *Klebsiella pneumoniae* outbreak including carbapenem and colistin resistance. J. Antimicrob. Chemother. 69:632–636. <http://dx.doi.org/10.1093/jac/dkt419>.
- Gunn JS. 2008. The *Salmonella* PmrAB regulon: lipopolysaccharide modifications, antimicrobial peptide resistance and more. Trends Microbiol. 16:284–290. <http://dx.doi.org/10.1016/j.tim.2008.03.007>.
- Breazeale SD, Ribeiro AA, McClarren AL, Raetz CR. 2005. A formyltransferase required for polymyxin resistance in *Escherichia coli* and the modification of lipid A with 4-amino-4-deoxy-L-arabinose. Identification and function of UDP-4-deoxy-4-formamido-L-arabinose. J. Biol. Chem. 280:14154–14167. <http://dx.doi.org/10.1074/jbc.M414265200>.
- Gunn JS, Ryan SS, Van Velkinburgh JC, Ernst RK, Miller SI. 2000. Genetic and functional analysis of a PmrA-PmrB-regulated locus necessary for lipopolysaccharide modification, antimicrobial peptide resistance, and oral virulence of *Salmonella enterica* serovar Typhimurium. Infect. Immun. 68:6139–6146. <http://dx.doi.org/10.1128/IAI.68.11.6139-6146.2000>.
- Hindler JA, Humphries RM. 2013. Colistin MIC variability by method for contemporary clinical isolates of multidrug-resistant Gram-negative bacilli. J. Clin. Microbiol. 51:1678–1684. <http://dx.doi.org/10.1128/JCM.03385-12>.
- Clinical and Laboratory Standards Institute. 2012. Methods for dilution of antimicrobial susceptibility tests for bacteria that grow aerobically; approved standard—9th ed. CLSI document M07-A9. Clinical and Laboratory Standards Institute, Wayne, PA.
- Clinical and Laboratory Standards Institute. 2014. Performance standards for antimicrobial susceptibility testing; 24th informational supplement. CLSI document M100-S24. Clinical and Laboratory Standards Institute, Wayne, PA.
- Sader HS, Rhomberg PR, Flamm RK, Jones RN. 2012. Use of a surfactant (polysorbate 80) to improve MIC susceptibility testing results for polymyxin B and colistin. Diagn. Microbiol. Infect. Dis. 74:412–414. <http://dx.doi.org/10.1016/j.diagmicrobio.2012.08.025>.
- Dortet L, Cuzon G, Nordmann P. 2014. Dissemination of carbapenemase-producing Enterobacteriaceae in France, 2012. J. Antimicrob. Chemother. 69:623–627. <http://dx.doi.org/10.1093/jac/dkt433>.
- Nordmann P, Naas T, Poirel L. 2011. Global spread of carbapenemase-producing Enterobacteriaceae. Emerg. Infect. Dis. 17:1791–1798. <http://dx.doi.org/10.3201/eid1710.110655>.
- Diancourt L, Passet V, Verhoef J, Grimont PA, Brisse S. 2005. Multilocus sequence typing of *Klebsiella pneumoniae* nosocomial isolates. J. Clin. Microbiol. 43:4178–4182. <http://dx.doi.org/10.1128/JCM.43.8.4178-4182.2005>.
- Carrèr A, Lassel L, Fortineau N, Mansouri M, Anguel N, Richard C, Nordmann P. 2009. Outbreak of CTX-M-15-producing *Klebsiella pneumoniae* in the intensive care unit of a French hospital. Microb. Drug Resist. 15:47–54. <http://dx.doi.org/10.1089/mdr.2009.0868>.
- Brink AJ, Coetzee J, Corcoran C, Clay CG, Hari-Makkan D, Jacobson RK, Richards GA, Feldman C, Nutt L, van Greune J, Deetlefs JD, Swart K, Denich L, Poirel L, Nordmann P. 2013. Emergence of OXA-48 and OXA-181 carbapenemases among *Enterobacteriaceae* in South Africa and evidence of *in vivo* selection of colistin resistance as a consequence of selective decontamination of the gastrointestinal tract. J. Clin. Microbiol. 51:369–372. <http://dx.doi.org/10.1128/JCM.02234-12>.
- Choi MJ, Ko KS. 2014. Mutant prevention concentrations of colistin for *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* clinical isolates. J. Antimicrob. Chemother. 69:275–277. <http://dx.doi.org/10.1093/jac/dkt315>.
- Moskowitz SM, Brannon MK, Dasgupta N, Pier M, Sgambati N, Miller AK, Selgrade SE, Miller SI, Denton M, Conway SP, Johansen HK, Hoiby N. 2012. PmrB mutations promote polymyxin resistance of *Pseudomonas aeruginosa* isolated from colistin-treated cystic fibrosis patients. Antimicrob. Agents Chemother. 56:1019–1030. <http://dx.doi.org/10.1128/AAC.05829-11>.
- Adams MD, Nickel GC, Bajaksouzian S, Lavender H, Murthy AR, Jacobs MR, Bonomo RA. 2009. Resistance to colistin in *Acinetobacter baumannii* associated with mutations in the PmrAB two-component system. Antimicrob. Agents Chemother. 53:3628–3634. <http://dx.doi.org/10.1128/AAC.00284-09>.
- Beceiro A, Llobet E, Aranda J, Bengoechea JA, Doumith M, Hornsey M, Dhanji H, Chart H, Bou G, Livermore DM, Woodford N. 2011. Phosphoethanolamine modification of lipid A in colistin-resistant variants of *Acinetobacter baumannii* mediated by the pmrAB two-component regulatory system. Antimicrob. Agents Chemother. 55:3370–3379. <http://dx.doi.org/10.1128/AAC.00079-11>.
- Breazeale SD, Ribeiro AA, Raetz CR. 2002. Oxidative decarboxylation of UDP-glucuronic acid in extracts of polymyxin-resistant *Escherichia coli*. Origin of lipid a species modified with 4-amino-4-deoxy-L-arabinose. J. Biol. Chem. 277:2886–2896. <http://dx.doi.org/10.1074/jbc.M109377200>.
- Mitrophanov AY, Jewett MW, Hadley TJ, Groisman EA. 2008. Evolution and dynamics of regulatory architectures controlling polymyxin B resistance in enteric bacteria. PLoS Genet. 4:e1000233. <http://dx.doi.org/10.1371/journal.pgen.1000233>.

Figure S1. Secondary structures of; panel A, the wild-type PmrB protein; panel B, protein PmrB exhibiting the Thr157Pro substitution. Position 157 is boxed in red.

(A)

```

      . 10 . 20 . 30 . 40 . 50
helix MALFAETWTRRRLLLTIGAILVQCQLISVFWLWHESEKQIQLLVASAI
sheet HHHHHHHHHHHHHHHH HHHH HHHHHHHHHHH
turns EEEEEEEEEEEEEEE T
coil CCC

      . 60 . 70 . 80 . 90 . 100
helix EGHNNQKHVEHEVREAVASLLVPSLLVGLALYISMLAVKRITRPLSRLQ
sheet HH HHHHHHHHHHHH H HHHHHHHH
turns EE EEEEEEE EEEE E
coil CCCCC C CCCCC

      . 110 . 120 . 130 . 140 . 150
SELENRTPDNLTPIVISESVPEVTAVTALNQLVSRINLPDRERLFTAD
helix HHHHHHHHH HHHHHHHH HHHHHHHH
sheet EEEEE EEEEE EEEEE
turns TTT TTT
coil CCC C C CCCC CCCC

      . 160 . 170 . 180 . 190 . 200
VAHELRIPlaglrlh1le1l1AKVHGVDPLIQRLDQMTTISQLLQLARV
helix HHHHHHHHHHHHHHHHHH H
sheet EEE EEE EEEEEEEEE
turns TT T
coil CCC CCCCC

      . 210 . 220 . 230 . 240 . 250
GQSFSAQSYQVLLDDVVKPLQDELEAMLAgrrqrl11TDIENEAVVSG
helix HHHHHHHHHHHHHH HHHHHHHH
sheet E EEEEEEE EEE EEE
turns T T
coil CCCC CC CCC

      . 260 . 270 . 280 . 290 . 300
DAPLIRVILLRNIVENARVSPGSTITRVSVKAGIMPMVAVEDEGPGIDEA
helix HHH HHH H H HHHH HHHH
sheet EEEEEEE EEEEE E EE
turns TTT TTT
coil CC CC C CCC CC

      . 310 . 320 . 330 . 340 . 350
KSGELSKAFVRMDSRYGGIGLGLSIVTRIAQLHDAQFLHNRRQPGPGVRA
helix HHHHHHHHH HHHHHHHH
sheet EE EEEEEEEEE EE EEE
turns TTTTTT TTTT
coil C C CCCCC

      . 360
WVLFPRGGQNVSTH
helix EEEE EEE
sheet TTTT
turns C CCC
coil

```



(B)

```

      . 10 . 20 . 30 . 40 . 50
MALFAITWTMRRLLLITGAILVCCQLISVFWLWHSKEQIQLLVASAI
helix HHHHHHHHHHHHHHHHHHH HHHH HHHHHHHHHHH
sheet EEEEEEEEEEEEEEE I
turns CCC
coil

      . 60 . 70 . 80 . 90 . 100
EGHNNQKHVEHEVREAVASLLVPSLLIVGLALYISMLAVKRIITRPLSLRQ
helix HH HHHHHHHHHHHHH H HHHHHHHHH
sheet EE EEEEEEE EEEE E
turns
coil CCCCC C CCCCC

      . 110 . 120 . 130 . 140 . 150
SELENRTPDNLTPIVISESVPEVTAVTTALNQLVSRINLPIDRERLFTAD
helix HHHHHHHHH HHHHHHHHH
sheet EEEEE EEEEE
turns TTT TTT
coil CCC C C CCCC CCCC

      . 160 . 170 . 180 . 190 . 200
VAHEIRPPIaglxrlh1le1lAKVHGVDPLIQRLDQMTTISIQLLQLARV
helix HHH HHHHHHHHHHHHH H H
sheet EEE EEE EEEEEEE
turns T T TT T
coil CCC CCC CCCCC

      . 210 . 220 . 230 . 240 . 250
GQSFAGSYQVILLDDVVKPLQDELEAMLAgqrqrl1ITDIENEAVVSQ
helix E EEEEEEE EEE EEE
sheet T T
turns T T
coil CCCC CC CCC

      . 260 . 270 . 280 . 290 . 300
DAPLIRVILLRNLVENARVSPGSTITIRVSKAGIMPVMAVEDEGPGIDEA
helix HHH HHH H H HHHHH HHHHH
sheet EEEEEEE EEEEE E EE
turns TTT TTT
coil CC CC C CCC CC

      . 310 . 320 . 330 . 340 . 350
KSGELSKAFVRMDSRYGGIGLGLSIVTRIAQLHDAQFLHNRRQPGPGVRA
helix HHHHHHHHH HHHHHHH
sheet EE EEEEEEE EE EEE
turns TTTTTT TTT
coil C C CCCCC

      . 360
WVLFPGRGQNVSTH
helix EEEE EEE
sheet TTTT
turns C CCC
coil
```

## **1.2. Identification d'altérations dans la protéine PhoP responsables de l'acquisition d'une hétérorésistance à la colistine**

### **Contexte :**

Lorsque ce travail a débuté, les mécanismes chromosomiques d'hétérorésistance à la colistine chez *K. pneumoniae* étaient inconnus. Des études réalisées chez *P. aeruginosa* et *A. baumannii* avaient révélé l'implication de mutations dans les gènes des systèmes à deux composants PmrA/PmrB et PhoP/PhoQ dans l'acquisition de résistances à la colistine (116–118), mais les mécanismes d'acquisition d'hétérorésistance à la colistine chez les BGN étaient inconnus.

### **Objectif de l'étude :**

L'objectif de cette étude était donc de rechercher le mécanisme responsable de l'acquisition d'une hétérorésistance à la colistine chez une souche clinique de *K. pneumoniae*.

### **Matériel et méthodes :**

Une souche de *K. pneumoniae* hétérorésistante à la colistine isolée en Afrique du Sud a fait l'objet de cette étude. Les CMI de la colistine ont été déterminées à l'aide de bandelettes Etest® et par dilutions en milieu liquide (méthode de référence). Les opérons *pmrAB* et *phoPQ* ont été séquencés pour rechercher des mutations. Une analyse *in silico* à l'aide du logiciel EMBOSS 6.3.1 a été réalisée pour prédire l'impact des mutations observées sur la modification de la structure secondaire de la protéine PhoP. Des expériences de complémentation ont été réalisées par clonage du gène sauvage *phoP* dans un plasmide vecteur et électroporation du plasmide recombinant dans les souches résistantes à la colistine. L'expression des différents gènes *pmr* et *pho* impliqués dans la modification du lipopolysaccharide a également été évaluée par RT-PCR.

### **Principaux résultats :**

Lors de la détermination des CMI de la colistine à l'aide de bandelette Etest®, une double zone a été observée suggérant la présence de deux sous-populations de sensibilités différentes à la colistine (hétérorésistance) dans cette souche de *K. pneumoniae*. Un séquençage des gènes *pmr* et *pho* a été réalisé sur chacune des sous-populations. Une substitution D191Y a été identifiée dans la protéine PhoP de la sous-population résistante à la colistine (CMI de la colistine égale à 128 mg/L selon la méthode de référence). D'après l'analyse *in silico*, cette mutation semblait être à l'origine d'une modification de la structure secondaire de la protéine (interruption de l'hélice alpha). L'expérience de complémentation de cette sous-population résistante avec le gène *phoP* sauvage a conduit à une restauration de la sensibilité (CMI = 0,12 mg/L selon la méthode de référence) et a ainsi confirmé l'implication de cette mutation dans l'acquisition de la résistance à la colistine. Des expériences de RT-PCR ont révélé que cette mutation entraînait la

surexpression de l'opéron *pmrHFIJKLM* responsable de l'ajout de groupements cationiques à la surface du lipopolysaccharide et donc de la résistance à la colistine. Par ailleurs, le séquençage du gène *phoP* de la sous-population sensible à la colistine (CMI = 0,12 mg/L selon la méthode de référence) a révélé la même substitution D191Y dans la protéine PhoP mais a aussi montré qu'une délétion partielle (25 pb) au sein du gène *phoP* s'était additionnée. Cette délétion décalait le cadre de lecture de *phoP*, conduisant à une protéine inactive plus longue (255 aa au lieu de 223 aa) et restaurait ainsi la sensibilité à la colistine. La présence de deux sous-populations (une sensible et une résistante) présentant des altérations différentes au sein du gène *phoP* était donc à l'origine de l'hétérorésistance observée.

### **Conclusion :**

Nous avons identifié pour la première fois un mécanisme d'hétérorésistance à la colistine chez *K. pneumoniae*. Cette étude suggère que l'hétérorésistance à la colistine dans la souche étudiée a été acquise en deux étapes : tout d'abord l'acquisition d'une résistance par mutation au sein du gène *phoP* puis la réversion de résistance d'une sous-population par délétion partielle du gène *phoP*.

**Ce travail a été publié dans *Antimicrobial Agents and Chemotherapy* en mars 2015 (IF = 4.415).**

# Heteroresistance to Colistin in *Klebsiella pneumoniae* Associated with Alterations in the PhoPQ Regulatory System

Aurélie Jayol,<sup>a</sup> Patrice Nordmann,<sup>a,b</sup> Adrian Brink,<sup>c</sup> Laurent Poirel<sup>a</sup>

Medical and Molecular Microbiology “Emerging Antibiotic Resistance” Unit, Department of Medicine, Faculty of Science, University of Fribourg,<sup>a</sup> and HFR–Hôpital Cantonal de Fribourg,<sup>b</sup> Fribourg, Switzerland; Department of Clinical Microbiology, Ampath National Laboratory Services, Milpark Hospital, Johannesburg, South Africa<sup>c</sup>

**A multidrug-resistant *Klebsiella pneumoniae* isolate exhibiting heteroresistance to colistin was investigated. The colistin-resistant subpopulation harbored a single amino acid change (Asp191Tyr) in protein PhoP, which is part of the PhoPQ two-component system that activates *pmrHFIJKLM* expression responsible for L-aminoarabinose synthesis and polymyxin resistance. Complementation assays with a wild-type *phoP* gene restored full susceptibility to colistin. Then, analysis of the colistin-susceptible subpopulation showed a partial deletion (25 bp) in the *phoP* gene compared to that in the colistin-resistant subpopulation. That deletion disrupted the reading frame of *phoP*, leading to a longer and inactive protein (255 versus 223 amino acids long). This is the first report showing the involvement of mutation(s) in PhoP in colistin resistance. Furthermore, this is the first study to decipher the mechanisms leading to colistin heteroresistance in *K. pneumoniae*.**

*Klebsiella pneumoniae* is a Gram-negative bacterium often associated with nosocomial infections, including urinary tract infection, pneumonia, and bloodstream infection (1). While multidrug resistance is increasingly reported in that species due to acquisition of numerous resistance traits, including extended-spectrum  $\beta$ -lactamase and carbapenemase genes, colistin is increasingly used for treating infections due to multidrug-resistant isolates. Colistin and polymyxin B correspond to important therapeutic options for treating infections caused by multidrug-resistant *K. pneumoniae*, particularly in countries with a high prevalence of carbapenemase producers (2). Those drugs are bactericidal for Gram-negative bacteria, interacting with the lipid A moiety of lipopolysaccharide (LPS) and subsequently causing disorganization of the outer membrane (3).

In *K. pneumoniae*, colistin resistance is being increasingly reported, and some cases of heteroresistant *K. pneumoniae* isolates have also been observed (4, 5). Colistin heteroresistance is defined as “the emergence of resistance to colistin by a subpopulation from an otherwise susceptible (MIC of  $\leq 2$  mg/liter) population” (6) that may be related to exposure to a suboptimal polymyxin concentration (7). A comparison of the *in vitro* susceptibility testing methods for colistin showed that the Etest and agar dilution methods are reliable for detecting resistant subpopulations in contrast to some automatic methods; nonetheless, the broth microdilution method remains the reference test for determination of colistin MICs (8).

Several studies have shown that mutations in the PmrAB or PhoPQ regulatory systems may confer colistin resistance in *K. pneumoniae* (9–12). In addition, inactivation of the *mgrB* gene, which encodes the MgrB protein known to negatively regulate the PhoPQ signaling system, may also be the source of acquired resistance to colistin (13–16).

Acquired resistance to polymyxins is mediated by the addition of 4-deoxyaminoarabinose (LARA4N) and/or phosphoethanolamine (pEtN) to lipid A. LARA4N synthesis requires the products of the *pmrE* gene and *pmrHFIJKLM* operon, and pEtN synthesis is encoded by the *pmrC* gene. These modifications create a more positively charged lipopolysaccharide and thus reduce the affinity

of LPS to positively charged polymyxins (7). Of note, mechanisms leading to heteroresistance in *K. pneumoniae* remain unknown.

PmrB and PhoQ are sensor cytoplasmic membrane-bound kinases activated by high concentrations of iron ( $\text{Fe}^{3+}$ ) and an acidic pH (pH 5.5) for the PmrAB system (17) and by low extracellular concentrations of magnesium ( $\text{Mg}^{2+}$ ) for the PhoPQ system (18). Upon activation, PmrB activates the response regulator PmrA, which in turn upregulates *pmrC* and *pmrHFIJKLM*. Likewise, PhoQ activates PhoP, which in turn activates the expression of the *pmrHFIJKLM* operon, directly by binding to the *pmrHFIJKLM* promoter and indirectly via PmrD-dependent activation of the PmrA protein also binding to the *pmrHFIJKLM* promoter (19).

The aim of this study was to determine the mechanism(s) responsible for heteroresistance to colistin in *K. pneumoniae*.

## MATERIALS AND METHODS

**Bacterial strains.** A multidrug-resistant *K. pneumoniae* clinical isolate named Kp75 was recovered in 2012 in South Africa. It produced the carbapenemase OXA-48, which has been reported elsewhere (20). The wild-type and colistin-susceptible *K. pneumoniae* ATCC 53153 strain was used as a control throughout the study.

**Antimicrobial susceptibility assays.** MICs were determined using Etest strips (AB bioMérieux, La Balme-les-Grottes, France) on Mueller-Hinton agar plates (Bio-Rad, Marnes-la-Coquette, France) with a 0.5 McFarland inoculum. The MICs of colistin were also determined by a broth

Received 15 December 2014 Returned for modification 11 January 2015

Accepted 23 February 2015

Accepted manuscript posted online 2 March 2015

**Citation** Jayol A, Nordmann P, Brink A, Poirel L. 2015. Heteroresistance to colistin in *Klebsiella pneumoniae* associated with alterations in the PhoPQ regulatory system. *Antimicrob Agents Chemother* 59:2780–2784. doi:10.1128/AAC.05055-14.

Address correspondence to Laurent Poirel, laurent.poirel@unifr.ch.

Supplemental material for this article may be found at <http://dx.doi.org/10.1128/AAC.05055-14>.

Copyright © 2015, American Society for Microbiology. All Rights Reserved.

doi:10.1128/AAC.05055-14



TABLE 1 Oligonucleotides used as primers in this study

Primer	Sequence (5' to 3')	Gene	Reference or study
pmrA ext F	CAT TTC CGC GCA CTG TCT GC	<i>pmrA</i>	12
pmrA ext R	CAG GTT TCA GTT GCA AAC AG	<i>pmrA</i>	12
pmrB ext F	ACC TAC GCG AAA AGA TTG GC	<i>pmrB</i>	12
pmrB ext R	GAT GAG GAT AGC GCC CAT GC	<i>pmrB</i>	12
phoP ext F	GAG CTT CAG ACT ACT ATC GA	<i>phoP</i>	12
phoP ext R	GGG AAG ATA TGC CGC AAC AG	<i>phoP</i>	12
phoQ ext F	ATA CCC ACA GGA CGT CAT CA	<i>phoQ</i>	12
phoQ ext R	CAG GTG TCT GAC AGG GAT TA	<i>phoQ</i>	12
mgrB ext F	TTA AGA AGG CCG TGC TAT CC	<i>mgrB</i>	13
mgrB ext R	AAG GCG TTC ATT CTA CCA CC	<i>mgrB</i>	13
mdh F	CCC AAC TCG CTT CAG GTT CAG	<i>mdh</i>	26
mdh R	CCG TTT TTC CCC AGC AGC AG	<i>mdh</i>	26
pmrC int F	GCG TGA TGA ATA TCC TCA CCA	<i>pmrC</i>	12
pmrC int R	CAC GCC AAA GTT CCA GAT GA	<i>pmrC</i>	12
pmrA int F	GAT GAA GAC GGG CTG CAT TT	<i>pmrA</i>	12
pmrA int R	ACC GCT AAT GCG ATC CTC AA	<i>pmrA</i>	12
pmrB int F	TGC CAG CTG ATA AGC GTC TT	<i>pmrB</i>	12
pmrB int R	TTC TGG TTG TTG TGC CCT TC	<i>pmrB</i>	12
pmrD int F	GAT CGC AGA GAT TGA AGC CT	<i>pmrD</i>	12
pmrD int R	GCG TTG CGG ATC TTC AAA GT	<i>pmrD</i>	12
pmrE int F	GCA TAC CGT AAT GCC GAC TA	<i>pmrE</i>	12
pmrE int R	GGG TTG ATC TCT GTG ACA TC	<i>pmrE</i>	12
pmrK int F	AGT ATC GGT CAG TGG CTG TT	<i>pmrK</i>	12
pmrK int R	CCG CTT ATC ACG AAA GAT CC	<i>pmrK</i>	12
phoP int F	GCG TCA CCA CCT CAA AGT TC	<i>phoP</i>	This study
phoP int R	GGC GAT ATC CGG GAG ATG TT	<i>phoP</i>	This study
phoQ int F	CTC AAG CGC AGC TAT ATG GT	<i>phoQ</i>	This study
phoQ int R	TCT TTG GCC AGC GAC TCA AT	<i>phoQ</i>	This study
rpsL int F	CCG TGG CGG TCG TGT TAA AGA	<i>rpsL</i>	13
rpsL int R	GCC GTA CTT GGA GCG AGC CTG	<i>rpsL</i>	13

culture microdilution method as recommended by the CLSI (21), with Tween 80 being added at 0.002% as suggested in several studies (22, 23). According to the EUCAST guidelines, isolates with MICs of  $\leq 2$   $\mu\text{g/ml}$  are categorized as susceptible, and those with MICs of  $> 2$   $\mu\text{g/ml}$  are categorized as resistant (24).

**PCR amplification and sequencing.** Whole-cell DNA was extracted using a commercially available kit (QIAquick; Qiagen, Valencia, CA) according to the manufacturer's instructions. The *pmrA*, *pmrB*, *phoP*, *phoQ*, and *mgrB* genes were amplified with specific oligonucleotide primers (the primers used in this study are listed in Table 1). Molecular detection of the  $\beta$ -lactam resistance genes was performed as reported previously (25). The amplified DNA fragments were purified with the QIAquick PCR purification kit (Qiagen, Courtaboeuf, France). Both strands of the amplification products obtained were sequenced with an ABI 3100 sequencer (Applied Biosystems, Foster City, CA). The nucleotide and deduced protein sequences were analyzed at the National Center for Biotechnology Information website ([www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)) by the Basic Local Alignment Search Tool (BLAST) program.

**Analysis of the primary and secondary structures of the PhoP protein.** The primary structure of PhoP was analyzed by using the Pfam website (<http://pfam.xfam.org/>). The predicted secondary structures of the proteins were obtained by the GOR method using the EMBOSS 6.3.1 software available on the Mobyly@Pasteur portal (<http://mobyly.pasteur.fr/cgi-bin/portal.py>).

**Complementation experiments.** The entire *phoP* and *phoQ* genes from the colistin-susceptible *K. pneumoniae* ATCC 53153 reference strain (MIC of colistin at 0.125  $\mu\text{g/ml}$ ) were amplified by PCR using 2 $\times$  Phusion HF master mix (Thermo Scientific Finnzymes, Illkirch, France) and primers sets phoP ext F/phoP ext R and phoQ ext F/phoQ ext R, respectively.

The noncoding sequence *mdh* was also amplified with primer set mdh F/mdh R as described previously (26) (the primers used are listed in Table 1). The amplified fragments were cloned into plasmid pCR-BluntII-TOPO (Applied Biosystems by Life Technologies, Carouge, Switzerland) and the resulting plasmids pTOPO-phoP, pTOPO-phoQ, and pTOPO-mdh were, respectively, introduced into the colistin-resistant isolate Kp75 by electroporation. Transformants were selected by overnight incubation at 37°C on Mueller-Hinton agar supplemented with 100  $\mu\text{g/ml}$  of zeocin. The MICs of colistin were determined by Etest for all *K. pneumoniae* transformants.

**Growth curves.** Growth kinetics were determined with subpopulations of isolate Kp75 in the absence of any selective pressure. Two hundred-milliliter volumes of Luria-Bertani broth were inoculated independently with  $10^8$  CFU of each strain, and cultures were grown for 24 h at 100 rpm and 37°C. Absorbance at 600 nm was measured every hour for the first 10 h and after 24 h. The colony counting was performed by serial dilution and final plating on solid medium.

**Transcriptional analysis by qRT-PCR.** Quantitative real-time PCR (qRT-PCR) was used to measure the expression of the *phoP*, *phoQ*, *pmrD*, *pmrC*, *pmrA*, *pmrB*, and *pmrK* genes, using specific oligonucleotide primers (Table 1). A culture volume of 0.5 ml was taken during the mid-log phase of growth (optical density at 600 nm [ $\text{OD}_{600\text{ nm}}$ ] = 1.1) and combined with 1 ml of RNAlater (Qiagen, Courtaboeuf, France). Total RNA was extracted from cell lysates using the RNeasy minikit (Qiagen), according to the manufacturer's instructions. qRT-PCR was carried out using a Rotor-Gene Q instrument (Qiagen), and QuantiFast SYBR green (Qiagen) was used as a signal reporter. In each run, a blank sample (distilled water) and a no reverse transcriptase control were included to exclude DNA contamination. Relative gene expression differences were cal-

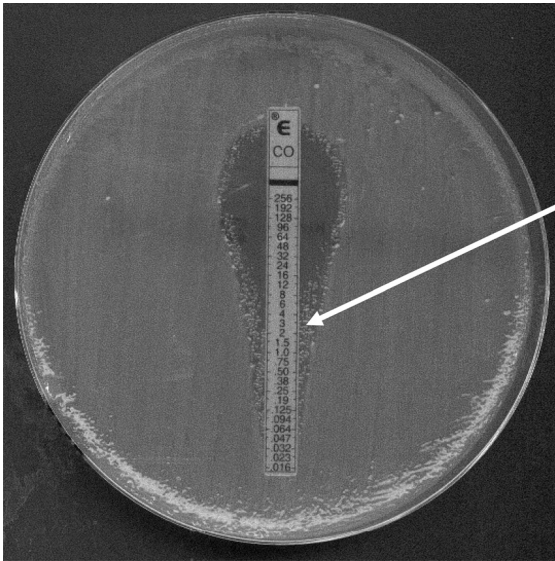


FIG 1 MIC determination using a colistin Etest strip for *K. pneumoniae* Kp75. The arrow indicates the colistin-resistant subpopulation (Kp75b).

culated by first normalizing the  $C_T$  values by subtracting the 12S rRNA control (*rpsL* gene) (13) and then comparing to the values obtained for the susceptible isolate.

**Nucleotide sequence accession numbers.** The nucleotide and protein sequences of the PhoP proteins of isolates Kp75a and Kp75b were registered in GenBank under accession numbers [KP742843](#) and [KP742844](#), respectively.

## RESULTS AND DISCUSSION

**Isolate Kp75 exhibits multidrug resistance, including resistance to colistin.** Isolate Kp75 was resistant to all  $\beta$ -lactams, including carbapenems (the MICs of imipenem, meropenem, and ertapenem were 3, 8, and  $>32$   $\mu\text{g/ml}$ , respectively). In addition, it was resistant to chloramphenicol, fluoroquinolones, gentamicin, tetracycline, and tobramycin. The MIC of rifampin was 16  $\mu\text{g/ml}$ , and the MIC of nitrofurantoin was  $>128$   $\mu\text{g/ml}$ . It remained susceptible only to sulfonamides, tigecycline, and amikacin. Determination of the MIC of colistin by using the broth culture microdilution method showed that isolate Kp75 was resistant to colistin (MIC of 128  $\mu\text{g/ml}$ ). Molecular detection of the  $\beta$ -lactamase resistance genes identified the narrow-spectrum  $\beta$ -lactamase *bla*<sub>TEM-1</sub> gene, the expanded-spectrum  $\beta$ -lactamase *bla*<sub>CTX-M-15</sub> gene, and the carbapenem-hydrolyzing class D  $\beta$ -lactamase *bla*<sub>OXA-48</sub> gene.

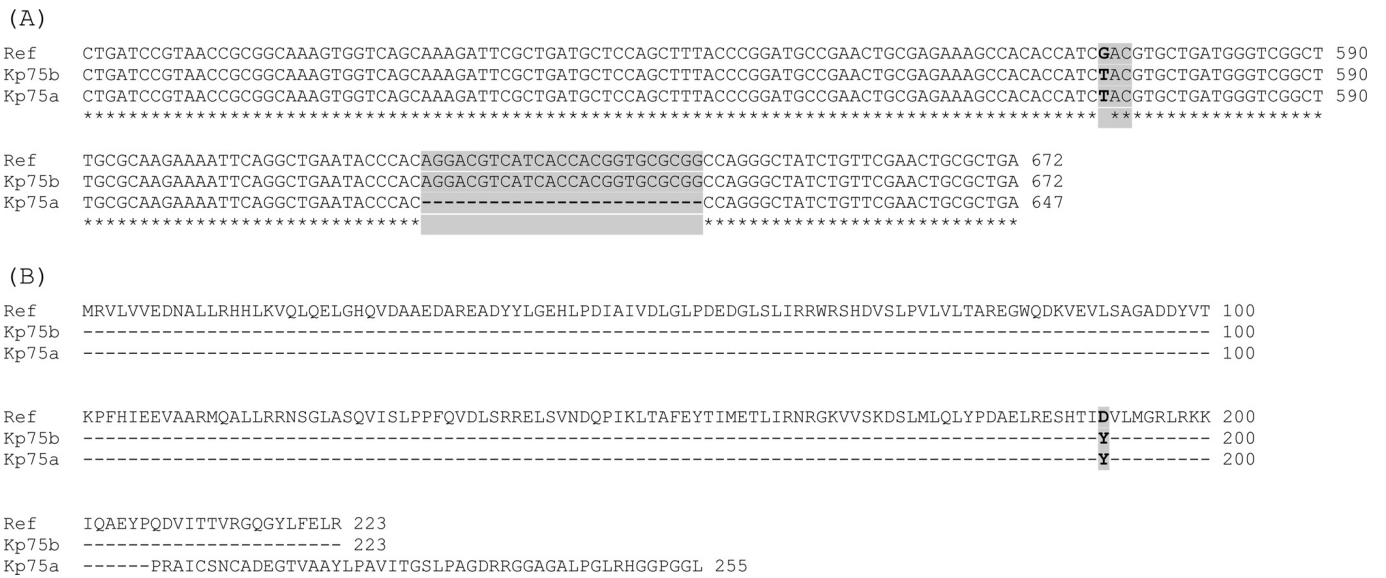
**Susceptibility testing by Etest revealed a heteroresistant phenotype.** When the MIC of colistin for isolate Kp75 was measured by using Etest strips, two subpopulations were identified close to the Etest strip (Fig. 1). Bacterial colonies located in the resistance and susceptible areas were picked up, and the two subpopulations were separated. The MICs for the two distinct subpopulations were again measured by Etest. The colistin-susceptible subpopulation (then termed Kp75a) and the colistin-resistant subpopulation (then termed Kp75b) had MICs of colistin at 0.12 and 12  $\mu\text{g/ml}$ , respectively. With use of the microdilution method, the MICs were found to be 0.12 and 128  $\mu\text{g/ml}$ , respectively. These data further highlight the discrepancies between values obtained by the two methods and reveal that the MICs obtained by the Etest

are significantly lower than those obtained by the microdilution method for such a resistant isolate.

**A single amino acid substitution in PhoP leads to colistin resistance in isolate Kp75b.** Total DNAs from isolate Kp75b and colistin-susceptible *K. pneumoniae* reference strain ATCC 53153 were used as the templates for PCRs to amplify the *pmrA*, *pmrB*, *phoP*, *phoQ*, and *mgrB* genes possibly involved in colistin resistance. A single base pair substitution was identified in the *phoP* gene of isolate Kp75b, leading to a single amino acid substitution (Asp191Tyr) compared to that of the PhoP proteins available in the GenBank databases and also compared to that of strain ATCC 53153 (Fig. 2). No substitution was identified in the *pmrA*, *pmrB*, *phoQ*, and *mgrB* genes.

The amino acid substitution was identified at position 191 of the PhoP protein, which is located in its C-terminal domain. We therefore speculate that the replacement of a polar negative amino acid by an aromatic amino acid partially hydrophobic in the C-terminal domain of PhoP might induce a constitutive activation of PhoP, deregulating the two-component regulatory system PhoPQ. The predicted secondary structures of two PhoP proteins, namely that of a wild-type strain and that of isolate Kp75b, were compared, and it was revealed that the Asp191Tyr amino acid substitution significantly modified the secondary structure of the protein, with an interruption of the  $\alpha$ -helix (see Fig. S1 in the supplemental material). It was therefore likely that resistance to colistin in isolate Kp75b was related to that single substitution in PhoP. To further assess this hypothesis, an experiment was conducted in order to complement isolate Kp75b with the entire *phoP* gene obtained from the wild-type strain ATCC 53153. The entire *phoP* and *phoQ* genes, together with a fragment of the *mdh* gene (a noncoding sequence as control), were amplified from the reference strain ATCC 53153 and cloned in plasmid pTOPO-BluntII, giving rise to, respectively, pTOPO-*phoP*, pTOPO-*phoQ*, and pTOPO-*mdh* and then transformed into the resistant-colistin clinical isolate Kp75b. The MIC determinations showed a complete reversion to susceptibility only for the transformant complemented with pTOPO-*phoP* (MIC at 0.125  $\mu\text{g/ml}$ ), whereas the MICs of transformants complemented with recombinant plasmids pTOPO-*phoQ* or pTOPO-*mdh* remained unchanged. This result strongly suggested that the amino acid substitution identified in PhoP was indeed responsible for the colistin resistance in Kp75b (Fig. 3).

**A partial deletion of the *phoP* gene is responsible for a reversion in colistin susceptibility.** Similarly to what was performed with isolate Kp75b, PCRs followed by sequencing were performed to amplify the *pmrA*, *pmrB*, *phoP*, *phoQ*, and *mgrB* genes of the colistin-susceptible isolate Kp75a. All sequences were found to be identical with those obtained with Kp75b, except for the *phoP* gene. Indeed, the *phoP* gene harbored not only a substitution leading to the Asp191Tyr amino acid substitution in the corresponding protein sequence but also a partial deletion (25 bp long) in the *phoP* gene of Kp75a, disrupting its reading frame and consequently giving rise to a longer protein of 255 amino acids instead of 223 amino acids as found in Kp75b and all other PhoP sequences in the databases (Fig. 2). This deletion was therefore considered to be responsible for the reversion into a colistin-susceptible phenotype. Figure 3 provides a summary of the evolution process observed in Kp75. This mechanism of partial gene deletion involved in reversion to colistin susceptibility was previously



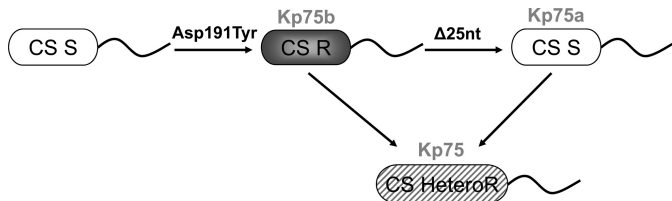
**FIG 2** Alignment of a fragment of the *phoP* gene nucleotide sequences (A) and amino acid PhoP protein sequences (B) of the colistin-resistant isolate Kp75b and the colistin-susceptible isolate Kp75a, on the basis of that of the wild-type reference strain ATCC 53153. In panel A, dashes indicate absent nucleotides in Kp75a, and stars indicate conserved nucleotides; in panel B, dashes indicate amino acid residues identical to those of the reference sequence. In the lower portion of panel A, the shaded nucleotides indicate the deleted 25-bp fragment identified in Kp75a. The amino acid substitution (Asp191Tyr) involved in resistance to colistin in Kp75b is shaded in panel B.

described in the PmrAB two-component system of *Acinetobacter baumannii* (27).

**Comparative growth rates of colistin-susceptible and colistin-resistant isogenic strains.** The growth kinetics of colistin-resistant (Kp75b) and colistin-susceptible (Kp75a) isogenic strains were determined in the absence of selective pressure. During a 24-h period, no significant difference was observed in growth rates between those two strains (data not shown).

**Upregulation of *phoP*, *phoQ*, *pmrD*, and *pmrK* gene expression in colistin-resistant strain Kp75b.** Expression of the *pmr* and *pho* genes was investigated to determine whether the expression of those genes involved in the PmrAB or PhoPQ regulatory systems might differ between the two Kp75 subpopulations. RT-PCR identified upregulation of the *phoP*, *phoQ*, *pmrD*, and *pmrK* genes in the resistant isolate Kp75b compared with that of the isogenic susceptible isolate Kp75a. In contrast, expression of the *pmrC*, *pmrA*, and *pmrB* genes did not differ significantly (Fig. 4).

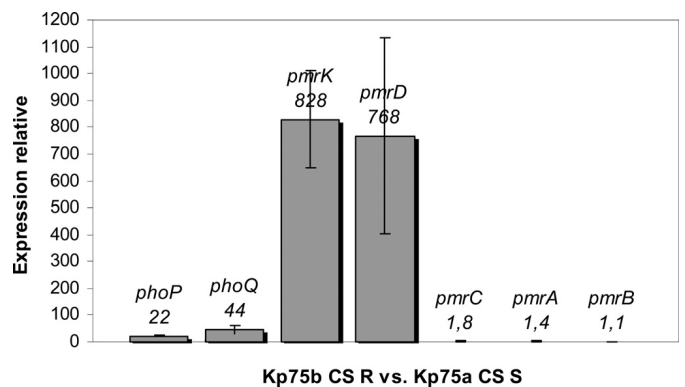
We may therefore consider that the mutated protein PhoP activates the transcription of the *pmrHFIIJKLM* operon, the product of which leads to synthesis of L-amino-arabinose and ultimately to



**FIG 3** Schematic representation of the mechanisms leading to heteroresistance in isolate Kp75. The colistin-susceptible (CS S) subpopulation (Kp75a) is represented in white, the colistin-resistant (CS R) subpopulation (Kp75b) in gray, and the mixed (CS HeteroR) population (Kp75) in gray stripes. The PhoP amino acid substitution (Asp191Tyr) and the deletion of the 25-bp fragment (Δ25 nucleotides [nt]) are indicated.

colistin resistance in *K. pneumoniae*. We also observed that PhoP activates the expression of the *PmrD*-encoding gene and that *PmrD* did not activate the expression of the *pmrA* gene, contrary to what was expected (19).

In conclusion, our study suggests that colistin heteroresistance in *K. pneumoniae* may be caused by alterations in the PhoPQ regulatory system. To the best of our knowledge, this is the first study investigating the genetic basis of colistin heteroresistance in that bacterial species. We first demonstrate here the involvement of the PhoP protein in resistance to colistin in *K. pneumoniae*. This additional mechanism adds to the diversity of genetic events that may lead to acquisition of resistance to colistin in that species. Interestingly, our data showed that the heteroresistance observed here was related to a reversion of resistance into susceptibility and not the opposite. Indeed, the amino acid substitution identified in PhoP to which the resistance was attributed was still identified in the susceptible isolate.



**FIG 4** Relative expression of the *phoP*, *phoQ*, *pmrD*, *pmrK*, *pmrC*, *pmrA*, and *pmrB* genes considering the colistin-resistant isolate Kp75b compared to the colistin-susceptible isolate Kp75a. Values and standard deviations are the means from three independent experiments.



In addition, we want to emphasize that the phenomenon of colistin heteroresistance remains very likely underestimated, considering that it may not be seen when the method (broth microdilution) recommended by the CLSI is used for the evaluation of susceptibility to polymyxins. We showed here that heteroresistance may be well demonstrated when only Etest strips are used. Notably, the clinical significance of colistin heteroresistance in *K. pneumoniae* is still unknown and remains to be evaluated.

## ACKNOWLEDGMENTS

This work was funded by the University of Fribourg, Switzerland, and by a grant from the European Community (R-GNOSIS, FP7/HEALTH-F3-2011-282512).

## REFERENCES

- Podschun R, Ullmann U. 1998. *Klebsiella* spp. as nosocomial pathogens: epidemiology, taxonomy, typing methods, and pathogenicity factors. *Clin Microbiol Rev* 11:589–603.
- Falagas ME, Lourida P, Poulikakos P, Rafailidis PI, Tansarli GS. 2014. Antibiotic treatment of infections due to carbapenem-resistant *Enterobacteriaceae*: systematic evaluation of the available evidence. *Antimicrob Agents Chemother* 58:654–663. <http://dx.doi.org/10.1128/AAC.01222-13>.
- Hancock RE. 1997. Peptide antibiotics. *Lancet* 349:418–422. [http://dx.doi.org/10.1016/S0140-6736\(97\)80051-7](http://dx.doi.org/10.1016/S0140-6736(97)80051-7).
- Meletis G, Tzampaz E, Sianou E, Tzavaras I, Sofianou D. 2011. Colistin heteroresistance in carbapenemase-producing *Klebsiella pneumoniae*. *J Antimicrob Chemother* 66:946–947. <http://dx.doi.org/10.1093/jac/dkr007>.
- Poudyal A, Howden BP, Bell JM, Gao W, Owen RJ, Turnidge JD, Nation RL, Li J. 2008. In vitro pharmacodynamics of colistin against multidrug-resistant *Klebsiella pneumoniae*. *J Antimicrob Chemother* 62:1311–1318. <http://dx.doi.org/10.1093/jac/dkn425>.
- Li J, Nation RL, Turnidge JD, Milne RW, Coulthard K, Rayner CR, Paterson DL. 2006. Colistin: the re-emerging antibiotic for multidrug-resistant Gram-negative bacterial infections. *Lancet Infect Dis* 6:589–601. [http://dx.doi.org/10.1016/S1473-3099\(06\)70580-1](http://dx.doi.org/10.1016/S1473-3099(06)70580-1).
- Falagas ME, Rafailidis PI, Matthaiou DK. 2010. Resistance to polymyxins: mechanisms, frequency and treatment options. *Drug Resist Updat* 13:132–138. <http://dx.doi.org/10.1016/j.drup.2010.05.002>.
- Humphries RM. 2015. Susceptibility testing of the polymyxins: where are we now? *Pharmacotherapy* 35:22–27. <http://dx.doi.org/10.1002/phar.1505>.
- Cannatelli A, Di Pilato V, Gianni T, Arena F, Ambretti S, Gaibani P, D'Andrea MM, Rossolini GM. 2014. In vivo evolution to colistin resistance by PmrB sensor kinase mutation in KPC-producing *Klebsiella pneumoniae* is associated with low-dosage colistin treatment. *Antimicrob Agents Chemother* 58:4399–4403. <http://dx.doi.org/10.1128/AAC.02555-14>.
- Cheng HY, Chen YF, Peng HL. 2010. Molecular characterization of the PhoPQ-PmrD-PmrAB mediated pathway regulating polymyxin B resistance in *Klebsiella pneumoniae* CG43. *J Biomed Sci* 17:60. <http://dx.doi.org/10.1186/1423-0127-17-60>.
- Choi MJ, Ko KS. 2014. Mutant prevention concentrations of colistin for *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* clinical isolates. *J Antimicrob Chemother* 69:275–277. <http://dx.doi.org/10.1093/jac/dkt315>.
- Jayol A, Poirel L, Brink A, Villegas MV, Yilmaz M, Nordmann P. 2014. Resistance to colistin associated with a single amino acid change in protein PmrB among *Klebsiella pneumoniae* isolates of worldwide origin. *Antimicrob Agents Chemother* 58:4762–4766. <http://dx.doi.org/10.1128/AAC.00084-14>.
- Cannatelli A, D'Andrea MM, Gianni T, Di Pilato V, Arena F, Ambretti S, Gaibani P, Rossolini GM. 2013. In vivo emergence of colistin resistance in *Klebsiella pneumoniae* producing KPC-type carbapenemase mediated by insertional inactivation of the PhoQ/PhoP *mgrB* regulator. *Antimicrob Agents Chemother* 57:5521–5526. <http://dx.doi.org/10.1128/AAC.01480-13>.
- Cannatelli A, Gianni T, D'Andrea MM, Di Pilato V, Arena F, Conte V, Tryfinopoulou K, Vatopoulos A, Rossolini GM. 2014. MgrB inactivation is a common mechanism of colistin resistance in KPC-producing *Klebsiella pneumoniae* of clinical origin. *Antimicrob Agents Chemother* 58:5696–5703. <http://dx.doi.org/10.1128/AAC.03110-14>.
- Poirel L, Jayol A, Bontron S, Villegas MV, Özdamar M, Turkoglu S, Nordmann P. 2015. The *mgrB* gene as a key target for acquired resistance to colistin in *Klebsiella pneumoniae*. *J Antimicrob Chemother* 70:75–80. <http://dx.doi.org/10.1093/jac/dku323>.
- Wright MS, Suzuki Y, Jones MB, Marshall SH, Rudin SD, van Duin D, Kaye K, Jacobs MR, Bonomo RA, Adams MD. 2015. Genomic and transcriptomic analyses of colistin-resistant clinical isolates of *Klebsiella pneumoniae* reveal multiple pathways of resistance. *Antimicrob Agents Chemother* 59:536–543. <http://dx.doi.org/10.1128/AAC.04037-14>.
- Gunn JS. 2008. The *Salmonella* PmrAB regulon: lipopolysaccharide modifications, antimicrobial peptide resistance and more. *Trends Microbiol* 16:284–290. <http://dx.doi.org/10.1016/j.tim.2008.03.007>.
- Groisman EA. 2001. The pleiotropic two-component regulatory system PhoP-PhoQ. *J Bacteriol* 183:1835–1842. <http://dx.doi.org/10.1128/JB.183.6.1835-1842.2001>.
- Mitrophanov AY, Jewett MW, Hadley TJ, Groisman EA. 2008. Evolution and dynamics of regulatory architectures controlling polymyxin B resistance in enteric bacteria. *PLoS Genet* 4:e1000233. <http://dx.doi.org/10.1371/journal.pgen.1000233>.
- Potron A, Poirel L, Rondinaud E, Nordmann P. 2013. Intercontinental spread of OXA-48  $\beta$ -lactamase-producing *Enterobacteriaceae* over a 11-year period, 2001 to 2011. *Euro Surveill* 18(31):pii=20549. <http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=20549>.
- Clinical and Laboratory Standards Institute. 2014. Performance standards for antimicrobial susceptibility testing; 24th informational supplement. CLSI document M100-S24. Clinical and Laboratory Standards Institute, Wayne, PA.
- Hindler JA, Humphries RM. 2013. Colistin MIC variability by method for contemporary clinical isolates of multidrug-resistant Gram-negative bacilli. *J Clin Microbiol* 51:1678–1684. <http://dx.doi.org/10.1128/JCM.03385-12>.
- Sader HS, Rhomberg PR, Flamm RK, Jones RN. 2012. Use of a surfactant (polysorbate 80) to improve MIC susceptibility testing results for polymyxin B and colistin. *Diagn Microbiol Infect Dis* 74:412–414. <http://dx.doi.org/10.1016/j.diagmicrobio.2012.08.025>.
- European Committee on Antimicrobial Susceptibility Testing. 2015. Breakpoint tables for interpretation of MICs and zone diameters. Version 2.0. [http://www.eucast.org/clinical\\_breakpoints/](http://www.eucast.org/clinical_breakpoints/).
- Nordmann P, Naas T, Poirel L. 2011. Global spread of carbapenemase-producing *Enterobacteriaceae*. *Emerg Infect Dis* 17:1791–1798. <http://dx.doi.org/10.3201/eid1710.110655>.
- Diancourt L, Passet V, Verhoef J, Grimont PA, Brisse S. 2005. Multi-locus sequence typing of *Klebsiella pneumoniae* nosocomial isolates. *J Clin Microbiol* 43:4178–4182. <http://dx.doi.org/10.1128/JCM.43.8.4178-4182.2005>.
- Adams MD, Nickel GC, Bajaksouzian S, Lavender H, Murthy AR, Jacobs MR, Bonomo RA. 2009. Resistance to colistin in *Acinetobacter baumannii* associated with mutations in the PmrAB two-component system. *Antimicrob Agents Chemother* 53:3628–3634. <http://dx.doi.org/10.1128/AAC.00284-09>.

**(A)**

```

      . 10 . 20 . 30 . 40 . 50
MRVLVVEDNALLRHHLKVQLQELGHQVDAEDAREADYLLGEHLPDIAIV
helix H HHHHHHHHHHHHHHHHHHH HHHHHHHHHHHHHHH HHH
sheet EEE
turns T
coil CC

      . 60 . 70 . 80 . 90 . 100
DLGLPDEDGLSLIRRWRSNDVSLPVLVLTAREGWQDKVEVLSAGADYVT
helix H HHHHH H HHH HHHH HHHHHHH
sheet EE E E E EEEEEEE EEE EEE EE
turns T TTTTTTT T T T
coil CCC C

      . 110 . 120 . 130 . 140 . 150
KPFHIEEVAARMQALLRRMSGLASQVISLPPFQVDSLRRRELSVNDQPIKL
helix HHHHHHHHHHHHHH
sheet EE EEEEEEE EEEE EEEE EEE
turns TT TT TTTTTTT
coil CCC CCC C

      . 160 . 170 . 180 . 190 . 200
TAFETYTIMETLIRNRGKVVSKDSLMLQLYPDALRESHTIDVLMGRLRKK
helix HHHHHHHHHHHHH HHH HHHHHHHHHHHHHHHHHHHHH
sheet E EEEEE EEEE EEEE
turns TTTT
coil CC C

      . 210 . 220
IOAEYPODVITTVRGQGYLFFELR H
helix
sheet EEE EEEEE EEEE
turns TTT TTT
coil C C CC

```

**(B)**

```

      . 10 . 20 . 30 . 40 . 50
MRVLVVEDNALLRHHLKVQLQELGHQVDAEDAREADYLLGEHLPDIAIV
helix H HHHHHHHHHHHHHHHHHHH HHHHHHHHHHHHHHH HHH
sheet EEE
turns T
coil CC

      . 60 . 70 . 80 . 90 . 100
DLGLPDEDGLSLIRRWRSNDVSLPVLVLTAREGWQDKVEVLSAGADYVT
helix H HHHHH H HHH HHHH HHHHHHH
sheet EE E E E EEEEEEE EEE EEE EE
turns T TTTTTTT T T T
coil CCC C

      . 110 . 120 . 130 . 140 . 150
KPFHIEEVAARMQALLRRMSGLASQVISLPPFQVDSLRRRELSVNDQPIKL
helix HHHHHHHHHHHHHH
sheet EE EEEEEEE EEEE EEEE EEE
turns TT TT TTTTTTT
coil CCC CCC C

      . 160 . 170 . 180 . 190 . 200
TAFETYTIMETLIRNRGKVVSKDSLMLQLYPDALRESHTIDVLMGRLRKK
helix HHHHHHHHHHHHH HHH HHHHHHHHHHHHHHHHHHHHH
sheet E EEEEE EEEE EEEE EEE
turns TTTT
coil CC C

      . 210 . 220
IOAEYPODVITTVRGQGYLFFELR H
helix
sheet EEE EEEEE EEEE
turns TTT TTT
coil C C CC

```

Figure S1

### **1.3. Identification d'altérations dans la protéine MgrB responsables de l'acquisition de résistances à la colistine**

#### **Contexte :**

En 2014, une étude a révélé que des altérations dans la protéine MgrB, un régulateur négatif du système à deux composants PhoPQ, étaient responsables de l'acquisition de résistances à la colistine chez *K. pneumoniae* (39).

#### **Objectif de l'étude :**

L'objectif des deux études qui suivent était donc de rechercher les altérations au sein du gène *mgrB* responsables de l'acquisition de résistances à la colistine dans une collection de souches cliniques de *K. pneumoniae*.

## **a. Les altérations dans la protéine MgrB sont un mécanisme fréquent d'acquisition de résistances à la colistine**

### **Matériel et méthodes :**

Quarante-sept souches de *K. pneumoniae* résistantes à la colistine isolées en Afrique du Sud, Colombie, France, et Turquie ont été incluses dans cette étude. Les CMI de la colistine ont été déterminées par dilutions en milieu liquide. Les opérons *pmrAB*, *phoPQ*, et *mgrB* ont été séquencés pour rechercher des mutations. Des expériences de complémentation ont été réalisées par clonage du gène sauvage *mgrB* dans un plasmide vecteur et électroporation du plasmide recombinant dans les souches résistantes à la colistine.

### **Principaux résultats :**

Des mutations ont été identifiées dans la protéine MgrB de douze souches de *K. pneumoniae* résistantes à la colistine (CMI de la colistine comprises entre 4 et 24 mg/L). Les mutations observées étaient variées : insertion de séquences nucléotidiques (IS5-like, IS*Kpn13*, IS*Kpn14*, et IS*10R*) au sein du gène *mgrB* ou de son promoteur, apparition d'un codon stop prématuré. Les expériences de complémentation de ces souches avec le gène *mgrB* sauvage ont conduit à une restauration de la sensibilité à la colistine (CMI = 0,032-0,047 mg/L) et ont ainsi confirmé l'implication des altérations au sein du gène *mgrB* et de son promoteur dans l'acquisition de la résistance à la colistine.

### **Conclusion :**

Nous avons identifié des altérations variées au sein du gène *mgrB* et de son promoteur responsables de l'acquisition de résistances à la colistine. La diversité des altérations observées suggère que le gène *mgrB* est une cible clé pour l'acquisition de résistances à la colistine chez *K. pneumoniae*.

**Ce travail a été publié dans *Antimicrobial Agents and Chemotherapy* en septembre 2014 (IF = 4.415).**

## The *mgrB* gene as a key target for acquired resistance to colistin in *Klebsiella pneumoniae*

Laurent Poirel<sup>1,2</sup>, Aurélie Jayol<sup>2</sup>, Séverine Bontron<sup>1</sup>, Maria-Virginia Villegas<sup>3</sup>, Melda Ozdamar<sup>4</sup>,  
Salih Türkoglu<sup>4</sup> and Patrice Nordmann<sup>1,2,5\*</sup>

<sup>1</sup>Medical and Molecular Microbiology Unit, Department of Medicine, Faculty of Science, University of Fribourg, Fribourg, Switzerland; <sup>2</sup>INSERM U914, South-Paris Medical School, K.-Bicêtre, Paris, France; <sup>3</sup>International Center for Medical Research and Training, CIDEIM, Cali, Colombia; <sup>4</sup>Medical Microbiology Department, School of Medicine, Istanbul Medipol University, Istanbul, Turkey; <sup>5</sup>Hôpital Fribourgeois—Hôpital Cantonal de Fribourg, Fribourg, Switzerland

\*Corresponding author. Medical and Molecular Microbiology Unit, Department of Medicine, Faculty of Science, University of Fribourg, rue Albert Gockel 3, CH-1700 Fribourg, Switzerland. Tel: +41-26-300-9581; E-mail: patrice.nordmann@unifr.ch

Received 14 April 2014; returned 30 May 2014; revised 10 July 2014; accepted 21 July 2014

**Objectives:** Alterations in the PhoPQ two-component regulatory system may be associated with colistin resistance in *Klebsiella pneumoniae*. MgrB is a small transmembrane protein produced upon activation of the PhoPQ signalling system, and acts as a negative regulator on this system. We investigated the role of the MgrB protein as a source of colistin resistance in a series of *K. pneumoniae*.

**Methods:** Colistin-resistant *K. pneumoniae* isolates were recovered from hospitalized patients worldwide (France, Turkey, Colombia and South Africa). The *mgrB* gene was amplified and sequenced. A wild-type *mgrB* gene was cloned and the corresponding recombinant plasmid was used for complementation assays. Clonal diversity was evaluated by MLST and Diversilab analysis.

**Results:** Of 47 colistin-resistant isolates, 12 were identified as having a mutated *mgrB* gene. Five clonally unrelated isolates had an *mgrB* gene truncated by an IS5-like IS, while one clone also harboured an insertional inactivation at the exact same position of the *mgrB* gene, but with IS*Kpn13*. Another clone harboured an insertional inactivation due to IS*Kpn14* at another location of the *mgrB* gene. Two clonally related isolates harboured an IS (IS10R) in the promoter region of *mgrB*. Finally, three clonally unrelated isolates harboured substitutions leading to anticipated stop codon in the MgrB protein. Complementation assays with a wild-type MgrB protein restored full susceptibility to colistin for all colistin-resistant isolates identified with qualitative or quantitative MgrB modifications.

**Conclusion:** The inactivation or down-regulation of the *mgrB* gene was shown to be a source of colistin resistance in *K. pneumoniae*. Interestingly, identical genetic events were identified among clonally unrelated isolates.

**Keywords:** gene inactivation, PhoPQ regulatory system, polymyxin, *Klebsiella pneumoniae*

### Introduction

Colistin and other polymyxins are cationic peptides synthesized by a Gram-positive species, namely *Paenibacillus polymyxa*. Those antibiotics act by disrupting the negatively charged outer membranes of Gram-negative bacteria.<sup>1</sup> Colistin is now often considered as the last option to treat multidrug-resistant Gram-negatives, and in particular carbapenemase-producing Enterobacteriaceae and *Acinetobacter baumannii*.<sup>2</sup> *Klebsiella pneumoniae* is the enterobacterial species in which acquisition of a carbapenemase is most frequently identified, and therefore multidrug-resistant *K. pneumoniae* are increasingly reported.<sup>3</sup>

Colistin is consequently increasingly used in endemic areas for carbapenemase-producing *K. pneumoniae*, and reports of colistin-resistant isolates in that species are on the rise.<sup>4</sup>

Alterations in the two-component regulatory systems PhoPQ and PmrAB,<sup>5,6</sup> as well as inactivation of the *mgrB* gene,<sup>7,8</sup> have been reported to be involved in polymyxin resistance in *K. pneumoniae*. The sensor kinase PhoQ is an integral membrane protein whose periplasmic domain is involved in signal detection. It is activated by low extracellular magnesium (Mg<sup>2+</sup>), acidic pH (pH 5.5) or the presence of cationic antimicrobial peptides.<sup>9,10</sup> Upon activation, it activates PhoP by phosphorylation. Then PhoP activates PmrHFIJKLM expression, directly by binding to



the *pmrHFJJKLM* promoter, and indirectly via PmrD-dependent activation of the PmrA protein also binding to the *pmrHFJJKLM* promoter.<sup>11</sup> The *pmrHFJJKLM* operon (also named *pmrF*, *pbg* or *arn* operon) encodes enzymes responsible for the synthesis and transfer of 4-amino-4-deoxy-L-arabinose (LAra4N). The addition of this cationic product (LAra4N) to lipid A leads to resistance to polymyxins by modification of the lipopolysaccharide target.<sup>10</sup>

MgrB, a small regulatory transmembrane protein of 47 amino acids, is produced upon activation of the PhoPQ signalling system.<sup>12</sup> Through interactions between the MgrB protein and the periplasmic domain of PhoQ, MgrB negatively regulates the PhoP/PhoQ system. MgrB spans the inner membrane and represses PhoP phosphorylation, probably by inhibiting the PhoQ kinase activity or stimulating phosphatase activity. Phosphorylation of PhoP increases transcription of the *mgrB* gene. MgrB is consequently part of a negative feedback loop in the PhoQ/PhoP signalling circuit.

Two recent pioneering studies showed that insertional inactivations in the *mgrB* gene were the source of acquired resistance to colistin in *K. pneumoniae*.<sup>7,8</sup> Such inactivation leads to an up-regulation of the PhoPQ system, leading to the up-regulation of the *pmrHFJJKLM* operon, and ultimately to colistin resistance.

The aim of our study was to analyse a collection of 47 colistin-resistant *K. pneumoniae* strains recovered from different geographical areas in order to identify whether there were alterations in the *mgrB* gene that may explain the resistance to colistin.

## Material and methods

### Bacterial strains

Forty-seven colistin-resistant *K. pneumoniae* clinical isolates recovered from hospitalized patients in France, Turkey, Colombia and South Africa were investigated. The isolates were identified using the API 20E system and the MALDI-TOF technique (bioMérieux, La Balme-les-Grottes, France).

### Antimicrobial susceptibility assays

MICs were determined using Etest strip (bioMérieux) according to the manufacturer's instructions on Mueller–Hinton agar plates (BioRad, Marnes-la-Coquette, France) with a 0.5 McFarland inoculum in sterile water, and interpreted as indicated by the EUCAST guidelines (EUCAST breakpoint tables for interpretation of MICs and zone diameters, version 3.1, 2013; <http://www.eucast.org>). Following the EUCAST breakpoints, isolates with MICs of colistin  $\leq 2$  mg/L were categorized as susceptible, although those with MICs  $> 2$  mg/L were resistant.

### PCR amplification and sequencing

Whole-cell DNA was extracted using the QIAquick kit (Qiagen, Courtaboeuf, France) according to the manufacturer's instructions. The entire *mgrB* gene was amplified with specific oligonucleotide primers *mgrB*-extF (5'-TTAAGAAGGCCGTGCTATCC-3', position -52 with respect to the *mgrB* start codon in order to include the promoter sequences) and *mgrB*-extR (5'-AAGGCGTTCATCTACCACC-3', located after the stop codon) as published.<sup>7</sup> PCR products were analysed by gel electrophoresis and sequenced with an automated ABI 3100 sequencer (Applied Biosystems,

Foster City, CA, USA). The nucleotide and deduced protein sequences were analysed at the National Center for Biotechnology Information web site ([www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)) using the Basic Local Alignment Search Tool program. ISs were analysed using the IS finder web site ([www-is.biotoul.fr](http://www-is.biotoul.fr)). Detection of  $\beta$ -lactamase genes was performed with primers as described previously.<sup>13</sup>

### Complementation assays

The entire *mgrB* gene from the colistin-susceptible *K. pneumoniae* reference strain ATCC 53153 (MIC at 0.125 mg/L) was amplified by PCR using the 2X Phusion HF Master Mix (Finnzymes, Life Technologies, Illkirch, France) and primers *mgrB*-extF/*mgrB*-extR.

The partial and therefore non-coding *mdh* sequence (supposed to encode a malate dehydrogenase) was PCR amplified with primers *mdh*-extF (5'-CCCAACTCGCTTCAGGTTCCAG-3')/*mdh*-extR (5'-CCGTTTTCCCGCAGCAGCAG-3').

The amplified fragments were respectively cloned into the high-copy plasmid pCR-BluntII-TOPO (Invitrogen, Illkirch, France) and the resulting plasmids pTOPO-*mgrB* and pTOPO-*mdh* (encoding resistance to zeocin) were separately transformed into the colistin-resistant isolates by electroporation. Electrotransformants were selected by overnight incubation at 37°C on Mueller–Hinton agar supplemented with 100 mg/L of zeocin. The colistin MICs of the *K. pneumoniae* transformants were measured using Etest.

Since pCR-BluntII-TOPO is a high-copy plasmid that may lead to a too high overexpression of the corresponding cloned genes and therefore constitute a bias in our experiments, we modified this plasmid backbone by replacing its pUCOri replication origin locus with the p15a replication of the low-copy plasmid pACYC184, leading to a new plasmid backbone we named pLow-TOPO. The exact same inserts were inserted into that low-copy plasmid and further experiments performed as above.

### Genotyping by MLST and Rep-PCR (Diversilab®)

MLST was carried out with seven standard housekeeping loci (*rpoB*, *gapA*, *mdh*, *pgi*, *phoE*, *infB* and *tonB*) according to Diancourt et al.<sup>14</sup> Sequence types were analysed using the database provided by the Institut Pasteur, Paris, France (<http://www.pasteur.fr/recherche/genopole/PF8/mlst/Kpneumoniae.html>).

DNA was extracted using UltraClean Microbial DNA Isolation kit (Mobio, Saint-Quentin-en-Yvelines, France). Genotyping was evaluated by rep-PCR, using the Diversilab *Klebsiella* kit (bioMérieux) following the manufacturer's recommendations. Amplicons were separated by microfluidic chip with the Agilent 2100 Bioanalyzer and the rep-PCR fingerprints were analysed with the coupled software.

## Results and discussion

### Analysis of the *mgrB* gene sequences

The entire *mgrB* gene of the 47 colistin-resistant isolates was amplified by PCR, and further sequence analysis of the amplicons showed that nine isolates generated a larger-in-size amplicon compared with a wild-type isolate. Identification of different fragment lengths compared with those obtained with a

colistin-susceptible strain (T1a) suggested the insertion of DNA sequences into the coding sequence or in the upstream vicinity of the *mgrB* gene in those nine isolates. Sequencing of the amplicons revealed that insertional inactivations had occurred at different positions into the *mgrB* gene in seven isolates and into the putative *mgrB* promoter in two isolates (Table 1). In addition, sequence analysis revealed a premature stop codon occurring in the sequence of *mgrB* for three additional isolates (Table 1).

**Characteristics of isolates**

The nine colistin-resistant isolates for which different-in-size amplicons were obtained were of different geographical origins, with six isolates from Colombia, two isolates from France and a single isolate from Turkey (Table 1). MICs of colistin for those isolates ranged from 4 to 24 mg/L (Table 1).

**Truncation of *mgrB* by an IS5-like IS is a key mechanism leading to colistin resistance**

In five isolates, the *mgrB* was truncated by the same 1195 bp IS. This IS was closely related (93% nucleotide identity) to IS5, with both transposases sharing 99% amino acid identity (3 substitutions out of 326 amino acids).<sup>15</sup> Surprisingly, the IS5-like insertion identified in the *mgrB* gene of those five distinct isolates always targeted the same location, between nucleotides 74 and 75 of the *mgrB* sequence, and was always in the same orientation (Table 1). Rep-PCR (Diversilab®) analysis revealed that four out of those five isolates were clonally unrelated, with only isolates C9 and C10 from Colombia being related, but different from all others (Figure 1a, Table 1). More surprisingly, this IS5-like element was identical to that recently identified in a colistin-resistant and KPC-3-producing *K. pneumoniae* isolate.<sup>7</sup> In that study, Cannatelli *et al.*<sup>7</sup> showed that the insertion of this IS5-like element into the *mgrB* gene was involved in resistance to colistin. Surprisingly, this IS5-like sequence was inserted at the exact same location compared with the isolates of our study, but in the opposite orientation (Figure 2b). A 4 bp target site duplication (TTAA), being the signature of the transposition event, was systematically identified at each extremity of the IS5-like element in all isolates (Figure 2b and c).

The colistin-susceptible isolate T1a and the resistant-colistin isolate T1b recovered from a same patient, respectively, before and after colistin therapy were found to be clonally related, as expected (Figure 1a, Table 1). Isolate T1a harboured a wild-type *mgrB* gene, indicating that insertion of the IS5-like element probably occurred *in vivo* and that the resistant isolate T1b had probably been selected under antibiotic selective pressure. Altogether, these data concur with the fact that the *mgrB* inactivation mediates acquired resistance to colistin.

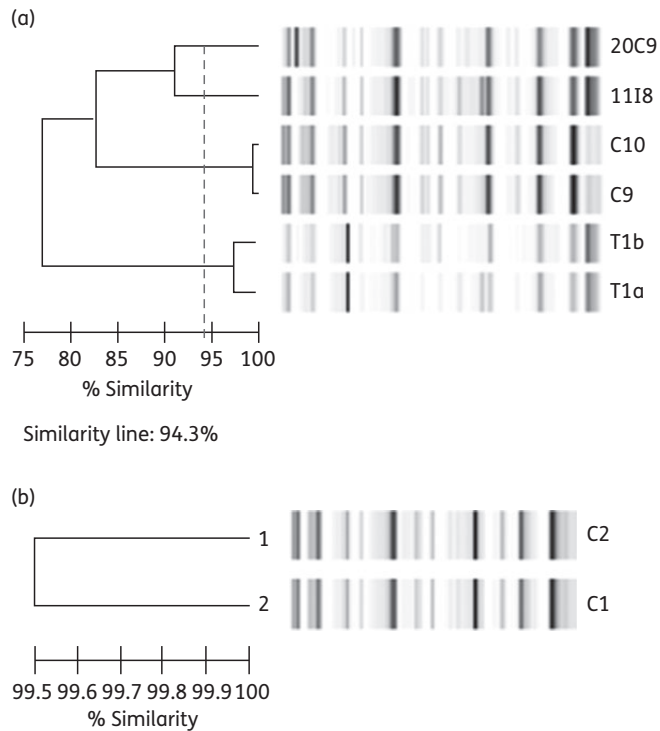
**Truncation of the *mgrB* gene by diverse ISs identified in colistin-resistant isolates**

In isolate C21, an IS of 1148 bp named ISKpn13 and belonging to the IS5 family was identified at nucleotide position 74 (Figure 2d, Table 1), which corresponds to the exact same position where the IS5-like targeted the *mgrB* gene in five other isolates (see above). ISKpn13 shared <30% nucleotide identity with the IS5-like element, their respective transposase proteins sharing 56% amino acid identity.<sup>16</sup> Detailed analysis of their inverted repeats revealed that

**Table 1.** Features of the colistin-resistant isolates in which disruption of the *mgrB* gene was identified

Isolate	Country of isolation	MICs (mg/L) by Etest						Colistin susceptibility	<i>mgrB</i> sequence	Insertion site between	Carbapenemase	Additional ESBL	Sequence type	Clone
		ERT	IPM	MEM	DOR	CST	S							
T1a	Turkey	3	12	>32	3	0.094	S	wild-type	—	KPC-2	none	ST258	I	
T1b	Turkey	>32	>32	>32	>32	4	R	truncated by IS5-like	+74 and +75	KPC-2	none	ST258	I	
1118	France	>32	>32	>32	>32	24	R	truncated by IS5-like	+74 and +75	KPC-3	none	ST258	II	
20C9	France	>32	>32	>32	>32	16	R	truncated by IS5-like	+74 and +75	KPC-3	none	ST512	III	
C9	Colombia	24	>32	>32	>32	12	R	truncated by IS5-like	+74 and +75	KPC-2	none	ST258	IV	
C10	Colombia	24	>32	>32	>32	8	R	truncated by IS5-like	+74 and +75	KPC-2	none	ST258	IV	
C21	Colombia	0.25	0.19	0.094	0.064	24	R	truncated by ISKpn13	+74 and +75	none	CTX-M-2	ST1271	V	
C22	Colombia	0.25	0.19	0.125	0.064	6	R	truncated by ISKpn14	+127 and +128	none	CTX-M-15	ST101	VI	
C1	Colombia	>32	>32	>32	>32	6	R	truncated by IS10R	-27 and -26	KPC-2	none	ST258	VII	
C2	Colombia	>32	>32	>32	>32	8	R	truncated by IS10R	-27 and -26	KPC-2	none	ST258	VII	
C11	Colombia	24	>32	>32	>32	12	R	truncated protein (29 aa)	—	KPC-2	none	ST258	VIII	
15I5	France	3	>32	6	1.5	12	R	truncated protein (29 aa)	—	none	none	ST70	IX	
Sa	France	>32	>32	>32	>32	24	R	truncated protein (27 aa)	—	KPC-2	none	ST258	X	

ERT, ertapenem; IPM, imipenem; MEM, meropenem; DOR, doripenem; CST, colistin; R, resistant; S, susceptible.



**Figure 1.** Dendrogram analysis and virtual gel images of rep-PCR fingerprint patterns of (a) the five *K. pneumoniae* isolates in which insertion of IS5-like into their *mgrB* gene has been identified; (b) the two *K. pneumoniae* isolates in which insertion of IS10R upstream of *mgrB* has been identified.

the first 11 bp were identical. In addition, the exact same target site duplication (TTAA) was identified on each extremity of ISKpn13. Thus, nucleotide positions 74–75 in *mgrB* might constitute a hot spot of integration for ISs belonging to the IS5 family.

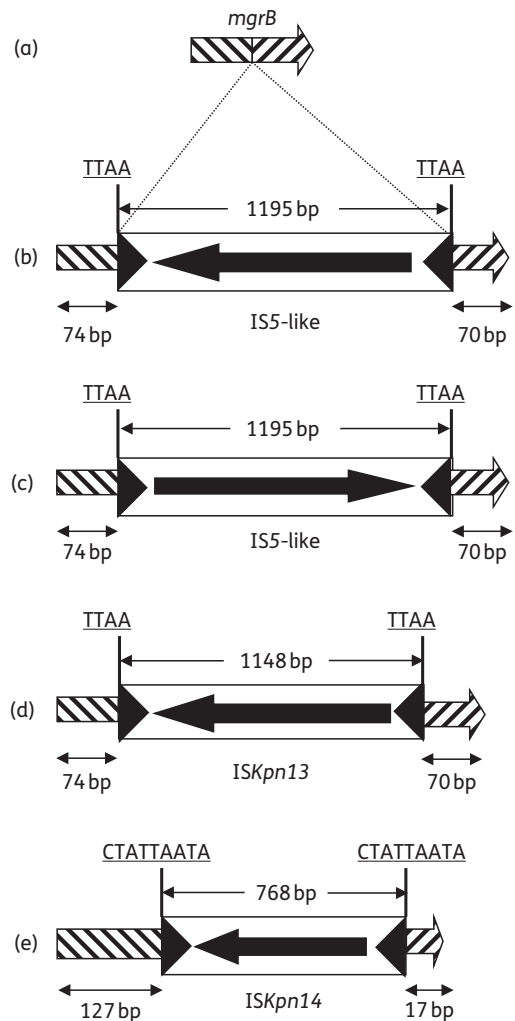
In isolate C22, a truncation of the *mgrB* gene was also found. This truncation resulted from the insertion of ISKpn14, a 768 bp IS belonging to the IS1 family that does not share any significant identity with IS5-like elements.<sup>17</sup> That insertion occurred between nucleotide positions 127 and 128 in the *mgrB* gene, and generated a 9 bp target site duplication (CTATTAATA) (Figure 2e, Table 1).

**Truncation of *mgrB* by a premature stop codon identified in three isolates**

In three isolates, a premature stop codon was identified in the sequence of the *mgrB* gene, leading to truncated proteins. The full-length MgrB protein that is usually 47 amino acids in wild-type isolates was therefore only 29 amino acids long in two unrelated isolates (1515 and C11), and it was 27 amino acids long in another isolate (Sa) (Table 1). Those amino acid substitutions were probably leading to a non-functional MgrB protein, and therefore were probably the source of colistin resistance.

**Modification of the *mgrB* gene expression through the insertion of IS elements**

In isolates C1 and C2, the IS10R element (also called ISVs<sub>a5</sub>) was identified between nucleotide positions –27 and –26 when



**Figure 2.** Schematic representation of the different insertion events identified in the *mgrB* gene. (a) The intact *mgrB* gene as found in wild-type isolates and isolate T1a; (b) *mgrB* truncated by IS5-like as identified by Cannatelli et al.<sup>7</sup>; (c) *mgrB* truncated by IS5-like in isolates 1118, 20C9, C9, C10 and T1b; (d) *mgrB* truncated by ISKpn13 in isolate C21; (e) *mgrB* truncated by ISKpn14 in isolate C22. The target site duplications are underlined. The left and right inverted repeats of each IS are represented as black triangles.

referring to the start codon of *mgrB* as +1. This 1152 bp IS belongs to the IS10 group, which itself belongs to the IS4 family.<sup>18</sup> This suggested that insertion of IS10R might have influenced the expression of *mgrB* in those two isolates. Analysis of the genetic relationship of isolates C1 and C2 performed by rep-PCR (Diversilab<sup>®</sup>) showed that they were clonally related (Figure 1b, Table 1).

*In silico* analysis of the sequence located upstream of a wild-type *mgrB* gene revealed putative promoter sequences made from –35 [TTTTGA] and –10 [TAAACT] boxes separated by 17 bp (Figure 3). The insertion of IS10R actually targeted the DNA sequence located between the *mgrB* start codon and this promoter region. Therefore, it may be hypothesized that this insertion led to an inactivation of this promoter with respect to the

TTATTTGTATGATCCCTGGCGTGATTTTGACACGAACACGTTTGAAACAAGTCGATGATTCCTAAACTTACCTTTCGTAATACAG  
 -35 PROM -10

↓

TTAGCCGCGGTTTAAGAAGGCCGTGCTATCCTGGCGACATTGCGTACTGATGCGGAGAGTGGAGTG  
 -> start codon mgrB

**Figure 3.** Sequences located at the 5'-extremity of the *mgrB* gene. The putative promoter (PROM) is indicated, with its respective –35 and –10 boxes underlined. The arrow indicates the target site for insertion of IS10R. The *mgrB* start codon is in bold.

**Table 2.** MICs results obtained by Etest before and after complementation

Isolates	<i>mgrB</i> genotype	Colistin MIC (mg/L) before complementation	Colistin MIC (mg/L) after complementation with			
			pTOPO- <i>mdh</i>	pTOPO- <i>mgrB</i>	pLow-TOPO- <i>mdh</i>	pLow-TOPO- <i>mgrB</i>
T1b	IS in <i>mgrB</i>	4	4	0.047	4	0.064
C1	IS in <i>mgrB</i> promoter region	6	8	0.032	8	0.016
Sa	truncated protein	24	24	0.047	24	0.032

*mgrB* expression, the corresponding –35 and –10 sequences being too far distant from the gene (Figure 3). That might explain a decrease in or even lack of expression of the *mgrB* gene, leading to a low production of the corresponding protein and consequently the acquired resistance to colistin.

### Complementation experiments

Complementation of three colistin-resistant isolates (one identified with an IS element inserted into the *mgrB* gene, one identified with an IS element inserted into the *mgrB* promoter region and one identified with a truncated *mgrB* gene) was performed. These three isolates were representative of the different isolates identified in this study. For that purpose, these three isolates were transformed with the high-copy recombinant plasmid pTOPO harbouring a wild-type *mgrB* gene (plasmid pTOPO-*mgrB*) in order to provide the wild-type MgrB protein *in trans*. As a result, a full susceptibility to colistin was fully recovered in those three isolates (Table 2). As expected, transformation with plasmid pTOPO-*mdh* used as negative control did not restore susceptibility to colistin. Similar if not identical results were actually obtained when using the low-copy plasmid pLow-TOPO harbouring the same inserts (Table 2). Those results confirmed that resistance to colistin was related to the lack of MgrB. Although we cannot exclude associated mechanisms in these *K. pneumoniae* isolates, results of this complementation assays assessed the role of MgrB in acquired colistin resistance.

### Conclusions

The truncation of the *mgrB* gene was recently identified as a source of acquired colistin resistance in *K. pneumoniae*. Here we further assessed the critical role of modifications in MgrB production in colistin resistance. By identifying a series of truncated *mgrB* genes among colistin-resistant *K. pneumoniae* isolates, we further confirmed that this gene might be considered as a key target for development of colistin resistance in *K. pneumoniae*. It is noteworthy that eight of those isolates belonged to ST258, which is

the most widespread clone producing KPC-type carbapenemase worldwide. Mutations were observed inside the coding sequence of the *mgrB* gene, but also in the promoter region, probably leading to a reduced expression of the *mgrB* gene in the latter case. Overall, those findings further confirm that MgrB makes a crucial contribution to the susceptibility to colistin usually observed in *K. pneumoniae*.

### Funding

This work was funded by the INSERM, France, by the University of Fribourg, Switzerland, and by grants from the European Community (R-GNOSIS, FP7/HEALTH-F3-2011-282512 and MAGIC-BULLET, FP7/HEALTH-F3-2001-278232).

### Transparency declarations

None to declare.

### References

- Falagas ME, Rafailidis PI, Matthaiou DK. Resistance to polymyxins: Mechanisms, frequency and treatment options. *Drug Resist Updat* 2010; **13**: 132–8.
- Yahav D, Farbman L, Leibovici L *et al*. Colistin: new lessons on an old antibiotic. *Clin Microbiol Infect* 2012; **18**: 18–29.
- Nordmann P, Naas T, Poirel L. Global spread of carbapenemase-producing Enterobacteriaceae. *Emerg Infect Dis* 2011; **17**: 1791–8.
- Biswas S, Brunel JM, Dubus JC *et al*. Colistin: an update on the antibiotic of the 21<sup>st</sup> century. *Expert Rev Anti Infect Ther* 2012; **10**: 917–34.
- Cheng HY, Chen YF, Peng HL. Molecular characterization of the PhoPQ-PmrD-PmrAB mediated pathway regulating polymyxin B resistance in *Klebsiella pneumoniae* CG43. *J Biomed Sci* 2010; **17**: 60.
- Cannatelli A, Pilato VD, Giani T *et al*. In vivo evolution to colistin resistance by PmrB sensor kinase mutation in KPC carbapenemase-producing *Klebsiella pneumoniae* associated with low-dosage colistin treatment. *Antimicrob Agents Chemother* 2014; **58**: 4399–403.

- 7** Cannatelli A, D'Andrea MM, Giani T et al. In vivo emergence of colistin resistance in *Klebsiella pneumoniae* producing KPC-type carbapenemase mediated by insertional inactivation of the PhoQ/PhoP mgrB regulator. *Antimicrob Agents Chemother* 2013; **57**: 5521–6.
- 8** Lopez-Camacho E, Gomez-Gil R, Tobes R et al. Genomic analysis of the emergence and evolution of multidrug resistance during a *Klebsiella pneumoniae* outbreak including carbapenem and colistin resistance. *J Antimicrob Chemother* 2014; **69**: 632–6.
- 9** Groisman EA. The pleiotropic two-component regulatory system PhoP-PhoQ. *J Bacteriol* 2001; **183**: 1835–42.
- 10** Kato A, Tanabe H, Utsumi R. Molecular characterization of the PhoP-PhoQ two-component system in *Escherichia coli* K-12: identification of extracellular Mg<sup>2+</sup>-responsive promoters. *J Bacteriol* 1999; **181**: 5516–20.
- 11** Mitrophanov AY, Jewett MW, Hadley TJ et al. Evolution and dynamics of regulatory architectures controlling polymyxin B resistance in enteric bacteria. *PLoS Genet* 2008; **4**: e1000233.
- 12** Lippa AM, Goulian M. Feedback inhibition in the PhoQ/PhoP signaling system by a membrane peptide. *PLoS Genet* 2009; **5**: e1000788.
- 13** Poirel L, Walsh TR, Cuvillier V et al. Multiplex PCR for detection of acquired carbapenemase genes. *Diagn Microbiol Infect Dis* 2011; **70**: 119–23.
- 14** Diancourt L, Passet V, Verhoef J et al. Multilocus sequence typing of *Klebsiella pneumoniae* nosocomial isolates. *J Clin Microbiol* 2005; **43**: 4178–82.
- 15** Blattner FR, Fianndt M, Hass KK et al. Deletions and insertions in the immunity region of coliphage lambda: revised measurement of the promoter-startpoint distance. *Virology* 1974; **62**: 458–71.
- 16** Marquez C, Labbate M, Raymondo C et al. Urinary tract infections in a South American population: dynamic spread of class 1 integrons and multidrug resistance by homologous and site-specific recombination. *J Clin Microbiol* 2008; **46**: 3417–25.
- 17** Sekine Y, Ohtsubo E. Frameshifting is required for production of the transposase encoded by insertion sequence 1. *Proc Natl Acad Sci USA* 1989; **86**: 4609–13.
- 18** Hjerde E, Lorentzen MS, Holden MT et al. The genome sequence of the fish pathogen *Aliivibrio salmonicida* strain LFI1238 shows extensive evidence of gene decay. *BMC Genom* 2008; **9**: 616.

## **b. L'insertion du gène *blaCTX-M* au sein du gène *mgrB* est responsable de l'acquisition d'une résistance croisée aux céphalosporines de troisième génération et à la colistine**

### **Matériel et méthodes :**

Une souche de *K. pneumoniae* résistante à la colistine (CMI = 64 mg/L) et aux céphalosporines de large spectre, isolée en France chez un patient jamais traité par de la colistine, a été incluse dans cette étude. Les CMI de la colistine ont été déterminées par dilutions en milieu liquide. Les opérons *pmrAB*, *phoPQ*, et *mgrB* ont été séquencés pour rechercher des mutations.

### **Principaux résultats :**

Nous avons identifié une séquence d'insertion au sein du gène *mgrB* responsable de l'acquisition de la résistance à la colistine. L'étude de cette séquence d'insertion a révélé qu'il s'agissait du gène *blaCTX-M-15* couplé à la séquence d'insertion *ISEcp1*. Le gène *blaCTX-M-15* code pour la BLSE CTX-M-15 responsable de l'acquisition de résistances aux céphalosporines de large spectre.

### **Conclusion :**

La transposition du gène *blaCTX-M-15* médiée par la séquence d'insertion *ISEcp1* au sein du gène *mgrB* est ainsi responsable de l'acquisition d'une résistance croisée aux céphalosporines de large spectre et à la colistine. Le patient avait été traité par du céfotaxime mais jamais par de la colistine. Nous ne savons pas si cette résistance à la colistine a été acquise *in vivo* mais étant donné la forte prévalence des gènes *blaCTX-M* dans les souches d'entérobactéries, une acquisition de la résistance à la colistine par simple transposition du gène *blaCTX-M* au sein du gène *mgrB* est très inquiétante.

**Ce travail a été publié dans *Antimicrobial Agents and Chemotherapy* en avril 2016 (IF = 4.415).**



# Acquisition of Broad-Spectrum Cephalosporin Resistance Leading to Colistin Resistance in *Klebsiella pneumoniae*

Aurélie Jayol,<sup>a</sup> Patrice Nordmann,<sup>a,b</sup> Marine Desroches,<sup>c,d</sup> Jean-Winoc Decousser,<sup>c,d</sup> Laurent Poirel<sup>a</sup>

Medical and Molecular Microbiology, Emerging Antibiotic Resistance Unit, Department of Medicine, Faculty of Science, University of Fribourg, Fribourg, Switzerland<sup>a</sup>; HFR - Hôpital Cantonal, Fribourg, Switzerland<sup>b</sup>; Department of Virology, Bacteriology-Infect Control, Parasitology-Myecology, Assistance Publique-Hôpitaux de Paris, University Hospital Henri Mondor, Créteil, France<sup>c</sup>; IAME, UMR 1137, INSERM, Paris, France<sup>d</sup>

**An extended-spectrum  $\beta$ -lactamase (ESBL)-producing and colistin-resistant *Klebsiella pneumoniae* clinical isolate was recovered from a patient who was treated with cefotaxime. This isolate harbored a *bla*<sub>CTX-M-15</sub> ESBL gene that was associated with an *ISEcp1* insertion sequence. Transposition of that tandem occurred within the chromosomal *mgrB* gene, leading to inactivation of the *mgrB* gene and consequently to acquired resistance to colistin. We showed here a coselection of colistin resistance as a result of a broad-spectrum cephalosporin selective pressure.**

Nosocomial infections caused by multidrug-resistant (MDR) *Klebsiella pneumoniae* represent a growing problem worldwide. The use of polymyxins (colistin and polymyxin B) is therefore being reconsidered as a last-line treatment option but also as a first-line treatment in some specific areas where MDR organisms are highly prevalent (Italy, Greece, and Brazil). However, an increasing number of studies are currently reporting resistance to colistin in *K. pneumoniae* (1).

Colistin is a polycationic antibiotic of the polymyxin family that interacts with the lipid A moiety of the Gram-negative bacterial lipopolysaccharide (LPS) and consequently disrupts the integrity of the outer membrane. The main mechanisms leading to polymyxin resistance are modifications to the bacterial outer membrane, such as modifications of lipid A with phosphoethanolamine and 4-amino-4-deoxy-L-arabinose (2). Truncation of the *mgrB* gene, which encodes a negative regulator of the PhoP/PhoQ signaling system, leads to upregulation of this system, leading to upregulation of the *pmrHFIJKLM* operon, the synthesis of 4-amino-4-deoxy-L-arabinose, and ultimately to colistin resistance (3, 4). Inactivation of the *mgrB* gene has been identified as a common source of acquired colistin resistance in *K. pneumoniae*, with diverse genetic events leading to alterations of the *mgrB* gene in both human and animal isolates (3–8). In addition, very recently, a plasmid-mediated resistance mechanism has been identified, corresponding to a phosphoethanolamine transferase identified in human and animal enterobacterial isolates worldwide, and also in food products (9–13).

CTX-M-type  $\beta$ -lactamases are extended-spectrum  $\beta$ -lactamases (ESBL) reported mostly in *Enterobacteriaceae* (14). CTX-M-15, which was first identified in 2001 from patients hospitalized in India (15), is the most common CTX-M variant worldwide, possessing significant activity against ceftazidime (16). The *bla*<sub>CTX-M-15</sub> gene is mostly located on large plasmids of various types and sizes, but it may also be identified on the chromosome of *K. pneumoniae* (17). The acquisition of both chromosomally encoded and plasmid-carried *bla*<sub>CTX-M-15</sub> genes has been shown to be related to the association with insertion sequence *ISEcp1* (18). *ISEcp1* is responsible for mobilization of the *bla*<sub>CTX-M-15</sub> gene and also for its high-level expression (19, 20). More generally, *ISEcp1*-like elements mobilize and promote the expression of *bla*<sub>CTX-M</sub> genes (21).

We report here the identification of a chromosomal insertion of the *ISEcp1*-*bla*<sub>CTX-M-15</sub> locus into the *mgrB* gene responsible for acquired colistin resistance in *K. pneumoniae*.

A patient with a history of prostate cancer (permanent urinary catheter) and chronic kidney disease was admitted for ischemic heart disease on 27 February 2015, at the Henri Mondor Hospital (Paris, France). The following day, he was transferred to the intensive care unit (ICU) since he developed septic shock secondary to a urinary infection with *Morganella morganii* and bacteremia with *Streptococcus mitis*. Those infections were treated with cefotaxime and gentamicin, and then ofloxacin was given for 12 days. *K. pneumoniae* isolate HM, which is resistant to broad-spectrum cephalosporins and colistin, was recovered on 25 March 2015 from a urine sample. A piperacillin-tazobactam combination was consequently initiated and maintained for 15 days. He was discharged but readmitted to the ICU on 15 May 2015 because of pyelonephritis complicated by acute renal failure. *K. pneumoniae* isolate HM was again recovered from urine cultures. Treatment with imipenem and amikacin was therefore initiated and maintained for 14 days, and the patient was discharged.

Susceptibility testing was performed by the disk diffusion method according to the EUCAST guidelines (22), except for colistin. The MIC of colistin was determined using broth microdilution, according to CLSI guidelines (23). The results were interpreted according to EUCAST guidelines (22). Although isolate HM was resistant to broad-spectrum cephalosporins, kanamycin, and gentamicin, and it remained susceptible to piperacillin-tazobactam, ceftazidime, carbapenems, amikacin, ciprofloxacin, trimethoprim-sulfamethoxazole, fosfomycin, and chloramphenicol. The isolates were resistant to colistin, with an MIC of 64 mg/liter.

Received 28 January 2016 Returned for modification 14 February 2016

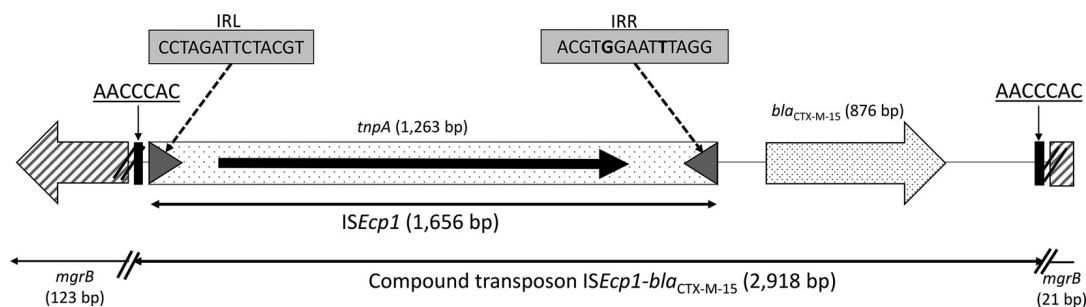
Accepted 25 February 2016

Accepted manuscript posted online 7 March 2016

Citation Jayol A, Nordmann P, Desroches M, Decousser J-W, Poirel L. 2016. Acquisition of broad-spectrum cephalosporin resistance leading to colistin resistance in *Klebsiella pneumoniae*. *Antimicrob Agents Chemother* 60:3199–3201. doi:10.1128/AAC.00237-16.

Address correspondence to Laurent Poirel, laurent.poirel@unifr.ch.

Copyright © 2016, American Society for Microbiology. All Rights Reserved.



**FIG 1** Schematic representation of the *mgrB* gene truncated by *ISEcp1-bla<sub>CTX-M-15</sub>* in *K. pneumoniae* isolate HM. Arrows indicate genes and their respective transcription orientations. IRL, inverted repeat left; IRR, inverted repeat right. The *mgrB* gene truncation is represented by diagonal dashes. *tnpA* corresponds to the transposase-encoding gene of *ISEcp1*. The 7-bp target site duplications are underlined.

Multilocus sequence typing analysis was performed on *K. pneumoniae* HM, as described previously (24), and revealed a novel *phoE* allele leading to a new sequence type (ST), namely, ST2003 (2-1-62-3-242-4-110), which differs from ST405 by only a single allele.

Sequence analysis of the genes encoding proteins involved in the modification of the LPS (*pmrA*, *pmrB*, *phoP*, *phoQ*, and *mgrB*) was performed as described previously (4). PCR and sequencing identified an integrated fragment of 2,918 bp in size truncating the *mgrB* gene (Fig. 1). This truncation occurred between nucleotides +21 and +22, and the inserted fragment consisted of an *ISEcp1-bla<sub>CTX-M-15</sub>* compound transposon (19). Noteworthy, even though the insertion of *ISEcp1* has been demonstrated to generate 5-bp target site duplications upon transposition (25), here, the *ISEcp1-bla<sub>CTX-M-15</sub>* compound transposon was unusually bracketed by a 7-bp direct repeat sequence (AACCCAC) (Fig. 1).

Conjugation and transformation experiments were performed in order to determine whether another copy of the *bla<sub>CTX-M-15</sub>* gene might also be plasmid-borne in that isolate. Conjugation experiments using *K. pneumoniae* isolate HM as a donor and azide-resistant *Escherichia coli* J53 as a recipient were therefore performed as described previously (26), with selection with ticarcillin (100 µg/ml) and sodium azide (100 µg/ml). No transconjugant was obtained despite repeated attempts. Plasmid extraction from *K. pneumoniae* HM was performed using the Kieser method (27). Electroporation assays were performed into *E. coli* TOP10 using this plasmid extract, and selection was done with ticarcillin (100 µg/ml), but no electrotransformant was obtained. In parallel, selection was performed onto plates containing colistin (3 µg/ml) in order to screen for a putative transferable colistin resistance determinant, but no transformant was obtained. In addition, S1 nuclease pulsed-field gel electrophoresis analysis was performed as described previously (28), but Southern hybridization with a *bla<sub>CTX-M-15</sub>*-specific probe failed to detect any positive plasmid. Finally, a search of the *mcr-1* gene by PCR, as described previously (13), remained negative.

The *bla<sub>CTX-M-15</sub>* gene was therefore chromosomally located and inserted into the *mgrB* gene. Noteworthy, the chromosomal insertion of the *ISEcp1-bla<sub>CTX-M-15</sub>* tandem into the *mgrB* gene resulted in acquired resistance to broad-spectrum cephalosporins, and concomitantly to colistin.

Although previous reports identified either premature stop codons, amino acid substitutions, or insertions of IS elements as a source of *mgrB* modifications, we identified here an original genetic event truncating the *mgrB* gene that led to the acquisition of

colistin resistance (3, 5, 6). What we actually observed here was the acquisition of a gene encoding acquired resistance to an antibiotic class leading to acquired resistance to another antibiotic class, which is noteworthy.

Here, we report that a cefotaxime- and gentamicin-containing regimen was followed by the selection of a *K. pneumoniae* isolate that was resistant to broad-spectrum cephalosporins, gentamicin, and colistin. Of note, the patient never received colistin. We do not know whether this colistin-resistant isolate was selected *in vivo* in this patient or whether it was acquired by cross-transmission. Considering the increasing rate of CTX-M-producing isolates worldwide, such acquisition of resistance to broad-spectrum cephalosporins being concomitantly a source of acquired resistance to colistin, which is now considered one of the last weapons of our armamentarium, is very worrying.

#### ACKNOWLEDGMENT

This work was financed by the University of Fribourg, Switzerland.

#### FUNDING INFORMATION

This work, including the efforts of Laurent Poirel, Aurélie Jayol, Patrice Nordmann, Marine Desroches, and Jean-Winoc Decusser, was funded by University of Fribourg.

#### REFERENCES

- Bialvaei AZ, Samadi Kafil H. 2015. Colistin, mechanisms and prevalence of resistance. *Curr Med Res Opin* 31:707–721. <http://dx.doi.org/10.1185/03007995.2015.1018989>.
- Olaitan AO, Morand S, Rolain JM. 2014. Mechanisms of polymyxin resistance: acquired and intrinsic resistance in bacteria. *Front Microbiol* 5:643.
- Poirel L, Jayol A, Bontron S, Villegas MV, Ozdamar M, Turkoglu S, Nordmann P. 2015. The *mgrB* gene as a key target for acquired resistance to colistin in *Klebsiella pneumoniae*. *J Antimicrob Chemother* 70:75–80. <http://dx.doi.org/10.1093/jac/dku323>.
- Cannatelli A, D'Andrea MM, Giani T, Di Pilato V, Arena F, Ambretti S, Gaibani P, Rossolini GM. 2013. *In vivo* emergence of colistin resistance in *Klebsiella pneumoniae* producing KPC-type carbapenemases mediated by insertional inactivation of the PhoQ/PhoP *mgrB* regulator. *Antimicrob Agents Chemother* 57:5521–5526. <http://dx.doi.org/10.1128/AAC.01480-13>.
- Kieffer N, Poirel L, Nordmann P, Madec JY, Haenni M. 2015. Emergence of colistin resistance in *Klebsiella pneumoniae* from veterinary medicine. *J Antimicrob Chemother* 70:1265–1267.
- Cannatelli A, Giani T, D'Andrea MM, Di Pilato V, Arena F, Conte V, Tryfinopoulou K, Vatopoulos A, Rossolini GM; COLGRIT Study Group. 2014. *MgrB* inactivation is a common mechanism of colistin resistance in KPC carbapenemase-producing *Klebsiella pneumoniae* of clinical origin. *Antimicrob Agents Chemother* 58:5696–5703. <http://dx.doi.org/10.1128/AAC.03110-14>.



7. Olaitan AO, Diene SM, Kempf M, Berrazeg M, Bakour S, Gupta SK, Thongmalayvong B, Akkhavong K, Somphavong S, Paboriboune P, Chaisiri K, Komalamisra C, Adelowo OO, Fagade OE, Banjo OA, Oke AJ, Adler A, Assous MV, Morand S, Raoult D, Rolain JM. 2014. Worldwide emergence of colistin resistance in *Klebsiella pneumoniae* from healthy humans and patients in Lao PDR, Thailand, Israel, Nigeria and France owing to inactivation of the PhoP/PhoQ regulator *mgrB*: an epidemiological and molecular study. *Int J Antimicrob Agents* 44:500–507. <http://dx.doi.org/10.1016/j.ijantimicag.2014.07.020>.
8. Cheng YH, Lin TL, Pan YJ, Wang YP, Lin YT, Wang JT. 2015. Colistin-resistant mechanisms in *Klebsiella pneumoniae* from Taiwan. *Antimicrob Agents Chemother* 59:2909–2913. <http://dx.doi.org/10.1128/AAC.04763-14>.
9. Liu YY, Wang Y, Walsh TR, Yi LX, Zhang R, Spencer J, Doi Y, Tian G, Dong B, Huang X, Yu LF, Gu D, Ren H, Chen X, Lv L, He D, Zhou H, Liang Z, Liu JH, Shen J. 2016. Emergence of plasmid-mediated colistin resistance mechanism MCR-1 in animals and human beings in China: a microbiological and molecular biological study. *Lancet Infect Dis* 16:161–168. [http://dx.doi.org/10.1016/S1473-3099\(15\)00424-7](http://dx.doi.org/10.1016/S1473-3099(15)00424-7).
10. Hasman H, Hammerum AM, Hansen F, Hendriksen RS, Olesen B, Agerø Y, Zankari E, Leekitcharoenphon P, Stegger M, Kaas RS, Cavaco LM, Hansen DS, Aarestrup FM, Skov RL. 2015. Detection of *mcr-1* encoding plasmid-mediated colistin-resistant *Escherichia coli* isolates from human bloodstream infection and imported chicken meat, Denmark 2015. *Euro Surveill* 20:pii=21331. <http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=21331>.
11. Haenni M, Poirel L, Kieffer N, Châtre P, Saras E, Métayer V, Dumoulin R, Nordmann P, Madec JY. Co-occurrence of extended spectrum  $\beta$ -lactamase and MCR-1 encoding genes on plasmids. *Lancet Infect Dis*, in press.
12. Malhotra-Kumar S, Xavier BB, Das AJ, Lammens C, Hoang HT, Pham NT, Goossens H. Colistin-resistant *Escherichia coli* harbouring *mcr-1* isolated from food animals in Hanoi, Vietnam. *Lancet Infect Dis*, in press.
13. Poirel L, Kieffer N, Liassine N, Thanh D, Nordmann P. Plasmid-mediated carbapenem and colistin resistance in a clinical isolate of *Escherichia coli*. *Lancet Infect Dis*, in press.
14. D'Andrea MM, Arena F, Pallechi L, Rossolini GM. 2013. CTX-M-type  $\beta$ -lactamases: a successful story of antibiotic resistance. *Int J Med Microbiol* 303:305–317. <http://dx.doi.org/10.1016/j.ijmm.2013.02.008>.
15. Karim A, Poirel L, Nagarajan S, Nordmann P. 2001. Plasmid-mediated extended-spectrum  $\beta$ -lactamase (CTX-M-3 like) from India and gene association with insertion sequence *ISEc1*. *FEMS Microbiol Lett* 201:237–241. <http://dx.doi.org/10.1111/j.1574-6968.2001.tb10762.x>.
16. Poirel L, Gniadkowski M, Nordmann P. 2002. Biochemical analysis of the ceftazidime-hydrolysing extended-spectrum  $\beta$ -lactamase CTX-M-15 and of its structurally related  $\beta$ -lactamase CTX-M-3. *J Antimicrob Chemother* 50:1031–1034. <http://dx.doi.org/10.1093/jac/dkf240>.
17. Coelho A, González-López JJ, Miró E, Alonso-Tarrés C, Mirelis B, Larrosa MN, Bartolomé RM, Andreu A, Navarro F, Johnson JR, Prats G. 2010. Characterisation of the CTX-M-15-encoding gene in *Klebsiella pneumoniae* strains from the Barcelona metropolitan area: plasmid diversity and chromosomal integration. *Int J Antimicrob Agents* 36:73–78. <http://dx.doi.org/10.1016/j.ijantimicag.2010.03.005>.
18. Lartigue MF, Poirel L, Nordmann P. 2004. Diversity of genetic environment of *bla*<sub>CTX-M</sub> genes. *FEMS Microbiol Lett* 234:201–207. <http://dx.doi.org/10.1111/j.1574-6968.2004.tb09534.x>.
19. Poirel L, Decousser JW, Nordmann P. 2003. Insertion sequence *ISEc1B* is involved in expression and mobilization of a *bla*<sub>CTX-M</sub>  $\beta$ -lactamase gene. *Antimicrob Agents Chemother* 47:2938–2945. <http://dx.doi.org/10.1128/AAC.47.9.2938-2945.2003>.
20. Poirel L, Lartigue MF, Decousser JW, Nordmann P. 2005. *ISEc1B*-mediated transposition of *bla*<sub>CTX-M</sub> in *Escherichia coli*. *Antimicrob Agents Chemother* 49:447–450. <http://dx.doi.org/10.1128/AAC.49.1.447-450.2005>.
21. Nordmann P, Lartigue MF, Poirel L. 2008. Beta-lactam induction of *ISEc1B*-mediated mobilization of the naturally occurring *bla*<sub>CTX-M</sub>  $\beta$ -lactamase gene of *Kluyvera ascorbata*. *FEMS Microbiol Lett* 288:247–249. <http://dx.doi.org/10.1111/j.1574-6968.2008.01359.x>.
22. European Committee on Antimicrobial Susceptibility Testing (EUCAST). 2016. Breakpoints tables for interpretation of MICs and zone diameters, version 6.0. European Committee on Antimicrobial Susceptibility Testing, Växjö, Sweden. [http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST\\_files/Breakpoint\\_tables/v\\_6.0\\_Breakpoint\\_table.pdf](http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Breakpoint_tables/v_6.0_Breakpoint_table.pdf).
23. Clinical and Laboratory Standards Institute (CLSI). 2015. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; approved standard, 10th ed. CLSI document M07-A10. Clinical and Laboratory Standards Institute, Wayne, PA.
24. Diancourt L, Passet V, Verhoef J, Grimont PA, Brisse S. 2005. Multilocus sequence typing of *Klebsiella pneumoniae* nosocomial isolates. *J Clin Microbiol* 43:4178–4182. <http://dx.doi.org/10.1128/JCM.43.8.4178-4182.2005>.
25. Cattoir V, Nordmann P, Silva-Sanchez J, Espinal P, Poirel L. 2008. *ISEc1*-mediated transposition of *qnrB*-like gene in *Escherichia coli*. *Antimicrob Agents Chemother* 52:2929–2932. <http://dx.doi.org/10.1128/AAC.00349-08>.
26. Potron A, Poirel L, Nordmann P. 2014. Derepressed transfer properties leading to the efficient spread of the plasmid encoding carbapenemase OXA-48. *Antimicrob Agents Chemother* 58:467–471. <http://dx.doi.org/10.1128/AAC.01344-13>.
27. Kieser T. 1984. Factors affecting the isolation of CCC DNA from *Streptomyces lividans* and *Escherichia coli*. *Plasmid* 12:19–36. [http://dx.doi.org/10.1016/0147-619X\(84\)90063-5](http://dx.doi.org/10.1016/0147-619X(84)90063-5).
28. Barton BM, Harding GP, Zuccarelli AJ. 1995. A general method for detecting and sizing large plasmids. *Anal Biochem* 226:235–240. <http://dx.doi.org/10.1006/abio.1995.1220>.

#### **1.4. Identification de mutations dans la protéine CrrB responsables de l'acquisition de résistances à la colistine**

##### **Contexte :**

En 2014, deux études ont révélé que des mutations dans la protéine CrrB, un régulateur positif du système à deux composants PmrAB, étaient responsables de l'acquisition de résistances à la colistine chez *K. pneumoniae* (30, 31).

##### **Objectif de l'étude :**

L'objectif de cette étude était donc de rechercher des altérations au sein du gène *crrB* responsables de l'acquisition de résistances à la colistine dans une collection de souches de *K. pneumoniae*.

##### **Matériel et méthodes :**

Trente-trois souches de *K. pneumoniae* résistantes à la colistine et ne présentant pas d'altérations au sein des gènes *pmrAB*, *phoPQ* et *mgrB*, ni de gènes plasmidiques *mcr-1* et *mcr-2*, ont été incluses dans cette étude. Les CMI de la colistine ont été déterminées par dilutions en milieu liquide. L'opéron *crrAB* a été séquencé pour rechercher des mutations. Des expériences de complémentation ont été réalisées par clonage du gène sauvage *crrB* dans un plasmide vecteur et électroporation du plasmide recombinant dans les souches résistantes à la colistine.

##### **Principaux résultats :**

Des mutations ont été identifiées dans la protéine CrrB de quatre souches de *K. pneumoniae* présentant une résistance de haut niveau à la colistine (CMI de la colistine > 128 mg/L). Les mutations observées étaient variées : F84S, N141Y, P151L, G183V. Trois de ces mutations étaient localisées dans le domaine phosphoaccepteur Histidine kinase A et deux d'entre elles étaient situées à la même position (acides aminés 141 et 151) que des mutations décrites comme étant responsables de la résistance à la colistine par Cheng et al (31) mais la substitution d'acide aminé était différente. Les expériences de complémentation de ces souches avec le gène *crrB* sauvage ont conduit à une restauration de la sensibilité à la colistine (CMI = 0,25-0,5 mg/L) et ont ainsi confirmé l'implication des 4 différentes mutations dans l'acquisition de la résistance à la colistine.

##### **Conclusion :**

Nous avons identifié quatre nouvelles mutations au sein du gène *crrB* responsables de l'acquisition d'un haut niveau de résistance à la colistine chez *K. pneumoniae*.

**Ce travail a été publié dans *Antimicrobial Agents and Chemotherapy* en novembre 2017 (IF = 4.415).**



# High-Level Resistance to Colistin Mediated by Various Mutations in the *crrB* Gene among Carbapenemase-Producing *Klebsiella pneumoniae*

Aurélie Jayol,<sup>a,b,c,d,e</sup> Patrice Nordmann,<sup>a,b,c,f</sup> Adrian Brink,<sup>g</sup>  
Maria-Virginia Villegas,<sup>h</sup> Véronique Dubois,<sup>d,e</sup> Laurent Poirel<sup>a,b,c</sup>

Emerging Antibiotic Resistance Unit, Medical and Molecular Microbiology, Department of Medicine, University of Fribourg, Fribourg, Switzerland<sup>a</sup>; INSERM European Unit (LEA Paris, IAME, France), University of Fribourg, Fribourg, Switzerland<sup>b</sup>; National Reference Center for Emerging Antibiotic Resistance, University of Fribourg, Fribourg, Switzerland<sup>c</sup>; Laboratory of Bacteriology, Bordeaux University Hospital, Bordeaux, France<sup>d</sup>; CNRS UMR5234, University of Bordeaux, Bordeaux, France<sup>e</sup>; University of Lausanne and University Hospital Center, Lausanne, Switzerland<sup>f</sup>; Department of Clinical Microbiology, Ampath National Laboratory Services, Milpark Hospital, Johannesburg, South Africa<sup>g</sup>; International Center for Medical Research and Training, CIDEIM, Cali, Colombia<sup>h</sup>

**ABSTRACT** Mutations in *crrAB* genes encoding a two-component regulator involved in modifications of lipopolysaccharide were searched for among a collection of colistin-resistant *Klebsiella pneumoniae* isolates. Four isolates, respectively, producing carbapenemases NDM-1, OXA-181, or KPC-2 showed mutated CrrB proteins compared with those in wild-type strains. Complementation assays with a wild-type CrrB protein restored the susceptibility to colistin in all cases, confirming the involvement of the identified substitutions in the resistance phenotype.

**KEYWORDS** polymyxin, rapid polymyxin NP test, susceptibility testing, resistance mechanisms, CrrAB, *Klebsiella pneumoniae*

The emergence and spread of carbapenemase-producing *Klebsiella pneumoniae* isolates worldwide has forced clinicians to reintroduce colistin as last-resort therapy (1). Besides the plasmid-mediated *mcr-1* and *mcr-2* genes in *K. pneumoniae* (2, 3), the chromosomally encoded alterations of the *mgrB* gene and the PmrAB and PhoPQ two-component systems are currently the most commonly reported mechanisms of acquisition of polymyxin resistance in this enterobacterial species (4). Recently, mutations in the *crrB* gene, belonging to a third two-component system (named CrrAB for colistin resistance regulation) and involved in lipopolysaccharide (LPS) modifications, were associated with colistin resistance (5, 6). Mutations in the *crrB* gene are responsible for the increase in *crrC* gene transcription, which in turn regulates the expression of the *pmrC* gene and the *pmrHFIJKLM* operon, through the PmrAB two-component system (6). Expression of these genes leads to the addition of cationic groups on the LPS and consequently to colistin resistance.

In previous studies, the colistin resistance mechanisms of a collection of 185 *K. pneumoniae* isolates recovered from human samples worldwide (Europe, Turkey, Colombia, South Africa) were analyzed. Chromosomally encoded modifications of LPS through alterations (mutation, truncation) of the *mgrB* gene (7–9), the *pmrAB* genes (10), or the *phoPQ* genes (11) were identified in 152 isolates. However, 33 isolates did not present any substitutions in these genes, and they were negative for the plasmid-mediated resistance genes *mcr-1* and *mcr-2*.

We analyzed the genes encoding the CrrAB system in these 33 isolates. Amplification of the *crrA* and *crrB* genes was performed using primers *crrAB-extF* (5'-GTGAGCCATCAAAT

Received 12 July 2017 Returned for  
modification 31 July 2017 Accepted 30  
August 2017

Accepted manuscript posted online 5  
September 2017

**Citation** Jayol A, Nordmann P, Brink A, Villegas M-V, Dubois V, Poirel L. 2017. High-level resistance to colistin mediated by various mutations in the *crrB* gene among carbapenemase-producing *Klebsiella pneumoniae*. *Antimicrob Agents Chemother* 61:e01423-17. <https://doi.org/10.1128/AAC.01423-17>.

**Copyright** © 2017 American Society for Microbiology. All Rights Reserved.

Address correspondence to Laurent Poirel, laurent.poirel@unifr.ch.

TCTCG-3') and *crrAB*-extR (5'-AAGTCCCAAAAGAGGCAAAC-3') located on each extremity of this operon. No amplification was obtained with the primers for 19 of the 33 isolates. We obtained the same result by using internal primers annealing into the *crrB* gene, namely, *crrB*-intF (5'-GTGACTATCTTACGTGGGAG-3') and *crrB*-intR (5'-CACTCAGCATCAAGGAGTAC-3'). This absence of amplification suggested an absence of the *crrAB* operon in these strains, which is in accordance with the variability of the lateral acquisition of the *crrAB* operon in *K. pneumoniae* (5). Amplification of the *crrAB* gene was obtained for the 14 remaining isolates. Subsequent sequencing identified mutations leading to amino acid changes (F84S, N141Y, P151L, and G183V) in the CrrB protein in 4 of the 14 colistin-resistant *K. pneumoniae* isolates (Table 1).

Three of the CrrB amino acid substitutions were located in the histidine kinase A (HisKA) phosphoacceptor domain (amino acids 136 to 200) (Table 1). Previously, four mutations in this HisKA domain were found to be involved in colistin resistance (6) (Table 1). Among them, two were at the same position as the mutations observed in our strains (amino acids 141 and 151), but the amino acid changes were different. The fourth strain presented a mutation in the HAMP domain of CrrB, whereas a single mutation in this domain has been shown (5). All of the substitutions in the CrrB protein, known to be responsible for colistin resistance, are shown in Table 1.

Determination of the colistin MICs by use of the reference broth microdilution method showed a high level of colistin resistance (MIC of colistin, >128  $\mu\text{g/ml}$  for all four isolates with mutated *crrB* genes) (Table 1). The high MICs of colistin in the *K. pneumoniae* strains are in accordance with results reported by Cheng et al. (6) (Table 1). Furthermore, Wright et al. (5) reported lower MICs of colistin (16  $\mu\text{g/ml}$ ) for two isolates exhibiting CrrB mutations. However, MICs were determined by use of the Etest strip technique, which is known to underestimate colistin MIC values (12).

We performed complementation assays to confirm the involvement of the mutated *crrB* gene in the colistin resistance phenotype. A recombinant plasmid (pTRIC) was built by cloning a triclosan resistance gene (*mFabI*) (13) into the low-copy-number plasmid pBR322. The wild-type *crrB* gene from the colistin-susceptible *K. pneumoniae* strain Af44a (MIC of colistin, 0.25  $\mu\text{g/ml}$ ) was amplified by PCR and cloned into this plasmid. The recombinant *crrB*-pTRIC plasmid and the pTRIC plasmid were separately transformed by electroporation into the four resistant strains presenting the mutations in the *crrB* gene. Transformants were selected by overnight incubation at 37°C on Mueller-Hinton agar supplemented with triclosan (1  $\mu\text{M}$ ), and the recombinant clones were checked by PCR and sequencing. MICs of colistin for the transformants revealed that production of a wild-type CrrB protein (*crrB*-pTRIC plasmid) restored the susceptibility to colistin in all isolates (MIC,  $\leq 0.5$   $\mu\text{g/ml}$ ) (Table 1), confirming that the different substitutions in CrrB were, respectively, responsible for the resistant phenotypes. As expected, transformation with the pTRIC plasmid used as a negative control did not restore any susceptibility to colistin.

All isolates presenting a CrrB amino acid change produced a carbapenemase (NDM-1, OXA-181, or KPC-2) (Table 1). They were recovered from patients who had been treated with colistin in Colombia, France, Greece, and South Africa. For the patient harboring the colistin-resistant isolate Af44b, one colistin-susceptible isolate (Af44a) was recovered before the colistin treatment, and pulsed-field gel electrophoresis analysis confirmed the clonal relationship of the two isolates (data not shown). Sequencing of the *crrB* gene of the Af44a isogenic susceptible strain identified a wild-type CrrB protein, reinforcing the hypothesis that the *in vivo* emergence of colistin resistance under colistin pressure might be related to CrrB mutations in some instances.

In conclusion, four novel mutations in the CrrB protein were identified at the sources of acquisition of high-level colistin resistance among carbapenemase-producing *K. pneumoniae*.

**TABLE 1** Features of colistin-resistant *K. pneumoniae* clinical isolates

Isolate <sup>a</sup>	Origin of isolate	MIC of colistin <sup>b</sup> ( $\mu\text{g/ml}$ )	Colistin susceptibility <sup>c</sup>	CrrB amino acid change in:																	MIC of colistin after complementation <sup>f</sup> ( $\mu\text{g/ml}$ )	Carbapenemase	Reference/source					
				TM <sup>d</sup>	HAMP <sup>d</sup>	HisKA <sup>d</sup>	10 <sup>e</sup>	Q	Y	F	L	W	140 <sup>e</sup>	N	P	151 <sup>e</sup>	G	S	183 <sup>e</sup>	195 <sup>e</sup>								
Af44a <sup>g</sup>	South Africa	0.25	S																								OXA-181	This study
UHKPC28	United States	16	R	L																							KPC-2	5
Col21	Taiwan	512	R	L																							No	6
Col28	Taiwan	>2,048	R	L																							No	6
Col44	Taiwan	512	R	H																						No	6	
20.70	France	>128	R	S																							NDM-1	This study
UHKPC26	United States	16	R	M																							KPC-2	5
Col36	Taiwan	2,048	R							R																No	6	
G104	Greece	>128	R							Y																	KPC-2	This study
Col4	Taiwan	>2,048	R							I																No	6	
Col20	Taiwan	2,048	R							I																No	6	
Af44b	South Africa	>128	R							L																OXA-181	This study	
Col7	Taiwan	1,024	R							S																No	6	
C7	Colombia	>128	R										V													KPC-2	This study	
Col22	Taiwan	2,048	R											N												No	6	

<sup>a</sup>Isolates from this study are indicated by shading.

<sup>b</sup>MICs of colistin were determined using the manual broth microdilution reference method for this study, Etest for the study by Wright et al. (5), and agar dilution for the study by Cheng et al. (6).

<sup>c</sup>S, susceptible (MIC,  $\leq 2$   $\mu\text{g/ml}$ ); R, resistant (MIC,  $> 2$   $\mu\text{g/ml}$ ), according to EUCAST breakpoints (<http://www.eucast.org/>).

<sup>d</sup>Domains of the CrrB protein predicted by SMART software are indicated as follows: TM, transmembrane domain (amino acids 12–34); HAMP, histidine kinase, adenyl cyclase, methyl binding protein, and phosphatase domain (amino acids 81–135); HisKA, histidine kinase A (phosphoacceptor) domain (amino acids 136–200).

<sup>e</sup>Amino acid positions where mutations have been detected.

<sup>f</sup>MICs of colistin after complementation with a wild-type CrrB protein (with plasmid crrB-pTRIC).

<sup>g</sup>The colistin-susceptible isolate Af44a is the isogenic colistin-susceptible counterpart of Af44b.

## ACKNOWLEDGMENTS

This work was funded by the University of Bordeaux and the University of Fribourg and by grants from the ANIWH A ERA-NET project, Switzerland, and the Office Fédéral de la Santé Publique, Bern, Switzerland (grant no. 16009294).

## REFERENCES

1. Falagas ME, Kasiakou SK. 2005. Colistin: the revival of polymyxins for the management of multidrug-resistant gram-negative bacterial infections. *Clin Infect Dis* 40:1333–1341. <https://doi.org/10.1086/429323>.
2. Liu YY, Wang Y, Walsh TR, Yi LX, Zhang R, Spencer J, Doi Y, Tian G, Dong B, Huang X, Yu LF, Gu D, Ren H, Chen X, Lv L, He D, Zhou H, Liang Z, Liu JH, Shen J. 2016. Emergence of plasmid-mediated colistin resistance mechanism MCR-1 in animals and human beings in China: a microbiological and molecular biological study. *Lancet Infect Dis* 16:161–168.
3. Xavier BB, Lammens C, Ruhel R, Kumar-Singh S, Butaye P, Goossens H, Malhotra-Kumar S. 2016. Identification of a novel plasmid-mediated colistin-resistance gene, *mcr-2*, in *Escherichia coli*, Belgium, June 2016. *Euro Surveill* 21:pil=30280. <http://www.eurosurveillance.org/images/dynamic/EE/V21N27/art22525.pdf>.
4. Poirel L, Jayol A, Nordmann P. 2017. Polymyxins: antibacterial activity, susceptibility testing, and resistance mechanisms encoded by plasmids or chromosomes. *Clin Microbiol Rev* 30:557–596. <https://doi.org/10.1128/CMR.00064-16>.
5. Wright MS, Suzuki Y, Jones MB, Marshall SH, Rudin SD, van Duin D, Kaye K, Jacobs MR, Bonomo RA, Adams MD. 2015. Genomic and transcriptomic analyses of colistin-resistant clinical isolates of *Klebsiella pneumoniae* reveal multiple pathways of resistance. *Antimicrob Agents Chemother* 59:536–543. <https://doi.org/10.1128/AAC.04037-14>.
6. Cheng YH, Lin TL, Lin YT, Wang JT. 2016. Amino acid substitutions of CrrB responsible for resistance to colistin through CrrC in *Klebsiella pneumoniae*. *Antimicrob Agents Chemother* 60:3709–3716. <https://doi.org/10.1128/AAC.00009-16>.
7. Poirel L, Jayol A, Bontron S, Villegas MV, Ozdamar M, Turkoglu S, Nordmann P. 2015. The *mgrB* gene as a key target for acquired resistance to colistin in *Klebsiella pneumoniae*. *J Antimicrob Chemother* 70:75–80.
8. Jayol A, Nordmann P, Desroches M, Decusser JW, Poirel L. 2016. Acquisition of broad-spectrum cephalosporin resistance leading to colistin resistance in *Klebsiella pneumoniae*. *Antimicrob Agents Chemother* 60:3199–3201. <https://doi.org/10.1128/AAC.00237-16>.
9. Nordmann P, Jayol A, Poirel L. 2016. Rapid detection of polymyxin resistance in *Enterobacteriaceae*. *Emerg Infect Dis* 22:1038–1043. <https://doi.org/10.3201/eid2206.151840>.
10. Jayol A, Poirel L, Brink A, Villegas MV, Yilmaz M, Nordmann P. 2014. Resistance to colistin associated with a single amino acid change in protein PmrB among *Klebsiella pneumoniae* isolates of worldwide origin. *Antimicrob Agents Chemother* 58:4762–4766. <https://doi.org/10.1128/AAC.00084-14>.
11. Jayol A, Nordmann P, Brink A, Poirel L. 2015. Heteroresistance to colistin in *Klebsiella pneumoniae* associated with alterations in the PhoPQ regulatory system. *Antimicrob Agents Chemother* 59:2780–2784. <https://doi.org/10.1128/AAC.05055-14>.
12. Humphries RM. 2015. Susceptibility testing of the polymyxins: where are we now? *Pharmacotherapy* 35:22–27.
13. Jang C-W, Magnuson T. 2013. A novel selection marker for efficient DNA cloning and recombineering in *E. coli*. *PLoS One* 8:e57075. <https://doi.org/10.1371/journal.pone.0057075>.

## **2. Mécanismes chromosomiques de résistance chez *K. oxytoca***

### **Contexte :**

Lorsque ce travail a débuté, les mécanismes chromosomiques de résistance à la colistine chez *K. oxytoca* étaient inconnus. Des travaux réalisés chez *K. pneumoniae* avaient révélé l'implication de mutations dans les gènes des systèmes à deux composants PmrA/PmrB et PhoP/PhoQ et au sein du gène *mgrB* dans l'acquisition de la résistance à la colistine.

### **Objectif de l'étude :**

L'objectif de cette étude était donc de rechercher les mécanismes responsables de l'acquisition de la résistance à la colistine dans une souche de *K. oxytoca*.

### **Matériel et méthodes :**

Une souche de *K. oxytoca* résistante à la colistine isolée en Colombie a fait l'objet de cette étude. Les CMI de la colistine ont été déterminées par dilutions en milieu liquide. Les opérons *pmrAB*, *phoPQ*, et *mgrB* ont été séquencés pour rechercher des mutations. Des expériences de complémentation ont été réalisées par clonage du gène sauvage *mgrB* de *K. oxytoca* dans un plasmide vecteur et électroporation du plasmide recombinant dans la souche résistante à la colistine.

### **Principaux résultats :**

Une séquence d'insertion IS*Kpn26*-like a été identifiée dans la région promotrice du gène *mgrB* de la souche de *K. oxytoca* résistante à la colistine (CMI de la colistine égale à 24 mg/L). L'expérience de complémentation avec le gène *mgrB* sauvage a conduit à une restauration de la sensibilité (CMI = 0,047 mg/L) et a ainsi confirmé l'implication de l'insertion de la séquence nucléotidique IS*Kpn26*-like au sein du promoteur du gène *mgrB* dans l'acquisition de la résistance à la colistine.

### **Conclusion :**

Nous avons identifié pour la première fois le mécanisme de résistance à la colistine d'une souche de *K. oxytoca*.

**Ce travail a été publié dans *International Journal of Antimicrobial Agents* en mars 2015 (IF = 4.097).**





## Short Communication

Modulation of *mgrB* gene expression as a source of colistin resistance in *Klebsiella oxytoca*Aurélie Jayol<sup>a</sup>, Laurent Poirel<sup>a,\*</sup>, Maria-Virginia Villegas<sup>b</sup>, Patrice Nordmann<sup>a,c</sup><sup>a</sup> Medical and Molecular Microbiology «Emerging Antibiotic Resistance» Unit, Department of Medicine, Faculty of Science, University of Fribourg, Rue Albert-Gockel 3, CH-1700 Fribourg, Switzerland<sup>b</sup> International Center for Medical Research and Training, CIDEIM, Cali, Colombia<sup>c</sup> Hôpital Fribourgeois – Hôpital Cantonal de Fribourg, Riaz, Switzerland

## ARTICLE INFO

## Article history:

Received 12 November 2014

Accepted 12 February 2015

## Keywords:

PhoPQ regulatory system

Polymyxin

Resistance

## ABSTRACT

Gene modifications in the PmrAB and PhoPQ two-component regulatory systems, as well as inactivation of the *mgrB* gene, are known to be causes of colistin resistance in *Klebsiella pneumoniae*. The objective of this study was to characterise the mechanism involved in colistin resistance in a *Klebsiella oxytoca* isolate. A *K. oxytoca* clinical isolate showing resistance to colistin was recovered in Cali, Colombia. The *pmrA*, *pmrB*, *phoP*, *phoQ* and *mgrB* genes were amplified and sequenced. Wild-type *mgrB* genes from *K. pneumoniae* and *K. oxytoca* were cloned, and corresponding recombinant plasmids were used for complementation assays. By analysing the *mgrB* gene of the *K. oxytoca* isolate and its flanking sequences, an insertion sequence (IS) of 1196 bp was identified in its promoter region. The insertion was located between nucleotides –39 and –38 when referring to the start codon of the *mgrB* gene, thus negatively interfering with expression of the *mgrB* gene by modifying its promoter structure. This IS was very similar to ISKpn26 (99% nucleotide identity) belonging to the IS5 family. Complementation assays with *mgrB* genes from wild-type *K. pneumoniae* or *K. oxytoca* restored full susceptibility to colistin. In conclusion, here we identified the mechanism involved in colistin resistance in a *K. oxytoca* isolate. Modulation of *mgrB* gene expression was the key factor for this acquired resistance to colistin.

© 2015 Published by Elsevier B.V.

## 1. Introduction

*Klebsiella oxytoca* and *Klebsiella pneumoniae* are frequent sources of nosocomial infections and the sources of nosocomial outbreaks [1,2]. The occurrence of multidrug resistance is increasingly observed among the Gram-negative pathogens *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and Enterobacteriaceae. Consequently, use of colistin, a polymyxin-type antibiotic, is being reconsidered in particular for treating critically ill patients infected with these pathogens [3–5]. Unfortunately, the increasing usage of colistin is being associated with the emergence of colistin resistance, in particular in *K. pneumoniae* [6–11].

Alterations in the two-component regulatory systems PmrAB and PhoPQ [8,9,12] are known to be involved in polymyxin resistance in *K. pneumoniae*. Inactivation of the *mgrB* gene,

encoding a negative feedback regulator of the PhoPQ two-component system, is also a common mechanism involved in colistin resistance in *K. pneumoniae* [10,13]. The reported alterations in the *mgrB* gene include insertion of several insertion sequences (IS5-like, IS1F-like, ISKpn13, ISKpn14) at different locations in the coding sequence of *mgrB* and in its promoter region. Non-silent point mutations, premature stop codons, small intragenic deletions and large deletion of the *mgrB* locus are also known. These alterations result in either reduced or even lack of expression of the *mgrB* gene leading to upregulation of the *phoPQ* and *pmrHFJKLM* operons that confer resistance to colistin.

Lippa and Goulian have identified MgrB homologues in the genome sequences of various enterobacterial species [14]. Therefore, alterations in *mgrB* gene expression might possibly be involved in acquisition of colistin resistance in different *Klebsiella* species.

The aim of this study was to identify the mechanism responsible for resistance to colistin in a *K. oxytoca* clinical isolate.

\* Corresponding author. Tel.: +41 26 300 9582.

E-mail address: [laurent.poirel@unifr.ch](mailto:laurent.poirel@unifr.ch) (L. Poirel).



## 2. Materials and methods

### 2.1. Bacterial strain

The colistin-resistant *K. oxytoca* clinical isolate was identified using an API20E system and the matrix-assisted laser desorption/ionisation time-of-flight (MALDI-TOF) technique (AB bioMérieux, La Balme-les-Grottes, France). A colistin-susceptible *K. oxytoca* clinical isolate was used as a wild-type control.

### 2.2. Antimicrobial susceptibility

Antimicrobial susceptibility testing was performed using Etest strips (AB bioMérieux) on Mueller–Hinton agar plates (Bio-Rad, Marnes-la-Coquette, France) with a 0.5 McFarland inoculum. Minimum inhibitory concentrations (MICs) were interpreted as indicated by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines (Breakpoint tables for interpretation of MICs and zone diameters. Version 4.0, 2014; <http://www.eucast.org>). Isolates with colistin MICs of  $\leq 2 \mu\text{g/mL}$  were categorised as susceptible whereas those with MICs of  $> 2 \mu\text{g/mL}$  were resistant.

### 2.3. PCR amplification and sequencing

Whole-cell DNA was extracted using a QIAquick Kit (QIAGEN, Courtaboeuf, France) according to the manufacturer's instructions. The entire *pmrA*, *pmrB*, *phoP*, *phoQ* and *mgrB* genes were amplified with specific oligonucleotides primers (Table 1) designed with sequences of *K. oxytoca* KCTC 1686 and E718 genomes available on the National Center for Biotechnology Information (NCBI) database (<http://www.ncbi.nlm.nih.gov/gate2.inist.fr/>). Amplified DNA fragments were purified with a QIAquick PCR Purification Kit (QIAGEN). Both strands of the amplification products obtained were sequenced with an ABI 3100 Sequencer (Applied Biosystems, Foster City, CA). The nucleotide and deduced protein sequences were analysed at the NCBI website (<http://www.ncbi.nlm.nih.gov>) by the Basic Local Alignment Search Tool (BLAST) programme. The insertion sequence (IS) was analysed using the IS finder website (<http://www-is.biotoul.fr>).

### 2.4. Complementation assays

The wild-type *mgrB* gene from a colistin-susceptible *K. oxytoca* strain and a *K. pneumoniae* strain, as well as the non-coding *mdh* sequence [15], were amplified by PCR using 2X Phusion<sup>®</sup> HF Master Mix (Finnzymes; Life Technologies, Illkirch, France) and the primers listed in Table 1. The amplified fragments were cloned into the plasmid pCR<sup>®</sup>-Blunt II-TOPO<sup>®</sup> (Invitrogen, Illkirch, France)

and the resulting plasmids pTOPO-*mgrBKox*, pTOPO-*mgrBKp* and pTOPO-*mdh* (encoding resistance to Zeocin<sup>™</sup>) were respectively transformed into an electrocompetent colistin-resistant *K. oxytoca* isolate by electroporation. Electrotransformants were selected by overnight incubation at 37 °C on Mueller–Hinton agar supplemented with 100  $\mu\text{g/mL}$  Zeocin<sup>™</sup>. MICs of colistin for the *K. oxytoca* transformants were determined by Etest (bioMérieux).

## 3. Results and discussion

### 3.1. Strain and patient features

The colistin-resistant *K. oxytoca* isolate C24 was recovered in February 2008 from a soft-tissue secretion from a 66-year-old male patient hospitalised for an intra-abdominal infection in Cali, Colombia. The isolate was resistant to broad-spectrum cephalosporins (MICs of ceftazidime and cefotaxime = 32  $\mu\text{g/mL}$ ), amikacin (MIC = 16  $\mu\text{g/mL}$ ) and colistin (MIC = 24  $\mu\text{g/mL}$ ) and was of intermediate susceptibility to ciprofloxacin (MIC = 1  $\mu\text{g/mL}$ ). The isolate was susceptible to carbapenems (MIC < 0.5  $\mu\text{g/mL}$ ), gentamicin (MIC = 0.5  $\mu\text{g/mL}$ ) and tigecycline (MIC = 0.5  $\mu\text{g/mL}$ ). It harboured a *bla*<sub>CTX-M-15</sub> gene but did not produce any carbapenemase according to the Carba NP test. To our knowledge, the patient had never been treated with any polymyxin.

### 3.2. Modification of *mgrB* gene expression through insertion of an insertion sequence element

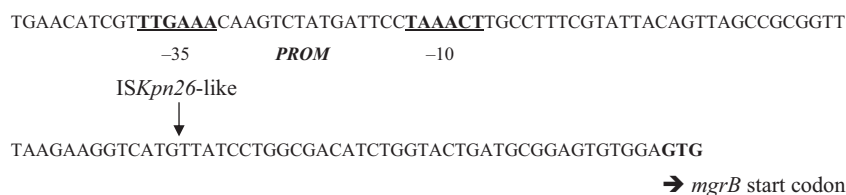
Sequence analysis of the *pmrA*, *pmrB*, *phoP* and *phoQ* genes known to be involved in lipopolysaccharide (LPS) synthesis showed 100% identity with the genes identified from colistin-susceptible wild-type *K. oxytoca* isolates (data not shown). Sequence analysis of the *mgrB* gene as well as upstream-located sequences revealed that an IS of 1196 bp had targeted the upstream vicinity of the *mgrB* gene between nucleotides –38 and –39 when referring to the start codon of *mgrB*. This IS was similar to ISKpn26 (differing by only 5 bp) and belonged to the IS5 family. It was bracketed by a 4-bp duplication (TTAT) being the likely signature of a transposition process. The origin of ISKpn26 is *K. pneumoniae* ([https://www-is.biotoul.fr/index.html?is\\_special\\_name=ISKpn26](https://www-is.biotoul.fr/index.html?is_special_name=ISKpn26)) and it has also been identified in *K. oxytoca* KONIH1 (GenBank accession no. CP008788.1). In silico analysis of the sequence located upstream of a wild-type *mgrB* gene revealed a putative promoter sequence made of –35 [TTGAAA] and –10 [TAAACT] boxes separated by 15 bp (Fig. 1). Insertion of ISKpn26 in isolate C24 led to displacement of this promoter with respect to the *mgrB* gene position, the corresponding –35 and –10 sequences being consequently very distant from it (Fig. 1). This suggests a decrease or even lack of expression of the *mgrB* gene, leading to low or absence of production of the corresponding protein and consequently to acquired resistance to colistin.

### 3.3. Complementation experiments

To confirm whether higher expression of *mgrB* might restore susceptibility to colistin, complementation of isolate C24 with a plasmid overexpressing *mgrB* was performed. Transformation assays were either performed with plasmids encoding the MgrB protein of *K. oxytoca* or *K. pneumoniae*, respectively. The *mgrB* gene was provided in trans together with its original promoter in order to ensure it would be expressed at a wild-type level. Following complementation, isolate C24 recovered full susceptibility to colistin with the wild-type MgrB of *K. oxytoca* (MIC = 0.047  $\mu\text{g/mL}$ ). Interestingly, the same MIC was obtained after complementation with the MgrB of *K. pneumoniae* (MIC = 0.047  $\mu\text{g/mL}$ ). As expected,

**Table 1**  
Primers used in this study.

Primer	Sequence (5'–3')	Gene	Ref.
Kox <i>pmrC</i> ext F	GCC GAT ATC GCA GGG GTT AA	<i>pmrC</i>	This study
Kox <i>pmrC</i> ext R	TAA CAG GAG CGC ATC GTC TT	<i>pmrC</i>	
Kox <i>pmrA</i> ext F	GCA AGC GTA TTC GCA GGA TA	<i>pmrA</i>	
Kox <i>pmrA</i> ext R	CTG ATG AGC TGA CAA ACG GC	<i>pmrA</i>	
Kox <i>pmrB</i> ext F	CAA CGA CAT TTA CAG CTG GG	<i>pmrB</i>	
Kox <i>pmrB</i> ext R	CTT TAC TGA GGA TAG CGC CA	<i>pmrB</i>	
Kox <i>phoP</i> ext F	CCA AGA GAC CGA GGT ACA AA	<i>phoP</i>	
Kox <i>phoP</i> ext R	GAG TGA CAA CAC CAG CAC TA	<i>phoP</i>	
Kox <i>phoQ</i> ext F	CCA TAC CAT CGA TGT GCT GA	<i>phoQ</i>	
Kox <i>phoQ</i> ext R	GCA GGT GTC TGT TAG GGA TT	<i>phoQ</i>	
Kox <i>mgrB</i> ext F	CGC GGT TTA AGA AGG TCA TG	<i>mgrB</i>	
Kox <i>mgrB</i> ext R	AGG CGT TTA TTC TAC CAC CC	<i>mgrB</i>	
<i>mdh</i> F	CCC AAC TCG CTT CAG GTT CAG	<i>mdh</i>	[15]
<i>mdh</i> R	CCG TTT TTC CCC AGC AGC AG	<i>mdh</i>	



**Fig. 1.** Sequences located at the 5'-extremity of the *mgrB* gene. The putative promoter (*PROM*) is indicated, with its respective -35 and -10 boxes underlined. The arrow indicates the target site for insertion of the insertion sequence ISKpn26-like. The *mgrB* start codon is in bold.

transformation with plasmid pTOPO-*mdh* used as a negative control did not restore susceptibility to colistin (MIC = 24 µg/mL).

### 3.4. Comparison of *mgrB* gene sequences of *K. oxytoca* and *K. pneumoniae*

Alignment of the *mgrB* sequences from wild-type *K. pneumoniae* MGH78578 and *K. oxytoca* KCTC1686 strains showed that the sequences were almost identical [only a single amino acid substitution (Cys to Tyr at amino acid position 28)]. This result explains why complementation with the *mgrB* gene of *K. pneumoniae* restored full susceptibility to colistin in the *K. oxytoca* isolate. Furthermore, it may suggest that all previously reported alterations of the *mgrB* gene [10,13] that have been shown to be the sources of acquired resistance to colistin in *K. pneumoniae* (such as insertions of IS elements at different locations in the coding sequence of *mgrB*, non-silent point mutations, premature stop codons, intragenic deletions, etc.) might also be a source of colistin resistance in *K. oxytoca*.

## 4. Conclusion

This study reports on the molecular identification of colistin resistance in *K. oxytoca*. This resistance mechanism is associated with modification of the biosynthesis pathway of LPS. It is similar to that reported in *K. pneumoniae*, underlining that common mechanisms of colistin resistance could be identified in different enterobacterial species.

### Nucleotide sequence accession number

The nucleotide sequences of the mutated *mgrB* genes identified in this study have been deposited at DDBJ/EMBL/GenBank under accession no. KP748172.

### Funding

This work was funded by the University of Fribourg (Fribourg, Switzerland) and by a grant from the European Community [MAGIC-BULLET, FP7/HEALTH-F3-2001-278232].

### Competing interests

None declared.

## Ethical approval

Not required.

## References

- [1] Hoenigl M, Valentini T, Zarfel G, Wuerstl B, Leitner E, Salzer HJ, et al. Nosocomial outbreak of *Klebsiella pneumoniae* carbapenemase-producing *Klebsiella oxytoca* in Austria. *Antimicrob Agents Chemother* 2012;56:2158–61.
- [2] Zarate MS, Gales AC, Picao RC, Pujol GS, Lanza A, Smayevsky J. Outbreak of OXY-2-producing *Klebsiella oxytoca* in a renal transplant unit. *J Clin Microbiol* 2008;46:2099–101.
- [3] Falagas ME, Rafailidis PI, Ioannidou E, Alexiou VG, Matthaïou DK, Karageorgopoulos DE, et al. Colistin therapy for microbiologically documented multidrug-resistant Gram-negative bacterial infections: a retrospective cohort study of 258 patients. *Int J Antimicrob Agents* 2010;35:194–9.
- [4] Landman D, Georgescu C, Martin DA, Quale J. Polymyxins revisited. *Clin Microbiol Rev* 2008;21:449–65.
- [5] Li J, Nation RL, Owen RJ, Wong S, Spelman D, Franklin C. Antibigrams of multidrug-resistant clinical *Acinetobacter baumannii*: promising therapeutic options for treatment of infection with colistin-resistant strains. *Clin Infect Dis* 2007;45:594–8.
- [6] Ah YM, Kim AJ, Lee JY. Colistin resistance in *Klebsiella pneumoniae*. *Int J Antimicrob Agents* 2014;44:8–15.
- [7] Cannatelli A, D'Andrea MM, Giani T, Di Pilato V, Arena F, Ambretti S, et al. In vivo emergence of colistin resistance in *Klebsiella pneumoniae* producing KPC-type carbapenemase mediated by insertional inactivation of the PhoQ/PhoP *mgrB* regulator. *Antimicrob Agents Chemother* 2013;57:5521–6.
- [8] Cannatelli A, Di Pilato V, Giani T, Arena F, Ambretti S, Gaibani P, et al. In vivo evolution to colistin resistance by PmrB sensor kinase mutation in KPC-producing *Klebsiella pneumoniae* is associated with low-dosage colistin treatment. *Antimicrob Agents Chemother* 2014;58:4399–403.
- [9] Jayol A, Poirel L, Brink A, Villegas MV, Yilmaz M, Nordmann P. Resistance to colistin associated with a single amino acid change in protein PmrB among *Klebsiella pneumoniae* isolates of worldwide origin. *Antimicrob Agents Chemother* 2014;58:4762–6.
- [10] Poirel L, Jayol A, Bontron S, Villegas MV, Özdamar M, Türkoglu S, et al. The *mgrB* gene as a key target for acquired resistance to colistin in *Klebsiella pneumoniae*. *J Antimicrob Chemother* 2015;70:75–80.
- [11] Monaco M, Giani T, Raffone M, Arena F, Garcia-Fernandez A, Pollini S, et al. Colistin resistance superimposed to endemic carbapenem-resistant *Klebsiella pneumoniae*: a rapidly evolving problem in Italy, November 2013 to April 2014. *Euro Surveill* 2014;19, pii:20939.
- [12] Cheng HY, Chen YF, Peng HL. Molecular characterization of the PhoQ–PmrD–PmrAB mediated pathway regulating polymyxin B resistance in *Klebsiella pneumoniae* CG43. *J Biomed Sci* 2010;17:60.
- [13] Cannatelli A, Giani T, D'Andrea MM, Di Pilato V, Arena F, Conte V, et al. MgrB inactivation is a common mechanism of colistin resistance in KPC carbapenemase-producing *Klebsiella pneumoniae* of clinical origin. *Antimicrob Agents Chemother* 2014;58:5696–703.
- [14] Lippa AM, Goulian M. Feedback inhibition in the PhoQ/PhoP signaling system by a membrane peptide. *PLoS Genet* 2009;5:e1000788.
- [15] Diancourt L, Passet V, Verhoef J, Grimont PA, Brisse S. Multilocus sequence typing of *Klebsiella pneumoniae* nosocomial isolates. *J Clin Microbiol* 2005;43:4178–82.

## **E. Impact de l'association des résistances chromosomique et plasmidique**

### **Contexte :**

Le gène plasmidique de résistance à la colistine, *mcr-1*, décrit dans le monde entier est responsable de l'acquisition d'un faible niveau de résistance à la colistine chez les entérobactéries (CMI comprises entre 4 et 8 mg/L dans la majorité des cas).

### **Objectif de l'étude :**

L'objectif de cette étude était de déterminer le niveau de résistance à la colistine résultant de la combinaison de mutations chromosomiques au sein du gène *pmrB* et du gène de résistance plasmidique *mcr-1* chez *E. coli*, pour déterminer si le gène *mcr-1* pouvait contribuer à l'acquisition d'un haut niveau de résistance à la colistine.

### **Matériel et méthodes :**

Un plasmide conjugatif (p41331) portant le gène de résistance à la colistine *mcr-1* et le gène de résistance à la ticarcilline et aux céphalosporines de large spectre *blaCTX-M-1* a été transféré par conjugaison dans une souche de référence *E. coli* K12 sensible à la colistine (CMI = 0,25 mg/L) et résistante à l'acide nalidixique et à la rifampicine. Les transconjugants ont été sélectionnés à l'aide de géloses LB supplémentées avec de la ticarcilline (sélection de p41331), de l'acide nalidixique et de la rifampicine (sélection de *E. coli* K12). Le plasmide p41331 a ensuite à nouveau été transféré dans deux souches de *E. coli* résistantes au cotrimoxazole et présentant des mutations chromosomiques au sein du gène *pmrB* (PmrB L110\* et T114P) (CMI = 2 et 8 mg/L, respectivement). Les transconjugants ont été sélectionnés à l'aide de géloses LB supplémentées avec de la ticarcilline (sélection de p41331), et du cotrimoxazole (sélection des souches de *E. coli* avec *pmrB* muté). Pour sélectionner le plasmide p41331, nous aurions aussi pu utiliser de la colistine à la place de la ticarcilline mais nous préférons éviter l'utilisation de colistine qui peut être responsable de l'acquisition de mutations additionnelles à l'origine d'une augmentation du niveau de résistance à la colistine (ceci aurait biaisé l'étude). La présence du gène *mcr-1* au sein des transconjugants a été confirmée par PCR. Les CMI de la colistine des souches parentales et des transconjugants ont été déterminées par dilutions en milieu liquide.

### **Principaux résultats :**

Le transconjugant de la souche de *E. coli* K12 avec le plasmide p41331 portant le gène *mcr-1* (p41331-K12) présentait un faible niveau de résistance à la colistine (CMI = 4 mg/L), tandis que les transconjugants des souches avec le gène *pmrB* muté (gène *mcr-1* + mutation dans PmrB) présentait des CMI plus élevées (8 et 32 mg/L). L'acquisition du gène de résistance plasmidique *mcr-1* dans les souches

possédant un gène *pmrB* muté entraînait ainsi une augmentation des CMI de 4 fois par rapport aux souches parentales.

**Conclusion :**

Cette étude indique qu'une augmentation du niveau de résistance à la colistine est possible par acquisition du gène plasmidique *mcr-1* chez des souches de *E. coli* présentant des mutations chromosomiques. Il s'agit ici d'un phénomène additif des résistances chromosomique et plasmidique.

**Ce travail a été publié dans *International Journal of Antimicrobial Agents* en juillet 2017 (IF = 4.097).**



ELSEVIER

Contents lists available at ScienceDirect

## International Journal of Antimicrobial Agents

journal homepage: [www.elsevier.com/locate/ijantimicag](http://www.elsevier.com/locate/ijantimicag)International Society of Chemotherapy  
for Infection and Cancer

## Letter to the Editor

**Increased colistin resistance upon acquisition of the plasmid-mediated *mcr-1* gene in *Escherichia coli* isolates with chromosomally encoded reduced susceptibility to polymyxins**



CrossMark

Sir,

Colistin is a last-resort antibiotic for treating infections due to multidrug-resistant enterobacterial isolates. The emergence of chromosomal mutations in genes involved in the modification of lipopolysaccharide, e.g. in *pmrAB*, is responsible for colistin resistance [1,2]. However, the major source of concern is related to the recent discovery of plasmid-mediated colistin resistance genes (*mcr-1* to -3) owing to the risk of spread of colistin resistance [2,3]. In *Escherichia coli*, chromosomally encoded PmrAB mutations and plasmid-mediated colistin resistance are responsible for low levels of colistin resistance [colistin minimum inhibitory concentrations (MIC) < 16 mg/L] [2,4,5].

The objective of this study was to determine the level of colistin resistance resulting from the combination of chromosomal mutations and plasmid-mediated *mcr-1* gene in *E. coli*.

Four *E. coli* isolates were used in this study, with isolates FRO and MAL being recovered from human urine samples and isolate 41331 from an animal sample. The colistin-susceptible *E. coli* reference strain K12 was used for mating-out assays. MICs of colistin were determined by broth microdilution method (BMD) as recommended by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines (<http://www.eucast.org>). Briefly, BMD manual panels were prepared extemporaneously in non-treated 96-well polystyrene microplates (Sarstedt, Nümbrecht, Germany). Dilutions of colistin sulfate (Sigma-Aldrich, St Louis, MO) ranging from 0.12–128 mg/L were made in cation-adjusted Mueller–Hinton broth. The results were interpreted based on EUCAST recommendations, with susceptibility and resistance breakpoints being at ≤2 mg/L and >2 mg/L, respectively.

Isolate FRO showed decreased susceptibility to colistin, with an MIC of 2 mg/L, and sequencing of the *pmrAB* genes identified an amino acid substitution (L110\*) in the PmrB protein known to be responsible for an increased colistin MIC [4]. Isolate MAL showed low-level colistin resistance (MIC = 8 mg/L) and sequencing identified an amino acid substitution (T114P) in the HAMP domain of the PmrB protein (Table 1). Neither of the isolates presented mutations in *phoP*, *phoQ* or *mgrB* genes.

Plasmid p41331 recovered from *E. coli* isolate 41331 (colistin MIC = 8 mg/L) carried both the *mcr-1* gene and the *bla*<sub>CTX-M-1</sub> gene encoding resistance to ticarcillin and broad-spectrum cephalosporins.

To determine the level of colistin resistance resulting from acquisition of the plasmid-mediated *mcr-1* gene in *E. coli* isolates MAL and FRO, plasmid p41331 was first transferred into *E. coli* K12 by mating-out assay and was then transferred into the colistin-resistant *E. coli* FRO and MAL isolates, respectively. To avoid selection

**Table 1**Molecular features and colistin minimum inhibitory concentrations (MICs) of the *Escherichia coli* parental strains and transconjugants.

Isolate <sup>a</sup>	Molecular features				Colistin MIC (µg/mL)	Other antimicrobial resistance
	PmrA	PmrB	MCR-1	CTX-M		
41331	WT	WT	+	+	8	NAL, CIP
K12	WT	WT	–	–	0.25	RIF, NAL
p41331-K12	WT	WT	+	+	4	RIF, NAL
FRO	WT	L110	–	–	2	None
P41331-FRO	WT	L110	+	+	8	None
MAL	WT	T114P	–	–	8	SXT, NAL, CIP
p41331-MAL	WT	T114P	+	+	32	SXT, NAL, CIP

WT, wild type; NAL, nalidixic acid; CIP, ciprofloxacin; RIF, rifampin; SXT, trimethoprim/sulfamethoxazole.

<sup>a</sup> 41331 was the donor strain for mating assay; K12, FRO and MAL were the recipients.

with colistin that could be responsible for acquisition of additional mutations, the *E. coli* transconjugants carrying plasmid p41331 were selected using Luria–Bertani agar plates supplemented with ticarcillin (100 mg/L). Presence of the *mcr-1* gene in transconjugants was confirmed by PCR using specific primers as described previously [6].

Determination of the MICs of the transconjugants revealed that the *E. coli* K12 transconjugant (p41331-K12) presented a low level of resistance (MIC = 4 mg/L), whereas *E. coli* FRO and MAL transconjugants (harbouring plasmid p41331 in addition to mutations in PmrB) exhibited higher MICs (8 mg/L and 32 mg/L, respectively) (Table 1). Acquisition of the plasmid-mediated *mcr-1* gene in isolates FRO and MAL was responsible for a four-fold increase in the MICs of colistin compared with the parental strains (2 mg/L to 8 mg/L for FRO and 8 mg/L to 32 mg/L for MAL).

This study indicates that an increase in resistance level to colistin may be achieved upon acquisition of the *mcr-1* gene in strains harbouring chromosomally encoded mutations. The two-step process leading to a higher level of resistance to colistin mirrors what is known for quinolone resistance, with a plasmid determinant conferring low-level resistance that may facilitate further selection of chromosomally encoding mechanisms eventually leading to high-level resistance [7]. These results further highlight that acquisition of the *mcr-1* gene may have a very significant clinical impact, contributing to a higher level of colistin resistance.

**Acknowledgment**

The authors thank Jean-Yves Madec for providing *E. coli* isolate 41331.

**Funding:** This work was funded by the University of Fribourg (Switzerland) and the University of Bordeaux (France).

<http://dx.doi.org/10.1016/j.ijantimicag.2017.07.006>

0924-8579/© 2017 Elsevier B.V. and International Society of Chemotherapy. All rights reserved.

Competing interests: None declared.

Ethical approval: Not required.

## References

- [1] Olaitan AO, Morand S, Rolain JM. Mechanisms of polymyxin resistance: acquired and intrinsic resistance in bacteria. *Front Microbiol* 2014;5:643.
- [2] Poirel L, Jayol A, Nordmann P. Polymyxins: antibacterial activity, susceptibility testing, and resistance mechanisms encoded by plasmids or chromosomes. *Clin Microbiol Rev* 2017;30:557–96.
- [3] Yin W, Li H, Shen Y, Liu Z, Wang S, Shen Z, et al. Novel plasmid-mediated colistin resistance gene *mcr-3* in *Escherichia coli*. *MBio* 2017;8:pii: e00543-17.
- [4] Hjort K, Nicoloff H, Andersson DI. Unstable tandem gene amplification generates heteroresistance (variation in resistance within a population) to colistin in *Salmonella enterica*. *Mol Microbiol* 2016;102:274–89.
- [5] Froelich JM, Tran K, Wall D. A *pmrA* constitutive mutant sensitizes *Escherichia coli* to deoxycholic acid. *J Bacteriol* 2006;188:1180–3.
- [6] Liu YY, Wang Y, Walsh TR, Yi LX, Zhang R, Spencer J, et al. Emergence of plasmid-mediated colistin resistance mechanism MCR-1 in animals and human beings in China: a microbiological and molecular biological study. *Lancet Infect Dis* 2015;16:161–8.
- [7] Poirel L, Pitout JDD, Calvo L, Rodriguez-Martinez J-M, Church D, Nordmann P. In vivo selection of fluoroquinolone-resistant *Escherichia coli* isolates expressing plasmid-mediated quinolone resistance and expanded-spectrum  $\beta$ -lactamase. *Antimicrob Agents Chemother* 2006;50:1525–7.

Aurélien Jayol

Emerging Antibiotic Resistance Unit,

Medical and Molecular Microbiology, Department of Medicine,

University of Fribourg, Fribourg, Switzerland

INSERM European Unit (LEA-IAME Paris, France),

University of Fribourg, Switzerland

National Reference Center for Emerging Antibiotic Resistance,

University of Fribourg, Switzerland

CNRS UMR5234, University of Bordeaux, France

Laboratory of Bacteriology, University Hospital of Bordeaux, France

Patrice Nordmann

Emerging Antibiotic Resistance Unit,

Medical and Molecular Microbiology, Department of Medicine,

University of Fribourg, Fribourg, Switzerland

INSERM European Unit (LEA-IAME Paris, France),

University of Fribourg, Switzerland

National Reference Center for Emerging Antibiotic Resistance,

University of Fribourg, Switzerland

University of Lausanne and University Hospital Center,

Lausanne, Switzerland

Catherine André

CNRS UMR5234, University of Bordeaux, France

Véronique Dubois

CNRS UMR5234, University of Bordeaux, France

Laboratory of Bacteriology, University Hospital of Bordeaux, France

Laurent Poirel \*

Emerging Antibiotic Resistance Unit,

Medical and Molecular Microbiology, Department of Medicine,

University of Fribourg, Fribourg, Switzerland

INSERM European Unit (LEA-IAME Paris, France),

University of Fribourg, Switzerland

National Reference Center for Emerging Antibiotic Resistance,

University of Fribourg, Switzerland

\* Corresponding author. Medical and Molecular Microbiology,

Department of Medicine, Faculty of Science,

University of Fribourg, rue Albert Gockel 3,

CH-1700 Fribourg, Switzerland.

E-mail address: [laurent.poirel@unifr.ch](mailto:laurent.poirel@unifr.ch) (L. Poirel)

13 April 2017

8 July 2017



## **F. Hafnia, un genre d'entérobactérie naturellement résistant à la colistine**

### **Contexte :**

A l'hôpital de Bordeaux, nous avons remarqué que le système automatisé BD Phoenix<sup>®</sup> catégorisait toutes les souches de *Hafnia* spp. comme résistantes à la colistine. Par ailleurs, des souches de *Hafnia* spp. cultivaient très fréquemment sur la gélose SuperPolymyxin lors de la recherche de portage fécal de souches résistantes à la colistine. Il y avait très peu de données dans la littérature sur la résistance à la colistine chez *Hafnia* spp. Seules trois publications décrivaient des souches de *Hafnia* résistantes à la colistine avec des prévalences allant de 25 à 80 % (119–121). Cependant, les méthodes utilisées pour la détection de la résistance à la colistine dans ces études étaient des méthodes qui sous-estimaient les CMI de la colistine (méthode des disques, bandelettes Etest<sup>®</sup>). Nous suspectons donc une résistance naturelle de bas niveau du genre *Hafnia* mal détectée. Par ailleurs, une nouvelle espèce, *Hafnia paralvei* avait récemment été identifiée (122) et une étude suggérait que *H. paralvei* pourrait être plus résistante à la colistine que *Hafnia alvei* (121).

### **Objectif de l'étude :**

L'objectif de cette étude était de déterminer la sensibilité à la colistine des souches de *H. alvei* et de *H. paralvei*.

### **Matériel et méthodes :**

La distribution par espèces de 25 souches cliniques de *Hafnia* spp. isolées entre 1996 et 2016 dans le Sud Ouest de la France a été analysée par séquençage partiel des gènes codant pour l'ARN16S et analyse des séquences à l'aide de la base de données internet Le Bibi (<https://umr5558-bibiserv.univ-lyon1.fr/lebibi/lebibi.cgi>). La sensibilité à la colistine des souches de *Hafnia* a été déterminée à l'aide de la méthode des disques, des bandelettes Etest<sup>®</sup>, du système automatisé BD Phoenix<sup>®</sup>, du Rapid Polymyxin NP test et les résultats obtenus ont été comparés à ceux obtenus avec la méthode de dilutions en milieu liquide de référence.

### **Principaux résultats :**

Quinze souches de *H. alvei* et dix souches de *H. paralvei* ont été identifiées. Une analyse phylogénétique a révélé une proximité du genre *Hafnia* avec des genres naturellement résistants à la colistine comme *Proteus*, *Providencia*, *Morganella* et *Serratia*. La détermination des CMI en milieu liquide par la technique de référence, le système automatisé BD Phoenix<sup>®</sup> et le test rapide Polymyxin NP a révélé un taux important de résistance à la colistine (96 %).

**Conclusion :**

Le taux de résistance important chez les souches de *Hafnia* associé à la forte proximité phylogénétique de ce genre bactérien avec des genres naturellement résistants à la colistine suggèrent que *Hafnia* est un genre d'entérobactéries naturellement résistant à la colistine. Cette résistance naturelle à la colistine est une résistance de bas niveau (CMI's comprises entre 4 et 32 mg/L). Les techniques utilisées pendant longtemps en routine dans les laboratoires, comme les bandelettes Etest<sup>®</sup> et la méthode des disques sous-estimaient largement cette résistance (72 % et 0 %, respectivement) expliquant pourquoi cette résistance naturelle était restée méconnue.

**Ce travail a été publié dans *Journal of Antimicrobial and Chemotherapy* en septembre 2017 (IF = 4.919).**



## Hafnia, an enterobacterial genus naturally resistant to colistin revealed by three susceptibility testing methods

Aurélie Jayol<sup>1–4</sup>, Marion Saly<sup>1,2</sup>, Patrice Nordmann<sup>3–5</sup>, Armelle Ménard<sup>1,6,7</sup>,  
Laurent Poirel<sup>3,4</sup> and Véronique Dubois<sup>1,2\*</sup>

<sup>1</sup>Laboratory of Bacteriology, University Hospital of Bordeaux, Bordeaux, France; <sup>2</sup>CNRS UMR5234, Microbiologie Fondamentale et Pathogénicité, University of Bordeaux, Bordeaux, France; <sup>3</sup>Emerging Antibiotic Resistance Unit, Medical and Molecular Microbiology, University of Fribourg, Fribourg, Switzerland; <sup>4</sup>INSERM European Unit (LEA Paris, IAME, France), University of Fribourg, Fribourg, Switzerland; <sup>5</sup>University Hospital and University of Lausanne, Lausanne, Switzerland; <sup>6</sup>UMR1053, Bordeaux Research in Translational Oncology BaRITOn, University of Bordeaux, Bordeaux, France; <sup>7</sup>INSERM Bordeaux Research in Translational Oncology BaRITOn, University of Bordeaux, Bordeaux, France

\*Corresponding author. CNRS UMR5234, Microbiologie Fondamentale et Pathogénicité, University of Bordeaux, 146 rue Léo Saignat, 33076 Bordeaux cedex, France. Tel: +33-5-57-57-17-42; Fax: +33-5-57-57-90-72; E-mail: veronique.dubois@u-bordeaux.fr

Received 5 January 2017; returned 27 February 2017; revised 8 April 2017; accepted 24 April 2017

**Objectives:** To determine the susceptibility to colistin of *Hafnia alvei* and *Hafnia paralvei*, and to compare methods for colistin resistance detection in the *Hafnia* genus.

**Methods:** A collection of 25 *Hafnia* isolates was studied. Species were identified by using 16S rRNA gene sequencing with subsequent phylogeny analysis. Susceptibility to colistin was determined using the broth microdilution (BMD) reference method, the Phoenix automated system, the Rapid Polymyxin NP test, the Etest system and the disc diffusion method.

**Results:** The collection consisted of 15 *H. alvei* and 10 *H. paralvei* isolates. Based on the 16S rRNA analysis, a close relationship of the *Hafnia* genus with naturally colistin-resistant enterobacterial genera (*Proteus*, *Morganella*, *Providencia* and *Serratia*) was identified. Susceptibility testing performed using the BMD method, the Phoenix automated system and the Rapid Polymyxin NP test revealed a high rate of colistin resistance (96%). Underestimation of colistin resistance using Etest strips (72%) and the disc diffusion method (0%) was observed.

**Conclusions:** The high rate of colistin resistance observed within the *Hafnia* genus and its close phylogenetic relationship with naturally colistin-resistant genera suggest that *Hafnia* is a naturally colistin-resistant enterobacterial genus.

### Introduction

*Hafnia* spp. are Gram-negative bacilli belonging to the Enterobacteriaceae family.<sup>1</sup> *Hafnia* isolates have been mainly recovered in mammalian guts and from food.<sup>1</sup> They are rarely isolated from human specimens, even though they may be occasionally opportunistic human pathogens. They are responsible for nosocomial infections including gastroenteritis, urinary tract infections, meningitis, pneumonia, wound infections, soft tissue infections, endophthalmitis and septicaemia.<sup>2,3</sup>

*Hafnia* spp. harbour a chromosomal *ampC* gene, encoding a class C  $\beta$ -lactamase that confers resistance to aminopenicillins and narrow-spectrum cephalosporins.<sup>4</sup> Natural antimicrobial susceptibility patterns of *Hafnia alvei* strains to 69 antimicrobial agents was reported by Stock et al.,<sup>4</sup> but polymyxin susceptibility was not tested. Colistin resistance within *Hafnia* isolates has been rarely reported.<sup>2,5–7</sup>

*H. alvei* was the unique *Hafnia* species described until a novel species, namely *Hafnia paralvei*, was introduced in the taxonomy.<sup>8</sup> A study suggested that *H. paralvei* could be more resistant to colistin than *H. alvei* isolates.<sup>5</sup>

Taking into account the recent subdivision of the *Hafnia* genus into *H. alvei* and *H. paralvei*, our aim was to analyse the distribution of *Hafnia* species among 25 clinical isolates of *Hafnia*, to determine their susceptibility to colistin with the broth microdilution (BMD) reference method, and to compare colistin susceptibility methods for determining colistin resistance in the *Hafnia* genus.

### Materials and methods

#### Bacterial strains and growth conditions

Twenty-five non-duplicated *Hafnia* isolates recovered from human samples between 1996 and 2016 were included in this study. Those isolates

have been collected from private laboratories (community patients) and at the university hospital of Bordeaux (hospitalized patients) (Table 1). They were kept at  $-80^{\circ}\text{C}$  because they were recovered from deep samples or because they were resistant to extended-spectrum cephalosporins. They were cultured on bromocresol purple (BCP) lactose agar and UriSelect 4 (URI4) chromogenic medium (Bio-Rad, Marnes-la-Coquette, France). The *Escherichia coli* ATCC 25922 strain, susceptible to colistin, was included in all experiments as a quality control.

### Bacterial species identification

Isolates were first identified as *H. alvei* using the MALDI-TOF mass spectrometer version 2.3 Microflex bench-top (Bruker, Champs-sur-Marne, France). As this spectrometer cannot distinguish between the two *Hafnia* species, the 16S rRNA gene of each isolate was partially amplified with primers 8F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1489R (5'-TACCTTGTTACGACTTCA-3') and sequenced (Eurofins Genomics, Les Ulis, France). Nucleotide sequences were analysed on the LeBiBi website (<https://umr5558-bibiserv.univ-lyon1.fr/lebibibi/lebibibi.cgi>) to identify the bacterial species.<sup>9</sup>

### Phylogenetic analysis

A phylogenetic tree based on 16S rRNA gene sequences was constructed for the 25 *Hafnia* isolates and two type strains (*H. alvei* ATCC 13337<sup>T</sup> and *H. paralvei* ATCC 29927<sup>T</sup>) using the neighbour-joining method.<sup>10</sup> The

evolutionary distances were computed using the Kimura two-parameter method<sup>11</sup> and evolutionary analyses were conducted using MEGA6.<sup>12</sup>

### Colistin susceptibility testing

Susceptibility testing of colistin was performed using five different methods. Following EUCAST 2017 breakpoints for Enterobacteriaceae,<sup>13</sup> isolates with an MIC of colistin  $\leq 2$  mg/L were categorized as susceptible although those with MICs  $> 2$  mg/L were categorized as resistant. For the disc diffusion method, EUCAST 2013 disc breakpoints were used, i.e. susceptible if inhibition zone  $\geq 15$  mm and resistant if  $< 15$  mm.

### BMD

The BMD method, which is the reference technique, was performed according to the CLSI/EUCAST common guidelines ([www.eucast.org](http://www.eucast.org)). BMD panels were prepared in untreated polystyrene trays (Sarstedt, Nümbrecht, Germany), and dilutions of colistin sulphate (Sigma-Aldrich, St Louis, MO, USA) were made in CAMHB (Bio-Rad). This procedure was performed in triplicate and the MICs were read after 16–20 h of incubation at  $37^{\circ}\text{C}$ .

### Disc diffusion method

The disc diffusion test was performed according to the EUCAST 2013 guidelines. The diameter of the inhibition zone was measured after an incubation of 16–24 h at  $37^{\circ}\text{C}$ .

**Table 1.** Origin, susceptibility results and PFGE profile of the *Hafnia* isolates

Isolate	Species <sup>a</sup>	Sample	Year of isolation	Colour on		Disc diameter (mm)	MIC (mg/L)			Rapid Polymyxin NP test	PFGE type
				BCP agar	URI4 agar		Etest	Phoenix	BMD		
1	<i>H. alvei</i>	urine	1996	purple	blue	<b>21</b>	<b>0.047</b>	$>4$	8	+	A
2	<i>H. alvei</i>	sputum	1999	purple	blue	<b>17</b>	3	4	8	+	A
3	<i>H. alvei</i>	pleural fluid	2002	purple	blue	<b>18</b>	8	$>4$	16	+	A
4	<i>H. alvei</i>	respiratory sample	2009	purple	blue	<b>19</b>	4	4	8	+	A
5	<i>H. alvei</i>	rectal swab	2016	purple	blue	<b>20</b>	3	4	16	+	A
6	<i>H. alvei</i>	rectal swab	2016	purple	blue	<b>20</b>	3	4	8	+	A
7	<i>H. alvei</i>	rectal swab	2016	purple	blue	<b>20</b>	4	4	16	+	A
8	<i>H. alvei</i>	bile	2013	purple	blue	<b>24</b>	<b>0.016</b>	$\leq 0.5$	<b>0.12</b>	–	B
9	<i>H. alvei</i>	urine	2015	purple	blue	<b>18</b>	8	$>4$	8	+	C
10	<i>H. alvei</i>	blood culture	2015	purple	blue	<b>17</b>	3	4	8	+	C
11	<i>H. alvei</i>	blood culture	2015	purple	blue	<b>18</b>	4	4	8	+	C
12	<i>H. alvei</i>	rectal swab	2015	purple	blue	<b>23</b>	<b>0.094</b>	$>4$	32	+	C
13	<i>H. alvei</i>	blood culture	2015	purple	blue	<b>20</b>	6	$>4$	16	+	C
14	<i>H. alvei</i>	rectal swab	2016	purple	blue	<b>20</b>	4	4	8	+	C
15	<i>H. alvei</i>	rectal swab	2016	purple	light pink	<b>20</b>	<b>2</b>	4	8	+	D
16	<i>H. paralvei</i>	drain	1998	yellow	pink	<b>18</b>	3	$>4$	8	+	E
17	<i>H. paralvei</i>	fluid sample	1999	yellow	pink	<b>17</b>	<b>2</b>	4	8	+	F
18	<i>H. paralvei</i>	urine	2002	yellow	pink	<b>20</b>	3	4	8	+	G
19	<i>H. paralvei</i>	urine	2006	yellow	pink	<b>20</b>	<b>2</b>	4	8	+	H
20	<i>H. paralvei</i>	urine	2009	yellow	pink	<b>21</b>	<b>0.064</b>	$>4$	8	+	I
21	<i>H. paralvei</i>	urine	2015	yellow	pink	<b>19</b>	6	4	8	+	J
22	<i>H. paralvei</i>	rectal swab	2016	yellow	pink	<b>20</b>	3	4	8	+	K
23	<i>H. paralvei</i>	rectal swab	2016	yellow	pink	<b>22</b>	4	4	8	+	L
24	<i>H. paralvei</i>	rectal swab	2016	purple	light pink	<b>19</b>	3	4	4	+	M
25	<i>H. paralvei</i>	rectal swab	2016	yellow	pink	<b>20</b>	3	4	8	+	N

Susceptible results are in bold.

<sup>a</sup>Identification of the species by 16S rRNA partial sequencing.

### Etest system

Etest strips (AB bioMérieux, La Balme-les-Grottes, France) containing a colistin concentration gradient ranging from 0.016 to 256 mg/L were employed as recommended by the manufacturer's guidelines. MICs were read after an incubation of 16–20 h at 37°C.

### Phoenix automated system

The principle of the BD Phoenix automated system (BD Diagnostic Systems, Le Pont de Claix, France) is based on the BMD technique. The bacterial suspension was prepared as recommended by the manufacturer and poured into the Gram-negative panel NMIC-93 (ref. 448282), which contains wells with colistin concentrations ranging from 0.5 to 4 mg/L. The panel was then incubated for 16 h at 37°C in the Phoenix automated system.

### Rapid Polymyxin NP test

Susceptibility testing of colistin was also assessed using the Rapid Polymyxin NP test, which is based on the detection of the glucose metabolism due to bacterial growth in the presence of colistin.<sup>14</sup> Formation of acid metabolites from this glucose metabolism is evidenced by a colour change (orange to yellow) of a pH indicator (red phenol). The Rapid Polymyxin NP test was performed as described previously with a bacterial inoculum with a turbidity equivalent to that of a 3 McFarland standard, a final colistin

concentration of 3.75 mg/L and a visual reading after 2 h of incubation at 37°C.<sup>14</sup>

### Molecular typing by PFGE analysis

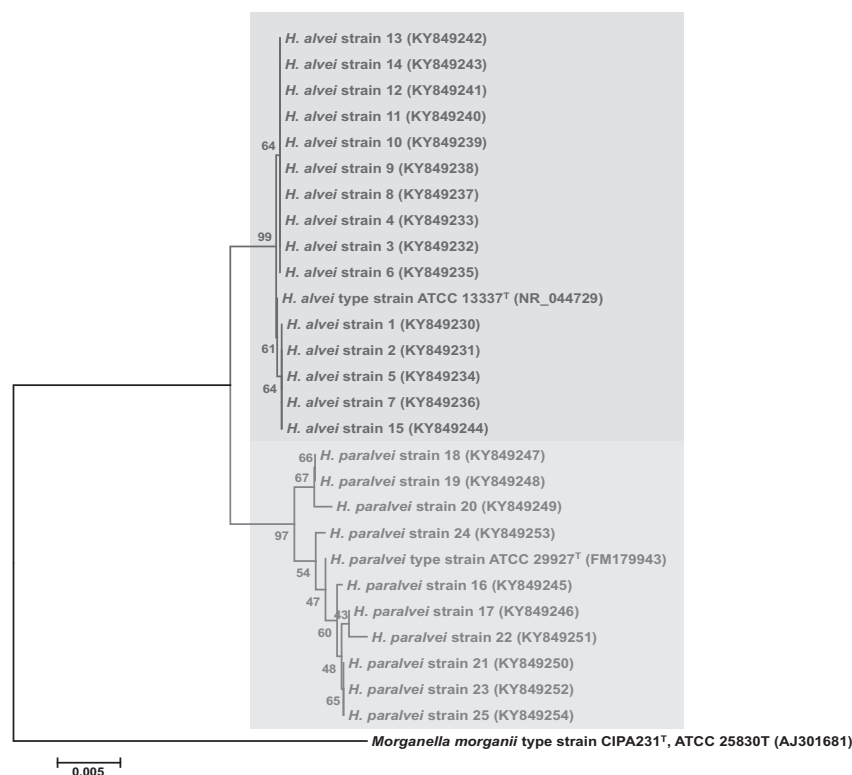
The genetic relatedness of the isolates was assessed by PFGE analysis with SfiI-digested genomic DNA.<sup>15</sup>

### Nucleotide sequences accession numbers

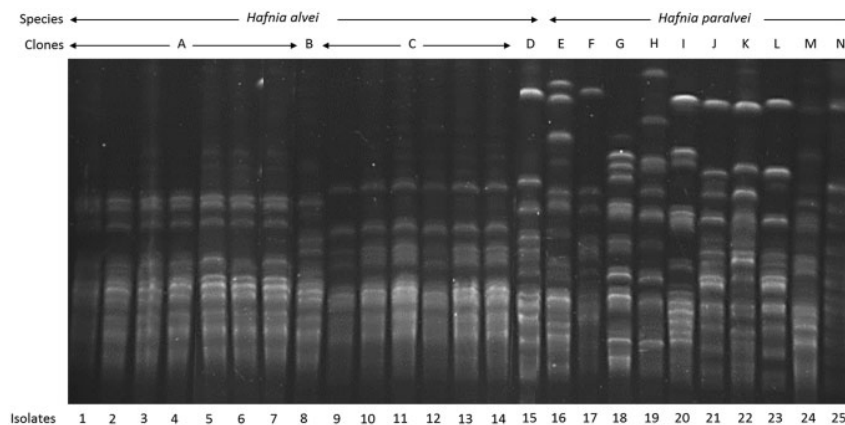
The nucleotide sequences of the rRNA 16S genes of the *H. alvei* and *H. paralvei* strains were registered in GenBank under accession numbers KY849230 to KY849254.

## Results and discussion

Fifteen *H. alvei* and 10 *H. paralvei* isolates were identified by using 16S rRNA gene sequencing (Table 1). The subsequent phylogeny analysis revealed that *H. alvei* and *H. paralvei* were clearly separated (two main branches highly stable to bootstrap resampling, with bootstrap values of 99 and 97 for *H. alvei* and *H. paralvei*, respectively) (Figure 1). It should be noted that *H. paralvei* isolates are more distant from each other than those of *H. alvei*.



**Figure 1.** Phylogenetic tree derived from 16S rRNA sequence comparisons of the *Hafnia* isolates. Phylogenetic analysis of approximately 1400 nt of the 16S rRNA gene of the 25 *Hafnia* isolates and two type strains was performed using MEGA software version 6.06<sup>12</sup> and the neighbour-joining method. The optimal tree with the sum of branch length = 0.06351829 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The tree is not rooted and is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura two-parameter method and are represented in units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated. There were a total of 1398 positions in the final dataset. The 16S rRNA gene of *Morganella morganii* was used as an unrelated outgroup sequence. All sequences are labelled by species, strain name and/or collection number and accession number in brackets. <sup>T</sup>, type strain.



**Figure 2.** PFGE analysis of *H. alvei* ( $n = 15$ ) and *H. paralvei* ( $n = 10$ ) isolates. Isolates are numbered from 1 to 25 and clones are numbered from A to N.

Features of the colonies on URI4 and BCP agar plates showed discrepant results between *H. alvei* and *H. paralvei* isolates, respectively (Table 1). The combination of those features seems therefore to be sufficient to distinguish *H. alvei* and *H. paralvei* species in routine laboratory testing.

The BMD reference method revealed a high rate of colistin resistance (96%) among the 25 *Hafnia* isolates recovered over a 20 year period (Table 1). Only a single isolate of *H. alvei* was susceptible to colistin (MIC 0.125 mg/L). The other isolates had colistin MIC values ranging from 4 to 32 mg/L with no *Hafnia* species-dependent variation in resistance level. This high rate of colistin resistance was not related to the spread of a clonal strain as the PFGE analysis identified a diversity of clones (14 clones) in our collection (Figure 2 and Table 1). Surprisingly, several isolates of *H. alvei* seemed to be clonally related (clone A and clone C, Table 1). Considering that isolates belonging to clone A have been recovered over a 20 year period in various laboratories in the Bordeaux area, it is unlikely that they correspond to a single disseminated strain. We therefore suppose that the genetic diversity of the *H. alvei* isolates is intrinsically less important than that of the *H. paralvei* isolates (none of the *H. paralvei* isolates was clonally related). Those results are in agreement with the 16S rRNA gene sequences analysis, which indicated a greater distance between the *H. paralvei* isolates compared with those of *H. alvei*.

As expected, a high rate of false susceptibility results (25%) was observed with Etest strips, compared with the BMD reference method. Moreover, none of the isolates was identified as colistin resistant with the disc diffusion method. Those resistance rates were similar to those reported in previous studies using the same diffusion methods.<sup>2,5</sup> This further highlights that those methods are not reliable for evaluating the colistin susceptibility (www.eucast.org) and may explain why resistance to colistin within the *Hafnia* genus has been previously underestimated. On the contrary, the results obtained with the Phoenix automated system and the new Rapid Polymyxin NP test fully correlated with the BMD reference method.

Analysis of the 16S rRNA gene sequences on the LeBiBi web site revealed that *H. alvei* and *H. paralvei* species belonged to the same cluster, which is closely related to the cluster of the naturally colistin-resistant *Serratia* genus (data not shown), thus suggesting

a natural resistance of *Hafnia* spp. The DNA relatedness of *Hafnia* spp. with genera naturally resistant to colistin has already been reported by Sproer et al.<sup>16</sup> (relatedness of *Hafnia* spp. and *Serratia* spp.) and Dauga<sup>17</sup> (relatedness of *Hafnia* spp. with the *Proteus-Providencia-Morganella* cluster).

This colistin-resistant trait may be related to the structural features of the lipopolysaccharide of *Hafnia* spp. as already described for *Serratia* spp.<sup>18</sup> and *Proteus* spp.<sup>19</sup> The unique strain determined as colistin susceptible with the BMD method might be a mutant not expressing its resistant phenotype. Ongoing investigations will contribute to understand the mechanism of intrinsic resistance in *Hafnia* genus and to identify to what extent the expression of colistin resistance can be modulated in colistin-susceptible strains.

## Conclusions

*Hafnia* spp. may be added to the list of enterobacterial species that are naturally resistant to colistin, along with *Proteus* spp., *Providencia* spp., *Morganella* spp. and *Serratia* spp.

## Acknowledgements

We are grateful for the technical assistance of Catherine André.

## Funding

This work was supported by internal funding of the University of Bordeaux, France, and the University of Fribourg, Switzerland.

## Transparency declarations

None to declare.

## References

- Janda JM, Abbott SL. The genus *Hafnia*: from soup to nuts. *Clin Microbiol Rev* 2006; **19**: 12–8.
- Gunthard H, Pennekamp A. Clinical significance of extraintestinal *Hafnia alvei* isolates from 61 patients and review of the literature. *Clin Infect Dis* 1996; **22**: 1040–5.

- 3** Stanic M, Meusburger E, Hartmann G *et al.* *Hafnia alvei* urosepsis in a kidney transplant patient. *Case Rep Transplant* 2015; **2015**: 863131.
- 4** Stock I, Rahman M, Sherwood KJ *et al.* Natural antimicrobial susceptibility patterns and biochemical identification of *Escherichia albertii* and *Hafnia alvei* strains. *Diagn Microbiol Infect Dis* 2005; **51**: 151–63.
- 5** Abbott SL, Moler S, Green N *et al.* Clinical and laboratory diagnostic characteristics and cytotoxic potential of *Hafnia alvei* and *Hafnia paralvei* strains. *J Clin Microbiol* 2011; **49**: 3122–6.
- 6** Savini V, Argentieri AV, Marrollo R *et al.* The Italian *Hafnia alvei* strain LMG 27376 is *Hafnia paralvei*. *Vet Microbiol* 2013; **167**: 742–3.
- 7** Savini V, Catavittello C, Talia M *et al.* Isolation of colistin-resistant *Hafnia alvei*. *J Med Microbiol* 2009; **58**: 278–80.
- 8** Huys G, Cnockaert M, Abbott SL *et al.* *Hafnia paralvei* sp. nov., formerly known as *Hafnia alvei* hybridization group 2. *Int J Syst Evol Microbiol* 2009; **60**: 1725–8.
- 9** Flandrois JP, Perriere G, Gouy M. leBIBIQBPP: a set of databases and a webtool for automatic phylogenetic analysis of prokaryotic sequences. *BMC Bioinformatics* 2016; **16**: 251.
- 10** Saitou N, Nei M. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* 1987; **4**: 406–25.
- 11** Kimura M. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J Mol Evol* 1980; **16**: 111–20.
- 12** Tamura K, Stecher G, Peterson D *et al.* MEGA6: molecular evolutionary genetics analysis version 6.0. *Mol Biol Evol* 2013; **30**: 2725–9.
- 13** EUCAST. *Breakpoints Tables for Interpretation of MICs and Zone Diameters, Version 1.0*. 2017. [www.eucast.org](http://www.eucast.org).
- 14** Nordmann P, Jayol A, Poirel L. Rapid detection of polymyxin resistance in Enterobacteriaceae. *Emerg Infect Dis* 2016; **22**: 1038–43.
- 15** Gamage SD, Luchansky JB, Ingham SC. Pulsed-field gel electrophoresis typing of *Hafnia alvei* isolated from chub-packed and retail ground beef. *Lett Appl Microbiol* 1998; **26**: 105–9.
- 16** Sproer C, Mendrock U, Swiderski J *et al.* The phylogenetic position of *Serratia*, *Buttiauxella* and some other genera of the family Enterobacteriaceae. *Int J Syst Bacteriol* 1999; **49**: 1433–8.
- 17** Dauga C. Evolution of the *gyrB* gene and the molecular phylogeny of Enterobacteriaceae: a model molecule for molecular systematic studies. *Int J Syst Evol Microbiol* 2002; **52**: 531–47.
- 18** Lin QY, Tsai YL, Liu MC *et al.* *Serratia marcescens* *arn*, a PhoP-regulated locus necessary for polymyxin B resistance. *Antimicrob Agents Chemother* 2014; **58**: 5181–90.
- 19** Jiang SS, Liu MC, Teng LJ *et al.* *Proteus mirabilis* *pmrI*, an RppA-regulated gene necessary for polymyxin B resistance, biofilm formation, and urothelial cell invasion. *Antimicrob Agents Chemother* 2010; **54**: 1564–71.

## **G. Options thérapeutiques pour le traitement des *K. pneumoniae* produisant une carbapénémase et résistantes à la colistine**

### **Contexte :**

Des épidémies avec des souches de *K. pneumoniae* productrices de carbapénémases et résistantes à la colistine ont été décrites dans le monde entier (9). La diffusion de ces souches limite les options thérapeutiques et augmente la mortalité des patients infectés avec ces souches (123). Récemment, une nouvelle association d'antibiotiques, le ceftazidime/avibactam, qui combine une céphalosporine de large spectre et un nouvel inhibiteur de bêta-lactamase, a été commercialisée (124). Cette association est active contre les BGN producteurs de carbapénémases de classe A (KPC) et certaines carbapénémases de classe D (OXA-48 et dérivés) mais n'est pas active contre les BGN producteurs de métallo-β-lactamases de classe B (124). Cependant, des études ont montré que l'association du ceftazidime/avibactam avec l'aztréonam était synergique sur les BGN producteurs de métallo-β-lactamases, mais seul un faible nombre de souches avait été testé (125, 126).

### **Objectifs de l'étude :**

L'objectif de cette étude était de déterminer l'activité *in vitro* du ceftazidime/avibactam, seul (pour les producteurs de carbapénémases de classe A ou D), et en association avec l'aztréonam (pour les producteurs de carbapénémases de classe B), sur une collection de souches de *K. pneumoniae* productrices de carbapénémases et résistantes à la colistine.

### **Matériel et méthodes :**

Une collection de 63 souches de *K. pneumoniae* résistantes à la colistine et productrices de carbapénémases (KPC, OXA-48, OXA-181, NDM, NDM+OXA-48 ou OXA-181) a été testée dans cette étude. Les CMI de la combinaison ceftazidime/avibactam ont été déterminées à l'aide de bandelettes MIC test strips<sup>®</sup> (Liofilchem). Pour les souches produisant une carbapénémase de classe D, les CMI de l'aztréonam ont également été déterminées à l'aide de bandelettes MIC test strips<sup>®</sup> et la combinaison ceftazidime/avibactam avec aztréonam a été testée par application d'une bandelette ceftazidime/avibactam sur la gélose MH, retrait après 5 minutes, puis application de la bandelette aztréonam au même endroit. Les CMI ont été lues après incubation de la gélose MH pendant 16 à 20 h à 35±2°C.

### **Principaux résultats :**

Toutes les souches produisant une carbapénémase de classe A (KPC) ou D (OXA-48 et OXA-181) étaient sensibles au ceftazidime/avibactam avec des CMI comprises entre 0,12 et 6 mg/L (sensible si ≤ 8 mg/L). Comme attendu, les souches produisant une carbapénémase de classe B (NDM seul ou en association avec une autre carbapénémase) présentaient une résistance de haut niveau au

ceftazidime/avibactam (CMI > 256 mg/L) mais étaient sensibles à l'association ceftazidime/avibactam/aztréonam avec des CMI inférieures à 2 mg/L.

### **Conclusion :**

Notre étude suggère que le ceftazidime/avibactam, seul ou en association avec l'aztréonam, est une option thérapeutique intéressante pour le traitement des souches productrices de carbapénèmases et résistantes à la colistine. Les carbapénèmases de classe B n'hydrolysent pas l'aztréonam mais les souches produisant ces carbapénèmases produisent également dans la plupart des cas des bêta-lactamases à spectre étendue (BLSE) qui hydrolysent l'aztréonam. Ainsi, lors de l'utilisation de l'association ceftazidime/avibactam/aztréonam, l'avibactam permet d'inhiber la BLSE, ce qui restaure ainsi la sensibilité de la souche à l'aztréonam. C'est donc l'association aztréonam/avibactam qui est ainsi responsable de l'activité observée.

**Ce travail a été publié dans *Journal of Antimicrobial and Chemotherapy* en février 2018 (IF = 4.919).**



MIC<sub>90</sub> values obtained for macrolides (erythromycin, azithromycin, clarithromycin) also ranged from 0.5 to 1.0 mg/L. Interestingly, decreasing susceptibility to azithromycin was observed within the ST1 isolates in comparison with non-ST1 isolates. All ST1 isolates showed MICs from 0.25 to 1 mg/L while 96% of non-ST1 isolates did not have MICs >0.25 mg/L. These data correlate with the previously recognized relationship between STs and azithromycin susceptibility of clinical *Legionella* isolates.<sup>8</sup> It was found that rifampicin is the most active drug against isolates with an MIC<sub>90</sub> of 0.032 mg/L under *in vitro* conditions. In contrast, doxycycline was found to be the least active drug, with an MIC<sub>90</sub> of 2.0 mg/L.

In summary, the *in vitro* activity of the tested antibiotics against the German clinical isolates was in agreement with results from other studies.<sup>8–10</sup>

The introduction of routine susceptibility testing seems necessary for better understanding of the development of antibiotic resistance *in vivo* within the *L. pneumophila* population. The obtained MIC values can be used as a reference for defining ECOFFs and establishing the clinical breakpoints by EUCAST.

## Acknowledgements

Part of this study was presented at the Annual Meeting of the European Study Group for Legionella Infections (ESGLI), Amsterdam, The Netherlands, 2016.

## Funding

This work was supported by the Robert Koch Institute on behalf of the Federal Ministry of Health (grant 1369–351).

## Transparency declarations

None to declare.

## References

- Moffie BG, Mouton RP. Sensitivity and resistance of *Legionella pneumophila* to some antibiotics and combinations of antibiotics. *J Antimicrob Chemother* 1988; **22**: 457–62.
- Nielsen K, Bangsbo JM, Hoiby N. Susceptibility of *Legionella* species to five antibiotics and development of resistance by exposure to erythromycin, ciprofloxacin, and rifampicin. *Diagn Microbiol Infect Dis* 2000; **36**: 43–8.
- Bruin JP, Koshkolda T, IJzerman EP *et al.* Isolation of ciprofloxacin-resistant *Legionella pneumophila* in a patient with severe pneumonia. *J Antimicrob Chemother* 2014; **69**: 2869–71.
- Shadoud L, Almahmoud I, Jarraud S *et al.* Hidden selection of bacterial resistance to fluoroquinolones *in vivo*: the case of *Legionella pneumophila* and humans. *EBioMedicine* 2015; **2**: 1179–85.
- Bruin JP, IJzerman EP, den Boer JW *et al.* Wild-type MIC distribution and epidemiological cut-off values in clinical *Legionella pneumophila* serogroup 1 isolates. *Diagn Microbiol Infect Dis* 2012; **72**: 103–8.
- EUCAST. *Antimicrobial Susceptibility Testing of Legionella pneumophila*. [http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST\\_files/General\\_documents/Legionella\\_guidance\\_document\\_20160909.pdf](http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/General_documents/Legionella_guidance_document_20160909.pdf).
- Descours G, Ginevra C, Jacotin N *et al.* Ribosomal mutations conferring macrolide resistance in *Legionella pneumophila*. *Antimicrob Agents Chemother* 2017; **61**: e02188–16.
- Mallegol J, Fernandes P, Melano RG *et al.* Antimicrobial activity of solithromycin against clinical isolates of *Legionella pneumophila* serogroup 1. *Antimicrob Agents Chemother* 2014; **58**: 909–15.
- Al-Matawah QA, Al-Zenki SF, Qasem JA *et al.* Detection and quantification of *Legionella pneumophila* from water systems in Kuwait residential facilities. *J Pathog* 2012; **2012**: 138389.
- De Giglio O, Napoli C, Lovero G *et al.* Antibiotic susceptibility of *Legionella pneumophila* strains isolated from hospital water systems in Southern Italy. *Environ Res* 2015; **142**: 586–90.

*J Antimicrob Chemother* 2018; **73**: 542–544

doi:10.1093/jac/dkx393

Advance Access publication 18 November 2017

## Ceftazidime/avibactam alone or in combination with aztreonam against colistin-resistant and carbapenemase-producing *Klebsiella pneumoniae*

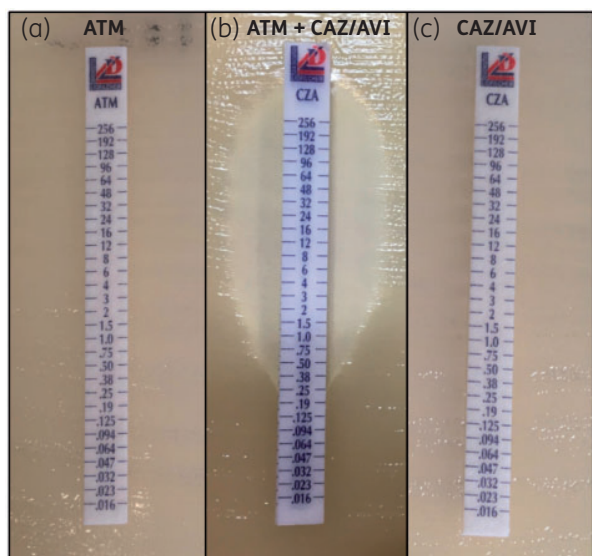
Aurélien Jayol<sup>1–4</sup>, Patrice Nordmann<sup>1–3,5</sup>,  
Laurent Poirel<sup>1–3\*</sup> and Véronique Dubois<sup>4,6</sup>

<sup>1</sup>Emerging Antibiotic Resistance Unit, Medical and Molecular Microbiology, Department of Medicine, University of Fribourg, Fribourg, Switzerland; <sup>2</sup>INSERM European Unit (LEA-IAME Paris, France), University of Fribourg, Fribourg, Switzerland; <sup>3</sup>National Reference Center for Emerging Antibiotic Resistance, University of Fribourg, Fribourg, Switzerland; <sup>4</sup>Laboratory of Bacteriology, University Hospital of Bordeaux, Bordeaux, France; <sup>5</sup>University of Lausanne and University Hospital Center, Lausanne, Switzerland; <sup>6</sup>CNRS UMR5234, University of Bordeaux, Bordeaux, France

\*Corresponding author. Medical and Molecular Microbiology Unit, Department of Medicine, Faculty of Science, University of Fribourg, rue Albert Gockel 3, CH-1700 Fribourg, Switzerland. Tel: +41-26-300-9582; E-mail: laurent.poirel@unifr.ch

Sir,

The spread of carbapenemase-producing *Klebsiella pneumoniae* is a major public health concern since such isolates are basically resistant to most available antibiotics, including  $\beta$ -lactams, fluoroquinolones and aminoglycosides.<sup>1</sup> Infections due to carbapenemase-producing *K. pneumoniae* are therefore commonly treated with a regimen containing colistin.<sup>1</sup> However, acquired



**Figure 1.** Example of synergistic combination of ceftazidime/avibactam (CAZ/AVI) and aztreonam (ATM) for an NDM + ESBL-producing *K. pneumoniae*. Susceptibility testing of ATM alone (a), combination of CAZ/AVI with ATM (b) and CAZ/AVI alone (c). This figure appears in colour in the online version of JAC and in black and white in the print version of JAC.

resistance to colistin now occurs frequently and has few therapeutic options.<sup>2</sup> Outbreaks with colistin-resistant and carbapenemase-producing *K. pneumoniae* isolates have been reported worldwide<sup>2</sup> and mortality rates are high owing to limited treatment options.<sup>3</sup>

Recently, a new therapeutic option, namely ceftazidime/avibactam, combining a broad-spectrum cephalosporin and a novel  $\beta$ -lactamase inhibitor, has been marketed. The addition of avibactam expands the spectrum of activity of ceftazidime to many MDR Enterobacteriaceae including producers of ESBLs and carbapenemases.<sup>4</sup> Indeed, avibactam is active against all types of ESBLs and against carbapenemases of class A (KPC) and of some class D (OXA-48 and its derivatives), but is not active against class B  $\beta$ -lactamases (MBLs).<sup>4</sup>

In two reports, the combination of ceftazidime/avibactam with aztreonam demonstrated a synergistic effect against MBL-producing Gram-negative pathogens, but only a small number of isolates were tested.<sup>5,6</sup>

The objective of this study was to determine the *in vitro* activity of ceftazidime/avibactam, alone (for class A and D carbapenemase producers) or in combination with aztreonam (for class B carbapenemase producers), against a collection of colistin-resistant and carbapenemase-producing *K. pneumoniae* isolates.

A collection of 63 *K. pneumoniae* isolates recovered from clinical samples in France, Colombia and Turkey were tested in this study. All the isolates were resistant to colistin (MICs of colistin ranging from 8 to >128 mg/L) and produced a carbapenemase. The nature of the carbapenemase and the mechanisms responsible for colistin resistance have been previously characterized. Our collection included 11 KPC-like producers, 32 OXA-48 producers, 5 OXA-181 producers, 8 NDM-like producers and 7 isolates that co-produced two carbapenemases (NDM and OXA-48 or NDM and OXA-181)

(Table S1, available as [Supplementary data](#) at JAC Online). The mechanisms responsible for colistin resistance were various and are indicated in Table S1.

MICs of ceftazidime/avibactam were determined using MIC test strips (Liofilchem, I2A, Montpellier, France) according to the manufacturer's guidelines. Following the EUCAST breakpoints (<http://www.eucast.org/>), isolates with an MIC of ceftazidime/avibactam of  $\leq 8$  mg/L were categorized as susceptible, whereas those with an MIC >8 mg/L were categorized as resistant. All the *K. pneumoniae* isolates producing a class A (KPC) or class D (OXA-48 and OXA-181) carbapenemase alone were susceptible to ceftazidime/avibactam with MICs ranging from 0.12 to 6 mg/L. As expected, the isolates producing a class B carbapenemase (NDM) alone or associated with another carbapenemase presented a high level of resistance to ceftazidime/avibactam (MIC  $\geq 256$  mg/L) (Table S1).

For the 15 isolates producing an MBL, MICs of aztreonam were also determined using MIC test strips (Liofilchem). According to EUCAST breakpoints, isolates with an MIC of  $\leq 1$  mg/L were categorized as susceptible, whereas those with an MIC >4 mg/L were categorized as resistant. Out of the 15 isolates, only a single isolate was actually susceptible to aztreonam. Resistance to aztreonam was mainly due to production of ESBLs (data not shown).

The *in vitro* synergy of ceftazidime/avibactam and aztreonam against MBL-positive isolates was studied using MIC test-strip-based synergy methods as previously described.<sup>6</sup> The combination was tested by first applying a ceftazidime/avibactam strip to the Mueller-Hinton agar, removing it after 5 min, then applying an aztreonam strip to the exact same location and replacing the ceftazidime/avibactam strip on top of the aztreonam strip.<sup>6</sup> Despite a high level of resistance to each antibiotic, the combination was synergistic for all the isolates with MICs of the combination <2 mg/L (Figure 1).

This study further suggests that ceftazidime/avibactam is an effective therapeutic option for treating infections caused by colistin-resistant and KPC- or OXA-48-producing *K. pneumoniae*. Moreover, the association of ceftazidime/avibactam and aztreonam is effective against NDM-producing *K. pneumoniae*. This combination was efficient in particular against *K. pneumoniae* isolates producing two carbapenemases. The synergy of the combination of ceftazidime/avibactam with aztreonam against NDM producers could be explained by the neutralization of the ESBL activity by avibactam allowing a restoration of the susceptibility to aztreonam. This study suggests that further commercialization of aztreonam/avibactam as a pharmaceutical preparation could be an interesting option to treat infections caused by MBL producers.

Notably, synergy of ceftazidime/avibactam and aztreonam can be easily evaluated using MIC test-strip synergy assays in clinical microbiology laboratories. Further investigations using experimental models and clinical trials are required to further confirm that this might be a relevant and effective therapeutic option in clinical practice.

## Acknowledgements

We thank Drs A. Brink, Z. Gülay, M. Yilmaz and M. V. Villegas who provided colistin-resistant and carbapenemase-producing *K. pneumoniae* isolates.

## Funding

This work was financed by the University of Bordeaux (France) and the University of Fribourg (Switzerland).

## Transparency declarations

None to declare.

## Supplementary data

Table S1 is available as [Supplementary data](#) at JAC Online.

## References

- Meletis G. Carbapenem resistance: overview of the problem and future perspectives. *Ther Adv Infect Dis* 2016; **3**: 15–21.
- Poirel L, Jayol A, Nordmann P. Polymyxins: antibacterial activity, susceptibility testing, and resistance mechanisms encoded by plasmids or chromosomes. *Clin Microbiol Rev* 2017; **30**: 557–96.
- Monaco M, Giani T, Raffone M *et al.* Colistin resistance superimposed to endemic carbapenem-resistant *Klebsiella pneumoniae*: a rapidly evolving problem in Italy, November 2013 to April 2014. *Euro Surveill* 2014; **19**: pii=20939.
- Lagacé-Wiens P, Walkty A, Karlowsky JA. Ceftazidime-avibactam: an evidence-based review of its pharmacology and potential use in the treatment of Gram-negative bacterial infections. *Core Evid* 2014; **9**: 13–25.
- Wenzler E, Deraedt MF, Harrington AT *et al.* Synergistic activity of ceftazidime-avibactam and aztreonam against serine and metallo- $\beta$ -lactamase-producing Gram-negative pathogens. *Diagn Microbiol Infect Dis* 2017; **88**: 352–4.
- David B, Fellous L, Lawrence C *et al.* Ceftazidime-avibactam and aztreonam, an interesting strategy to overcome  $\beta$ -lactam resistance conferred by metallo- $\beta$ -lactamases in Enterobacteriaceae and *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother* 2017; **61**: e01008–17.

*J Antimicrob Chemother* 2018; **73**: 544–546

doi:10.1093/jac/dkx411

Advance Access publication 9 November 2017

## Emergence of multiple carbapenemase-producing organisms in single patients: an increasing threat to treatment of infection

Jingna An, Kaisheng Lai, Yanning Ma, Ling Guo, Liyan Ye, Yanping Luo and Jiyong Yang\*

Department of Microbiology, Chinese PLA General Hospital, Beijing, China

\*Corresponding author. Microbiology Department, 301 Hospital, 28# Fuxing Road, Beijing 100853, China. Tel: +86-10-66936575; Fax: +86-10-68232198; E-mail: yangjy301@hotmail.com

Sir,

The global spread of carbapenemase-producing organisms (CPOs) poses a growing threat to health services worldwide. The coexistence of various CPOs in single patients has been reported.<sup>1–4</sup> Recently, a study was conducted on a hospitalized patient from whom five CPOs were identified. The patient was a male in his 80s and he was admitted to the respiratory ICU (RICU) with lung and urinary tract infections in autumn 2014 and died 10 months later. While in the RICU he received multiple antibiotics, including piperacillin/tazobactam, ceftazidime, minocycline, moxifloxacin, meropenem and imipenem. This study did not require formal ethical approval, because we have only analysed the characteristics of clinical isolates that were collected

**Table 1.** Characteristics of carbapenemase-producing Gram-negative organisms from the same patient

Isolate no.	Species	Source	Isolation date (dd/mm/yyyy)	PFGE type	Carbapenemase(s)	MIC (mg/L)								
						CTX	CAZ	FEP	TZP	IPM	MEM	ETP	AMK	CIP
IRA001	<i>A. baumannii</i>	sputum	18/10/2014	ABA1	OXA-23, OXA-51	>64	>64	>64	>128	>16	>16	>8	>64	>4
IRP001	<i>P. aeruginosa</i>	sputum	05/11/2014	PAE1	VIM-1	>64	>64	>64	>128	>16	>16	>8	>64	>4
IR1247	<i>K. pneumoniae</i>	urine	04/02/2015	X	KPC-2, OXA-48	>64	>64	>64	>128	>16	>16	>8	>64	>4
IR5690	<i>K. pneumoniae</i>	urine	04/06/2015	A	OXA-48	>64	4	2	>128	2	2	>8	>64	>4
IR5691	<i>K. pneumoniae</i>	sputum	19/06/2015	A	OXA-48	>64	4	2	>128	2	2	>8	>64	>4
IR5692	<i>K. pneumoniae</i>	sputum	22/07/2015	A	OXA-48	>64	4	2	>128	2	2	>8	>64	>4
IR5693	<i>K. pneumoniae</i>	sputum	25/08/2015	A	OXA-48	>64	4	2	>128	2	2	>8	>64	>4
IR53017	<i>E. coli</i>	urine	01/07/2015	E	OXA-48	>64	16	>64	64	<1	<1	>8	>64	>4
IR5283	<i>E. cloacae</i>	sputum	21/08/2015	N/A	OXA-48	>64	4	16	64	8	4	4	>64	>4

CTX, cefotaxime; CAZ, ceftazidime; FEP, cefepime; TZP, piperacillin/tazobactam; IPM, imipenem; MEM, meropenem; ETP, ertapenem; AMK, amikacin; CIP, ciprofloxacin; N/A, not applicable.

© The Author 2017. Published by Oxford University Press on behalf of the British Society for Antimicrobial Chemotherapy. All rights reserved. For Permissions, please email: [journals.permissions@oup.com](mailto:journals.permissions@oup.com).

## Supplementary data

**Table S1.** Mechanisms of colistin resistance, carbapenemase content and MICs of ceftazidime-avibactam, aztreonam and combination of ceftazidime/avibactam + aztreonam of the colistin-resistant and carbapenemase-producing *K. pneumoniae* isolates.

Isolates	Resistance mechanisms to colistin	Carbapenemase	MICs (mg/L)		
			CZA	ATM	CZA+ATM
11 I8	IS5 between +74 and +75 of <i>mgrB</i> gene	KPC-3	6	-	-
20 C9	IS5 between +74 and +75 of <i>mgrB</i> gene	KPC-3	2	-	-
13.45	ISKpn26-like between +74 and +75 of <i>mgrB</i> gene	KPC-2	1.5	-	-
16.72	Full deletion of <i>mgrB</i> gene	KPC-2	1.5	-	-
21.43	Premature termination of MgrB	KPC-2	1	-	-
C1	IS10R into <i>mgrB</i> promotor between -26 and -27	KPC-2	1.5	-	-
C3	T157P substitution in PmrB	KPC-3	1	-	-
C7	G183V substitution in CrrB	KPC-2	0.75	-	-
C9	IS5 between +74 and +75 of <i>mgrB</i> gene	KPC-2	1.5	-	-
C11	Premature termination of MgrB	KPC-2	1.5	-	-
T1b	IS5 between +74 and +75 of <i>mgrB</i> gene	KPC-2	1	-	-
12 E2	N42Y and K43I substitutions in MgrB	OXA-48	0.5	-	-
15.72	G53S substitution in PmrA	OXA-48	0.5	-	-
12.32	IS1R into <i>mgrB</i> promotor between -45 and -46	OXA-48	1	-	-
14.5	Deletion of one nucleotide in <i>mgrB</i> at position 74	OXA-48	0.5	-	-
14.22	Deletion of one nucleotide in <i>mgrB</i> at position 23	OXA-48	2	-	-
15.20	IS1R between +21 and +22 of <i>mgrB</i> gene	OXA-48	0.38	-	-
16.43	IS5-like between +74 and +75 of <i>mgrB</i> gene	OXA-48	0.12	-	-
18.5	IS1R into <i>mgrB</i> promotor between -45 and -46	OXA-48	0.75	-	-
18.60	Full deletion of <i>mgrB</i> gene	OXA-48	0.5	-	-
19.3	ISKpn14-like into <i>mgrB</i> promotor between -45 and -46	OXA-48	1.5	-	-
20.10	Duplication of 19 nt between +84 and +85 of <i>mgrB</i> gene	OXA-48	0.19	-	-
20.16	IS102-like between +36 and +37 of <i>mgrB</i> gene	OXA-48	0.25	-	-
21.19	M27K substitution in MgrB	OXA-48	1.5	-	-
22.53	IS1R into <i>mgrB</i> promotor between -61 and -62	OXA-48	1	-	-
22.76	Premature termination of MgrB	OXA-48	0.38	-	-
Af47	Full deletion of <i>mgrB</i> gene	OXA-48	0.75	-	-
T2	T157P substitution in PmrB	OXA-48	1.5	-	-
T7	L17Q substitution in PmrB	OXA-48	0.5	-	-
T153	IS2 between +64 and +65 of <i>mgrB</i> gene	OXA-48	0.75	-	-
T163	IS2 between +44 and +45 of <i>mgrB</i> gene	OXA-48	0.75	-	-
T166	IS1R between +123 and +124 of <i>mgrB</i> gene	OXA-48	1	-	-
T167	ISKpn14 between +77 and +78 of <i>mgrB</i> gene	OXA-48	0.5	-	-
T191	T157P substitution in PmrB	OXA-48	0.12	-	-
T232	Premature termination of MgrB	OXA-48	0.75	-	-
T286	W47R substitution in MgrB	OXA-48	0.75	-	-
T65	IS1R into <i>mgrB</i> promotor between -45 and -46	OXA-48	1.5	-	-
T82	T157P substitution in PmrB	OXA-48	3	-	-
T83	IS1R between +44 and +45 of <i>mgrB</i> gene	OXA-48	2	-	-
T101	R16C substitution in PhoQ	OXA-48	3	-	-
T104	IS1R between +44 and +45 of <i>mgrB</i> gene	OXA-48	3	-	-
T106	ISKpn14 into <i>mgrB</i> promotor between -28 and -29	OXA-48	0.38	-	-
T332A	ISKpn14 into <i>mgrB</i> promotor between -28 and -29	OXA-48	0.75	-	-
Af18	Deletion of one nucleotide in <i>mgrB</i> at position 70	OXA-181	1.5	-	-
Af39	IS903B between +76 and +77 of <i>mgrB</i> gene	OXA-181	0.75	-	-
Af44	P151L substitution in CrrB	OXA-181	0.25	-	-

# ***DISCUSSION ET PERSPECTIVES***

L'émergence et la diffusion des entérobactéries productrices de carbapénèmases dans le monde entier sont considérées comme un risque de santé publique majeur puisque les options thérapeutiques pour traiter les patients infectés par ces souches sont limitées. La colistine, un antibiotique de la famille des polymyxines, fait partie des molécules de derniers recours potentiellement utilisables pour le traitement des patients infectés par ces souches multirésistantes. L'utilisation de la colistine est ainsi en augmentation constante, ce qui a engendré l'émergence de résistances. Afin de mieux comprendre et de contrôler la dissémination de cette résistance, plusieurs points d'actions devaient être menés :

- Déterminer les performances des systèmes existants pour tester la sensibilité à la colistine
- Développer de nouveaux outils diagnostiques (test rapide, milieu de culture sélectif) pour la détection de la résistance à la colistine
- Etendre les connaissances sur les mécanismes d'acquisition de résistance à la colistine chez les entérobactéries
- Proposer des options thérapeutiques pour lutter contre les entérobactéries productrices de carbapénèmases et résistantes à la colistine

### **Quelles sont les performances des automates et des systèmes commerciaux pour déterminer les CMI de la colistine ?**

La méthode actuellement recommandée par l'EUCAST et le CLSI pour tester la sensibilité à la colistine est la détermination des CMI par dilutions en milieu liquide. Cette méthode n'est pas vraiment adaptée à la Microbiologie Clinique car elle nécessite une préparation longue et fastidieuse (pesée de poudres, dilutions d'antibiotique dans une plaque 96 puits) mais aussi parce que cette préparation manuelle peut engendrer des erreurs. Pour déterminer les CMI de la colistine, des automates et des panels commerciaux testant des concentrations variées de colistine sont disponibles et représentent une alternative à la méthode de référence manuelle. Lorsque nous avons débuté ce travail, l'absence de fiabilité de certains systèmes et automates, comme les bandelettes Etest<sup>®</sup> ou le système automatisé Vitek<sup>®</sup> 2 (bioMérieux), pour tester la sensibilité à la colistine avait été soulignée (111). Des études étaient nécessaires pour préciser les performances de chaque système en comparant les résultats obtenus à ceux de la technique de référence de dilutions en milieu liquide. Lors de ce travail, nous avons évalué un automate, le BD Phoenix<sup>®</sup> (Becton Dickinson), et trois panels commerciaux de dilutions en milieu liquide : le panel UMIC<sup>®</sup> (réf UM-COL-040, Biocentric), le panel MicroScan<sup>®</sup> (réf NM44, Beckman Coulter) et le panel Sensititre<sup>®</sup> (réf FRCOL, ThermoFisher Diagnostics). Nous avons montré la fiabilité du système automatisé BD Phoenix<sup>®</sup> pour la détection des résistances plasmidiques mais nous avons cependant montré les limites de ce système concernant la détection de l'hétérorésistance de certaines souches de *K. pneumoniae* et de *Enterobacter* spp. Nous avons montré que les performances du panel UMIC<sup>®</sup> étaient insuffisantes pour détecter les

souches résistantes à la colistine (sensibilité = 88,7 %, avec échec de détection de certaines résistances plasmidiques). Nous avons également évalué le panel Microscan<sup>®</sup> et avons montré que les CMI de la colistine devaient être vérifiées par une autre méthode pour les souches de *P. aeruginosa* et *A. baumannii* catégorisées comme résistantes, puisque ce panel donnait un taux élevé de résultats de fausses résistances pour les BGN non fermentants (spécificité = 73%). Notre étude a révélé que le panel Sensititre<sup>®</sup> était le panel de dilutions en milieu liquide qui présentait les meilleures performances (sensibilité = 97% et spécificité = 100%) parmi ceux que nous avons testés pour détecter les souches résistantes à la colistine. Ces résultats ont été confirmés lors d'une étude réalisée par le laboratoire de développement EUCAST (99). Ces résultats indiquent donc que des résultats variables de CMI à la colistine sont obtenus selon le système utilisé. Le panel Sensititre<sup>®</sup> semble être la meilleure alternative pour la détermination des CMI lorsque la méthode de référence manuelle n'est pas réalisable. De nouveaux panels et notamment un panel Microscan<sup>®</sup> doivent prochainement être commercialisés et une étude devra être réalisée afin de déterminer si les taux de résultats de fausses résistances sont moins importants avec ce nouveau panel qu'avec le panel Microscan<sup>®</sup> NM44 que nous avons testé.

### **Quel est l'apport du Rapid Polymyxin NP test et de la gélose SuperPolymyxin pour tester la sensibilité à la colistine par rapport aux méthodes pré-existantes ?**

Avec les techniques décrites précédemment (méthode de référence de dilutions en milieu liquide, systèmes automatisés et panels commerciaux pour la détermination des CMI), les résultats sont obtenus en 18h au minimum. Nous avons développé un test colorimétrique, le Rapid Polymyxin NP test, qui permet la détection des souches résistantes à la colistine à partir de colonies bactériennes en moins de 2h; les résultats sont ainsi obtenus jusqu'à 16h plus tôt si l'on compare avec la méthode de référence. Contrairement à la méthode de dilutions en milieu liquide, ce test est très facile à réaliser car il nécessite seulement la préparation d'un milieu et d'une solution de colistine qui peuvent être congelés pour les utilisations ultérieures. Les performances du test que nous avons développé sont très bonnes (sensibilité = 99,3 % et spécificité = 95,4 %) et donc supérieures à de nombreux systèmes préexistants. Le Rapid Polymyxin NP test est peu coûteux (moins de 10 euros par test) et peut ainsi être utilisé dans n'importe quel laboratoire de routine et pour la réalisation d'études épidémiologiques incluant un nombre important de souches. Le développement de ce test est donc une avancée importante en terme d'amélioration du diagnostic de la résistance à la colistine (rapide, simple à réaliser, excellentes sensibilité et spécificité, peu coûteux). Ses limites sont l'absence de détermination des CMI (catégorisation sensibilité/résistance seulement), la lecture visuelle (et donc potentiellement erronée car subjective) du changement de couleur (orange à jaune), et son utilisation restreinte aux entérobactéries (le test ne peut pas être utilisé pour les bacilles à Gram négatif qui ne fermentent pas le glucose tels que *P. aeruginosa* et *A. baumannii*).



Nous avons également montré que le Rapid Polymyxin NP test pouvait être réalisé directement à partir d'hémocultures pour détecter les souches d'entérobactéries résistantes à la colistine responsables de septicémie, et ce en moins de 4h (au lieu de 48h avec les méthodes standards). Or l'initiation d'une antibiothérapie inappropriée chez les patients présentant une septicémie est responsable d'une diminution de survie (127) et d'une augmentation du temps d'hospitalisation (128). Dans les pays où les carbapénèmases sont endémiques, une détection rapide de la résistance à la colistine chez les souches d'entérobactéries responsables de septicémies pourra donc contribuer à initier une antibiothérapie appropriée dans un délai très court (quelques heures après la positivité de l'hémoculture) et ainsi améliorer la prise en charge des patients bactériémiques. Ce test permettra également d'implémenter des précautions contacts pour isoler les patients infectés par des souches multirésistantes productrices de carbapénèmases et résistantes à la colistine pour éviter le développement d'épidémies hospitalières.

L'incidence croissante de la résistance acquise à la colistine chez les BGN et la description d'épidémies hospitalières de souches de *K. pneumoniae* productrices de carbapénèmases et résistantes à la colistine est un phénomène inquiétant (9). C'est pourquoi la diffusion des BGN résistants à la colistine doit être monitorée afin d'entreprendre toutes les actions nécessaires pour la préservation de cet antibiotique de dernier recours (utilisation rationnée pour éviter des pressions de sélections inutiles, isolement des patients porteurs de souches résistantes). La découverte récente des gènes de résistance plasmidiques à la colistine (*mcr-1* à *mcr-5*) aggrave encore la situation puisque la résistance à la colistine peut être transmissible d'une espèce bactérienne à une autre par transfert de gènes plasmidiques. La lutte contre la diffusion de ces résistances plasmidiques, comme cela est déjà pratiqué pour les gènes de carbapénèmases dans certains pays, pourrait prévenir la diffusion de ces gènes de résistance. Dans cet objectif, nous avons développé une gélose sélective, la gélose SuperPolymyxin, qui permet la détection des BGN résistants à la colistine à partir de prélèvements cliniques (selles ou écouvillons rectaux notamment). Cette gélose sélective est un outil important pour lutter contre la diffusion de la résistance plasmidique à la colistine chez les BGN car, couplée aux méthodes de biologie moléculaire pour la détection des gènes *mcr-1* à *5*, elle permet le dépistage des patients porteurs de souches d'entérobactéries présentant une résistance plasmidique à la colistine. Ce dépistage pourrait permettre l'isolement précoce des patients porteurs de ces souches et la mise en place de mesures d'hygiène pour éviter la diffusion à bas bruit de la résistance à la colistine dans les hôpitaux. Cette gélose est également un outil intéressant pour la réalisation d'études épidémiologiques (screening de la résistance à la colistine chez l'Homme, l'animal et dans l'environnement, par exemple l'eau) afin d'identifier les réservoirs et de mieux comprendre la diffusion des résistances plasmidiques de l'animal à l'homme. Depuis la finalisation de notre étude de mise au point de cette gélose sélective, trois autres géloses sélectives ont été développées pour la détection de la résistance à la colistine, la gélose LBJMR de Bardet et al (129), la gélose CHROMagar COL-APSE® (CHROMagar, Paris, France) (130) et

la gélose CHROMID Colistin R<sup>®</sup> (bioMérieux). Une étude comparative de ces géloses serait intéressante afin de comparer leurs performances respectives.

La commercialisation récente du test Rapid Polymyxin NP et de la gélose de screening SuperPolymyxin par la société ELITech Microbiology (Signes, France) en Europe facilitera l'accès des laboratoires hospitaliers à ces deux nouveaux outils pour la détection de la résistance à la colistine et contribuera ainsi à une meilleure détection de la résistance à la colistine chez les BGN.

### **Quelles sont les mécanismes de résistances à la colistine chez *K. pneumoniae* et *K. oxytoca* ?**

En raison du manque de fiabilité des tests pour détecter la résistance à la colistine, l'épidémiologie de la résistance à cet antibiotique chez l'Homme (prévalence et mécanismes de résistance) était très mal connue lorsque nous avons débuté cette étude. Grâce aux tests développés (test Rapid Polymyxin NP et gélose SuperPolymyxin), nous avons screené des collections de centaines de souches de *K. pneumoniae* provenant de plusieurs pays (Afrique du Sud, Colombie, France, Suisse, Turquie) et avons isolé près de 300 souches de *K. pneumoniae* résistantes à la colistine. A l'initiation de ce travail, les mécanismes de résistance à la colistine chez *K. pneumoniae* étaient inconnus. Puisque les mutations dans les gènes chromosomiques *pmrA*, *pmrB*, *phoP*, et *phoQ* avaient déjà été décrites chez *P. aeruginosa* comme responsables de l'acquisition de résistance à la colistine (131), nous avons séquencé ces gènes dans les souches de *K. pneumoniae* afin de rechercher des mutations pouvant être responsables de l'acquisition de résistance à la colistine. L'implication des mutations observées dans le phénotype de résistance a été confirmée par des expériences de complémentation.

Nous avons identifié pour la première fois une mutation au sein du gène *pmrB* conduisant à une substitution T157P dans la protéine PmrB responsable de l'acquisition de la résistance à la colistine chez *K. pneumoniae*. Cette mutation semblait être un hot spot de mutation dans la protéine PmrB car nous l'avons identifiée dans des souches d'Afrique du Sud, de Colombie et de Turquie non reliées clonalement. L'implication de cette mutation dans le phénotype de résistance à la colistine chez *K. pneumoniae* a par la suite été confirmée par deux autres équipes (132, 133).

Cette étude a également permis l'identification d'un mécanisme d'hétérorésistance à la colistine chez *K. pneumoniae*. Dans la souche étudiée, l'hétérorésistance à la colistine aurait été acquise en deux étapes : tout d'abord l'acquisition d'une résistance par mutation au sein du gène *phoP* puis la réversion de résistance d'une sous-population par délétion partielle du gène *phoP*. La signification clinique de l'hétérorésistance à la colistine chez *K. pneumoniae* reste à préciser mais une étude chez la souris suggère que la présence de sous-populations résistantes à la colistine serait à l'origine d'échecs thérapeutiques en cas de traitement par de la colistine (17).

En 2013, une équipe italienne a démontré que des mutations dans le gène *mgrB*, un régulateur négatif du système à deux composants PhoPQ, étaient responsables de l'acquisition de résistances à la colistine chez *K. pneumoniae* (39). Nous avons alors séquencé le gène *mgrB* des souches de *K. pneumoniae* et d'une souche de *K. oxytoca* résistantes à la colistine de notre collection et avons identifié des altérations au sein du gène *mgrB* et de son promoteur à l'origine de l'acquisition de la résistance. Ces mutations étaient très variées (substitutions à différentes positions, insertions de séquences variées, délétions de séquences...).

En 2014, une équipe américaine a démontré que des mutations dans le gène *crrB*, qui régule positivement le système à deux composants PmrAB, étaient responsables de l'acquisition de résistance à la colistine chez *K. pneumoniae* (30). Nous avons alors séquencé l'opéron *crrAB* des souches de *K. pneumoniae* résistantes à la colistine de notre collection et avons identifié de nouvelles mutations au sein du gène *crrB* responsables de l'acquisition d'une résistance de haut niveau à la colistine.

Nous avons ainsi identifié de nombreuses mutations dans des gènes variés responsables de l'acquisition de résistance à la colistine chez *K. pneumoniae* et *K. oxytoca*. Le mécanisme de résistance d'une dizaine de souches de *K. pneumoniae* reste encore à déterminer (pas de mutations chromosomiques connues, ni d'acquisition de gènes de résistance plasmidiques). Une analyse par chromatographie du LPS pourrait permettre de déterminer si la résistance à la colistine est due à l'ajout de groupements cationiques sur le LPS. Une analyse de la transcription des gènes par RT-PCR pourrait permettre de préciser si la résistance à la colistine est liée à une surexpression des gènes codant pour des protéines qui régulent ou modifient le LPS (gènes *pmr*, *pho*, *mgrB*, *crrAB*). Enfin, un séquençage du génome complet de ces souches et une analyse bioinformatique comparative avec des souches sensibles à la colistine pourrait permettre d'identifier de nouveaux gènes de résistance (chromosomiques ou plasmidiques).

### **Est-ce que le gène plasmidique *mcr-1* peut contribuer à l'acquisition d'une résistance de haut niveau à la colistine chez *E. coli* ?**

De nombreuses études ont révélé que le gène plasmidique *mcr-1* était responsable de l'acquisition d'un faible niveau de résistance à la colistine (CMI's comprises entre 4 et 16 mg/L) (9). Nous voulions savoir si cette résistance plasmidique pouvait s'ajouter aux résistances chromosomiques pour obtenir une résistance de haut niveau comme déjà décrit pour la résistance aux quinolones (134). Notre étude a révélé qu'une augmentation du niveau de résistance à la colistine est possible par acquisition du gène plasmidique *mcr-1* chez des souches de *E. coli* présentant des mutations chromosomiques dans le gène *pmrB*. Ces résultats soulignent le fait que l'acquisition du gène plasmidique *mcr-1* peut avoir un impact clinique significatif en contribuant à l'acquisition d'une résistance de haut niveau à la colistine et que la lutte contre sa diffusion est un élément essentiel pour préserver cet antibiotique de dernier recours.

## Quels sont les genres d'entérobactéries naturellement résistants à la colistine ?

*Proteus*, *Providencia*, *Morganella* et *Serratia* sont des genres d'entérobactéries connus pour leurs résistances naturelles de haut niveau à la colistine (4). De plus, une équipe française a récemment montré que certains clusters de *Enterobacter cloacae* seraient naturellement résistants à la colistine (16). Dans notre étude, nous avons démontré que le genre *Hafnia* était un autre genre d'entérobactéries naturellement résistant à la colistine. Cette résistance intrinsèque était méconnue car les méthodes utilisées pour détecter la résistance à la colistine jusqu'à il y a quelques années (méthode des disques et bandelettes Etest®) ne détectaient pas la résistance à la colistine chez ce genre d'entérobactéries en raison d'un faible niveau de résistance (CMI entre 4 et 16 mg/L). Le mécanisme de résistance intrinsèque à la colistine chez *Hafnia* reste encore à déterminer.

## Quelle est l'épidémiologie de la résistance à la colistine chez les entérobactéries productrices de carbapénèmes en France ?

Afin de préciser l'épidémiologie de la résistance à la colistine chez les souches d'entérobactéries multirésistantes en France (espèces présentant des résistances acquises, taux de résistance dans la collection étudiée, mécanismes de résistances et clonalité des souches résistantes), nous avons étudié une collection de souches d'entérobactéries productrices de carbapénémase isolées en France en 2014. Nous avons trouvé un taux relativement élevé de résistance à la colistine chez *K. pneumoniae* et *E. cloacae* (6,2 % et 7,7 %, respectivement). Les mécanismes de résistance à la colistine chez *K. pneumoniae* étaient majoritairement des altérations du gène *mgrB*, et aucune souche ne produisait de gènes plasmidiques de résistance. Nous avons identifié de façon rétrospective une épidémie de *K. pneumoniae* productrices de la carbapénémase OXA-48 et de la BLSE CTX-M-15 et résistantes à la colistine impliquant 15 cas dans le Nord de la France. Le taux de résistance observé chez *K. pneumoniae* (6,2 %) parmi les souches productrices de carbapénémase était plus faible que ce qui avait été observé à la même époque dans d'autres pays européens comme l'Italie (43 %) (111) ou l'Espagne (20 %) (135). La limite de cette étude correspond au fait que seules les souches d'entérobactéries productrices de carbapénèmes envoyées au CNR en 2014 ont pu être étudiées (collection non exhaustive). On ne peut donc pas parler ici d'une prévalence en France mais seulement d'un taux de résistance dans notre collection.

## **Quelles sont les options thérapeutiques actives sur les entérobactéries productrices de carbapénèmases et résistantes à la colistine ?**

L'émergence et la diffusion de la résistance à la colistine chez les souches productrices de carbapénèmases conduit à des impasses thérapeutiques. Nous avons montré que le ceftazidime-avibactam, seul ou en association avec l'aztréonam, était une option thérapeutique à considérer pour le traitement des infections à *K. pneumoniae* productrices de carbapénèmases et résistantes à la colistine. Une autre étude réalisée dans notre équipe a révélé que la plazomicine, un aminoglycoside de dernière génération, était actif sur les souches d'entérobactéries résistantes à la colistine, y compris celles produisant le gène de résistance plasmidique *mcr-1* (136). D'autres molécules devront être testées afin d'élargir les options thérapeutiques pour traiter ces souches multirésistantes. Le céfidérocol, une céphalosporine sidérophore qui n'est pas hydrolysée par les carbapénèmases, devrait être testé (137). Plusieurs études ont révélé que la combinaison de la colistine avec des molécules anti-Gram positive, comme la vancomycine, la teicoplanine ou la rifampicine, était synergique contre les souches de *A. baumannii* résistantes à la colistine (138, 139). Ces associations devront également être testées chez les souches d'entérobactéries productrices de carbapénémase et résistantes à la colistine.

***REFERENCES***  
***BIBLIOGRAPHIQUES***

1. Yahav D, Farbman L, Leibovici L, Paul M. 2012. Colistin: new lessons on an old antibiotic. *Clin Microbiol Infect* 18:18-29.
2. Koch-Weser J, Sidel VW, Federman EB, Kanarek P, Finer DC, Eaton AE. 1970. Adverse effects of sodium colistimethate. Manifestations and specific reaction rates during 317 courses of therapy. *Ann Intern Med* 72:857-68.
3. Biswas S, Brunel JM, Dubus JC, Reynaud-Gaubert M, Rolain JM. 2012. Colistin: an update on the antibiotic of the 21st century. *Expert Rev Anti Infect Ther* 10:917-34.
4. Falagas ME, Kasiakou SK. 2005. Colistin: the revival of polymyxins for the management of multidrug-resistant gram-negative bacterial infections. *Clin Infect Dis* 40:1333-41.
5. Li J, Nation RL, Turnidge JD, Milne RW, Coulthard K, Rayner CR, Paterson DL. 2006. Colistin: the re-emerging antibiotic for multidrug-resistant Gram-negative bacterial infections. *Lancet Infect Dis* 6:589-601.
6. Hancock RE. 1997. Peptide antibiotics. *Lancet* 349:418-22.
7. Falagas ME, Rafailidis PI, Ioannidou E, Alexiou VG, Matthaïou DK, Karageorgopoulos DE, Kapaskelis A, Nikita D, Michalopoulos A. 2010. Colistin therapy for microbiologically documented multidrug-resistant Gram-negative bacterial infections: a retrospective cohort study of 258 patients. *Int J Antimicrob Agents* 35:194-9.
8. Kwa A, Kasiakou SK, Tam VH, Falagas ME. 2007. Polymyxin B: similarities to and differences from colistin (polymyxin E). *Expert Rev Anti Infect Ther* 5:811-21.
9. Poirel L, Jayol A, Nordmann P. 2017. Polymyxins: Antibacterial activity, susceptibility testing, and resistance mechanisms encoded by plasmids or chromosomes. *Clin Microbiol Rev* 30:557-596.
10. Deris ZZ, Swarbrick JD, Roberts KD, Azad MAK, Akter J, Horne AS, Nation RL, Rogers KL, Thompson PE, Velkov T, Li J. 2014. Probing the penetration of antimicrobial polymyxin lipopeptides into gram-negative bacteria. *Bioconjug Chem* 25:750-760.
11. Poudyal A, Howden BP, Bell JM, Gao W, Owen RJ, Turnidge JD, Nation RL, Li J. 2008. In vitro pharmacodynamics of colistin against multidrug-resistant *Klebsiella pneumoniae*. *J Antimicrob Chemother* 62:1311-8.
12. Ito-Kagawa M, Koyama Y. 1980. Selective cleavage of a peptide antibiotic, colistin by colistinase. *J Antibiot Tokyo* 33:1551-5.
13. Aquilini E, Merino S, Knirel YA, Regue M, Tomas JM. 2014. Functional identification of *Proteus mirabilis eptC* gene encoding a core lipopolysaccharide phosphoethanolamine transferase. *Int J Mol Sci* 15:6689-702.
14. Jiang SS, Liu MC, Teng LJ, Wang WB, Hsueh PR, Liaw SJ. 2010. *Proteus mirabilis pmrI*, an RppA-regulated gene necessary for polymyxin B resistance, biofilm formation, and urothelial cell invasion. *Antimicrob Agents Chemother* 54:1564-71.
15. Lin QY, Tsai YL, Liu MC, Lin WC, Hsueh PR, Liaw SJ. 2014. *Serratia marcescens arn*, a PhoP-regulated locus necessary for polymyxin B resistance. *Antimicrob Agents Chemother* 58:5181-90.



16. Guerin F, Isnard C, Sinel C, Morand P, Dhalluin A, Cattoir V, Giard JC. 2016. Cluster-dependent colistin hetero-resistance in *Enterobacter cloacae* complex. *J Antimicrob Chemother* 71:3058-61.
17. Band VI, Crispell EK, Napier BA, Herrera CM, Tharp GK, Vavikolanu K, Pohl J, Read TD, Bosinger SE, Trent MS, Burd EM, Weiss DS. 2016. Antibiotic failure mediated by a resistant subpopulation in *Enterobacter cloacae*. *Nat Microbiol* 1:16053.
18. Telke AA, Olaitan AO, Morand S, Rolain J-M. 2017. *soxRS* induces colistin hetero-resistance in *Enterobacter asburiae* and *Enterobacter cloacae* by regulating the *acrAB-tolC* efflux pump. *J Antimicrob Chemother* 72:2715-21.
19. Jeannot K, Bolard A, Plésiat P. 2017. Resistance to polymyxins in Gram-negative organisms. *Int J Antimicrob Agents* 49:526-35.
20. Gunn JS. 2008. The Salmonella PmrAB regulon: lipopolysaccharide modifications, antimicrobial peptide resistance and more. *Trends Microbiol* 16:284-90.
21. Yan A, Guan Z, Raetz CR. 2007. An undecaprenyl phosphate-aminoarabinose flippase required for polymyxin resistance in *Escherichia coli*. *J Biol Chem* 282:36077-89.
22. Chin C-Y, Gregg KA, Napier BA, Ernst RK, Weiss DS. 2015. A PmrB-regulated deacetylase required for lipid A modification and polymyxin resistance in *Acinetobacter baumannii*. *Antimicrob Agents Chemother* 59:7911-4.
23. Cheng HY, Chen YF, Peng HL. 2010. Molecular characterization of the PhoPQ-PmrD-PmrAB mediated pathway regulating polymyxin B resistance in *Klebsiella pneumoniae* CG43. *J Biomed Sci* 17:60.
24. Gunn JS, Ryan SS, Van Velkinburgh JC, Ernst RK, Miller SI. 2000. Genetic and functional analysis of a PmrA-PmrB-regulated locus necessary for lipopolysaccharide modification, antimicrobial peptide resistance, and oral virulence of *Salmonella enterica* serovar typhimurium. *Infect Immun* 68:6139-46.
25. Kato A, Latifi T, Groisman EA. 2003. Closing the loop: the PmrA/PmrB two-component system negatively controls expression of its posttranscriptional activator PmrD. *Proc Natl Acad Sci U S A* 100:4706-11.
26. Kato A, Tanabe H, Utsumi R. 1999. Molecular characterization of the PhoP-PhoQ two-component system in *Escherichia coli* K-12: identification of extracellular Mg<sup>2+</sup>-responsive promoters. *J Bacteriol* 181:5516-20.
27. Roland KL, Martin LE, Esther CR, Spitznagel JK. 1993. Spontaneous *pmrA* mutants of *Salmonella typhimurium* LT2 define a new two-component regulatory system with a possible role in virulence. *J Bacteriol* 183:1835-42.
28. Groisman EA. 2001. The pleiotropic two-component regulatory system PhoP-PhoQ. *J Bacteriol* 183:1835-42.
29. Park SY, Groisman EA. 2014. Signal-specific temporal response by the *Salmonella* PhoP/PhoQ regulatory system. *Mol Microbiol* 91:135-44.
30. Wright MS, Suzuki Y, Jones MB, Marshall SH, Rudin SD, van Duin D, Kaye K, Jacobs MR, Bonomo RA, Adams MD. 2014. Genomic and transcriptomic analyses of colistin-

- resistant clinical isolates of *Klebsiella pneumoniae* reveal multiple pathways of resistance. *Antimicrob Agents Chemother* 59:536-43.
31. Cheng YH, Lin TL, Lin YT, Wang JT. 2016. Amino acid substitutions of CrrB responsible for resistance to colistin through CrrC in *Klebsiella pneumoniae*. *Antimicrob Agents Chemother* 60:3709-16.
  32. Cheng YH, Lin TL, Lin YT, Wang JT. 2018. A putative RND-type efflux pump, H239\_3064, contributes to colistin resistance through CrrB in *Klebsiella pneumoniae*. *J Antimicrob Chemother* 73:1509-16.
  33. Fernandez L, Gooderham WJ, Bains M, McPhee JB, Wiegand I, Hancock RE. 2010. Adaptive resistance to the “last hope” antibiotics polymyxin B and colistin in *Pseudomonas aeruginosa* is mediated by the novel two-component regulatory system ParR-ParS. *Antimicrob Agents Chemother* 54:3372-82.
  34. Lee J-Y, Chung ES, Na IY, Kim H, Shin D, Ko KS. 2014. Development of colistin resistance in *pmrA*-, *phoP*-, *parR*- and *cprR*-inactivated mutants of *Pseudomonas aeruginosa*. *J Antimicrob Chemother* 69:2966-71.
  35. Muller C, Plesiat P, Jeannot K. 2011. A two-component regulatory system interconnects resistance to polymyxins, aminoglycosides, fluoroquinolones, and beta-lactams in *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother* 55:1211-21.
  36. Gutu AD, Sgambati N, Strasbourger P, Brannon MK, Jacobs MA, Haugen E, Kaul RK, Johansen HK, Hoiby N, Moskowitz SM. 2013. Polymyxin resistance of *Pseudomonas aeruginosa phoQ* mutants is dependent on additional two-component regulatory systems. *Antimicrob Agents Chemother* 57:2204-15.
  37. Fernández L, Jenssen H, Bains M, Wiegand I, Gooderham WJ, Hancock REW. 2012. The two-component system CprRS senses cationic peptides and triggers adaptive resistance in *Pseudomonas aeruginosa* independently of ParRS. *Antimicrob Agents Chemother* 56:6212-22.
  38. Lippa AM, Goulian M. 2009. Feedback inhibition in the PhoQ/PhoP signaling system by a membrane peptide. *PLoS Genet* 5:e1000788.
  39. Cannatelli A, D’Andrea MM, Giani T, Di Pilato V, Arena F, Ambretti S, Gaibani P, Rossolini GM. 2013. *In vivo* emergence of colistin resistance in *Klebsiella pneumoniae* producing KPC-type carbapenemases mediated by insertional inactivation of the PhoQ/PhoP *mgrB* regulator. *Antimicrob Agents Chemother* 57:5521-6.
  40. Lopez-Camacho E, Gomez-Gil R, Tobes R, Manrique M, Lorenzo M, Galvan B, Salvarelli E, Moatassim Y, Salanueva IJ, Pareja E, Codoner FM, Alvarez-Tejado M, Garcillan-Barcia MP, De la Cruz F, Mingorance J. 2013. Genomic analysis of the emergence and evolution of multidrug resistance during a *Klebsiella pneumoniae* outbreak including carbapenem and colistin resistance. *J Antimicrob Chemother* 69:632-6.
  41. Mitrophanov AY, Jewett MW, Hadley TJ, Groisman EA. 2008. Evolution and dynamics of regulatory architectures controlling polymyxin B resistance in enteric bacteria. *PLoS Genet* 4:e1000233.

42. Hjort K, Nicoloff H, Andersson DI. 2016. Unstable tandem gene amplification generates heteroresistance (variation in resistance within a population) to colistin in *Salmonella enterica*. *Mol Microbiol* 102:274-89.
43. Halaby T, Kucukkose E, Janssen AB, Rogers MRC, Doorduyn DJ, van der Zanden AGM, Al Naiemi N, Vandenbroucke-Grauls CMJE, van Schaik W. 2016. Genomic characterization of colistin heteroresistance in *Klebsiella pneumoniae* during a nosocomial outbreak. *Antimicrob Agents Chemother* 60:6837-43.
44. Moffatt JH, Harper M, Harrison P, Hale JD, Vinogradov E, Seemann T, Henry R, Crane B, St Michael F, Cox AD, Adler B, Nation RL, Li J, Boyce JD. 2010. Colistin resistance in *Acinetobacter baumannii* is mediated by complete loss of lipopolysaccharide production. *Antimicrob Agents Chemother* 54:4971-7.
45. Moffatt JH, Harper M, Adler B, Nation RL, Li J, Boyce JD. 2011. Insertion sequence ISAbal1 is involved in colistin resistance and loss of lipopolysaccharide in *Acinetobacter baumannii*. *Antimicrob Agents Chemother* 55:3022-4.
46. Beceiro A, Moreno A, Fernandez N, Vallejo JA, Aranda J, Adler B, Harper M, Boyce JD, Bou G. 2013. Biological cost of different mechanisms of colistin resistance and their impact on virulence in *Acinetobacter baumannii*. *Antimicrob Agents Chemother* 58:518-26.
47. Hood MI, Becker KW, Roux CM, Dunman PM, Skaar EP. 2013. Genetic determinants of intrinsic colistin tolerance in *Acinetobacter baumannii*. *Infect Immun* 81:542-51.
48. Lean SS, Suhaili Z, Ismail S, Rahman NI, Othman N, Abdullah FH, Jusoh Z, Yeo CC, Thong KL. 2014. Prevalence and genetic characterization of carbapenem- and polymyxin-resistant *Acinetobacter baumannii* isolated from a tertiary hospital in Terengganu, Malaysia. *ISRN Microbiol* 2014:953417.
49. Lean SS, Yeo CC, Suhaili Z, Thong KL. 2015. Comparative genomics of two ST 195 carbapenem-resistant *Acinetobacter baumannii* with different susceptibility to polymyxin revealed underlying resistance mechanism. *Front Microbiol* 6:1445.
50. Campos MA, Vargas MA, Regueiro V, Llompert CM, Alberti S, Bengoechea JA. 2004. Capsule polysaccharide mediates bacterial resistance to antimicrobial peptides. *Infect Immun* 72:7107-14.
51. Llobet E, Tomas JM, Bengoechea JA. 2008. Capsule polysaccharide is a bacterial decoy for antimicrobial peptides. *Microbiology* 154:3877-86.
52. Fresno S, Jimenez N, Izquierdo L, Merino S, Corsaro MM, De Castro C, Parrilli M, Naldi T, Regue M, Tomas JM. 2006. The ionic interaction of *Klebsiella pneumoniae* K2 capsule and core lipopolysaccharide. *Microbiology* 152:1807-18.
53. Pilonieta MC, Erickson KD, Ernst RK, Detweiler CS. 2009. A protein important for antimicrobial peptide resistance, YdeI/OmdA, is in the periplasm and interacts with OmpD/NmpC. *J Bacteriol* 191:7243-52.
54. Kocsis B, Kádár B, Fullár A, Felso P, Kocsis B, Böddi K, Kristóf K, Tóth Á, Nagy K, Szabó D. 2014. The role of outer membrane protein loss in colistin-resistant *Klebsiella pneumoniae* ST258 and *Enterobacter asburiae* clinical isolates. 24th ECCMID Poster eP225.

55. Bengoechea JA, Skurnik M. 2000. Temperature-regulated efflux pump/potassium antiporter system mediates resistance to cationic antimicrobial peptides in *Yersinia*. *Mol Microbiol* 37:67-80.
56. Srinivasan VB, Rajamohan G. 2013. KpnEF, a new member of the *Klebsiella pneumoniae* cell envelope stress response regulon, is an SMR-type efflux pump involved in broad-spectrum antimicrobial resistance. *Antimicrob Agents Chemother* 57:4449-62.
57. Osei Sekyere J, Amoako DG. 2017. Carbonyl Cyanide m-Chlorophenylhydrazine (CCCP) reverses resistance to colistin, but not to carbapenems and tigecycline in multidrug-resistant *Enterobacteriaceae*. *Front Microbiol* 8:228.
58. Padilla E, Llobet E, Domenech-Sanchez A, Martinez-Martinez L, Bengoechea JA, Alberti S. 2009. *Klebsiella pneumoniae* AcrAB efflux pump contributes to antimicrobial resistance and virulence. *Antimicrob Agents Chemother* 54:177-83.
59. Warner DM, Levy SB. 2010. Different effects of transcriptional regulators MarA, SoxS and Rob on susceptibility of *Escherichia coli* to cationic antimicrobial peptides (CAMPs): Rob-dependent CAMP induction of the *marRAB* operon. *Microbiol Read Engl* 156:570-8.
60. Liu YY, Wang Y, Walsh TR, Yi LX, Zhang R, Spencer J, Doi Y, Tian G, Dong B, Huang X, Yu LF, Gu D, Ren H, Chen X, Lv L, He D, Zhou H, Liang Z, Liu JH, Shen J. 2015. Emergence of plasmid-mediated colistin resistance mechanism MCR-1 in animals and human beings in China: a microbiological and molecular biological study. *Lancet Infect Dis* 16:161-8.
61. Xavier BB, Lammens C, Ruhel R, Kumar-Singh S, Butaye P, Goossens H, Malhotra-Kumar S. 2016. Identification of a novel plasmid-mediated colistin-resistance gene, *mcr-2*, in *Escherichia coli*, Belgium, June 2016. *Euro Surveill* 21.
62. Yin W, Li H, Shen Y, Liu Z, Wang S, Shen Z, Zhang R, Walsh TR, Shen J, Wang Y. 2017. Novel plasmid-mediated colistin resistance gene *mcr-3* in *Escherichia coli*. *mBio* 8.
63. Carattoli A, Villa L, Feudi C, Curcio L, Orsini S, Luppi A, Pezzotti G, Magistrali CF. 2017. Novel plasmid-mediated colistin resistance *mcr-4* gene in *Salmonella* and *Escherichia coli*, Italy 2013, Spain and Belgium, 2015 to 2016. *Euro Surveill* 22.
64. Borowiak M, Fischer J, Hammerl JA, Hendriksen RS, Szabo I, Malorny B. 2017. Identification of a novel transposon-associated phosphoethanolamine transferase gene, *mcr-5*, conferring colistin resistance in d-tartrate fermenting *Salmonella enterica* subsp. *enterica* serovar Paratyphi B. *J Antimicrob Chemother* 72:3317-24.
65. Di Pilato V, Arena F, Tascini C, Cannatelli A, Henrici De Angelis L, Fortunato S, Giani T, Menichetti F, Rossolini GM. 2016. *mcr-1.2*, a new *mcr* variant carried on a transferable plasmid from a colistin-resistant KPC carbapenemase-producing *Klebsiella pneumoniae* strain of Sequence Type 512. *Antimicrob Agents Chemother* 60:5612-5.
66. Yang YQ, Li YX, Song T, Yang YX, Jiang W, Zhang AY, Guo XY, Liu BH, Wang X, Lei CW, Xiang R, Wang HN. 2017. Colistin resistance gene *mcr-1* and its variant in *Escherichia coli* isolates from chickens in China. *Antimicrob Agents Chemother* 61.

67. Tijet N, Sheth PM, Lastovetska O, Chung C, Patel SN, Melano RG. 2014. Molecular characterization of *Klebsiella pneumoniae* carbapenemase (KPC)-producing *Enterobacteriaceae* in Ontario, Canada, 2008-2011. PLoS One 9:e116421.
68. Lu X, Hu Y, Luo M, Zhou H, Wang X, Du Y, Li Z, Xu J, Zhu B, Xu X, Kan B. 2017. MCR-1.6, a new MCR variant carried by an IncP plasmid in a colistin-resistant *Salmonella enterica* serovar Typhimurium isolate from a healthy individual. Antimicrob Agents Chemother 61.
69. Poirel L, Kieffer N, Fernandez-Garayzabal JF, Vela AI, Larpin Y, Nordmann P. 2017. MCR-2-mediated plasmid-borne polymyxin resistance most likely originates from *Moraxella pluranimalium*. J Antimicrob Chemother 72:2947-9.
70. Liu L, Feng Y, Zhang X, McNally A, Zong Z. 2017. New variant of *mcr-3* in an extensively drug-resistant *Escherichia coli* clinical isolate carrying *mcr-1* and *bla*NDM-5. Antimicrob Agents Chemother 61.
71. Carretto E, Brovarone F, Nardini P, Russello G, Barbarini D, Pongolini S, Gagliotti C, Carattoli A, Sarti M. 2018. Detection of *mcr-4* positive *Salmonella enterica* serovar Typhimurium in clinical isolates of human origin, Italy, October to November 2016. Euro Surveill 23.
72. Rebelo AR, Bortolaia V, Kjeldgaard JS, Pedersen SK, Leekitcharoenphon P, Hansen IM, Guerra B, Malorny B, Borowiak M, Hammerl JA, Battisti A, Franco A, Alba P, Perrin-Guyomard A, Granier SA, De Frutos Escobar C, Malhotra-Kumar S, Villa L, Carattoli A, Hendriksen RS. 2018. Multiplex PCR for detection of plasmid-mediated colistin resistance determinants, *mcr-1*, *mcr-2*, *mcr-3*, *mcr-4* and *mcr-5* for surveillance purposes. Euro Surveill 23.
73. García V, García-Meniño I, Mora A, Flament-Simon SC, Díaz-Jiménez D, Blanco JE, Alonso MP, Blanco J. 2018. Co-occurrence of *mcr-1*, *mcr-4* and *mcr-5* genes in multidrug-resistant ST10 Enterotoxigenic and Shiga toxin-producing *Escherichia coli* in Spain (2006-2017). Int J Antimicrob Agents 52:104-8.
74. Ma S, Sun C, Hulth A, Li J, Nilsson LE, Zhou Y, Börjesson S, Bi Z, Bi Z, Sun Q, Wang Y. 2018. Mobile colistin resistance gene *mcr-5* in porcine *Aeromonas hydrophila*. J Antimicrob Chemother 11.
75. Antoniadou A, Kontopidou F, Poulakou G, Koratzanis E, Galani I, Papadomichelakis E, Kopterides P, Souli M, Armaganidis A, Giamarellou H. 2007. Colistin-resistant isolates of *Klebsiella pneumoniae* emerging in intensive care unit patients: first report of a multiclonal cluster. J Antimicrob Chemother 59:786-80.
76. Matthaiou DK, Michalopoulos A, Rafailidis PI, Karageorgopoulos DE, Papaioannou V, Ntani G, Samonis G, Falagas ME. 2008. Risk factors associated with the isolation of colistin-resistant gram-negative bacteria: a matched case-control study. Crit Care Med 36:807-11.
77. Brink AJ, Coetzee J, Corcoran C, Clay CG, Hari-Makkan D, Jacobson RK, Richards GA, Feldman C, Nutt L, van Greune J, Deetlefs JD, Swart K, Devenish L, Poirel L, Nordmann P. 2012. Emergence of OXA-48 and OXA-181 carbapenemases among *Enterobacteriaceae* in South Africa and evidence of *in vivo* selection of colistin resistance as a consequence of selective decontamination of the gastrointestinal tract. J Clin Microbiol 51:369-72.

78. Halaby T, Al Naiemi N, Kluytmans J, van der Palen J, Vandenbroucke-Grauls CM. 2013. Emergence of colistin resistance in *Enterobacteriaceae* after the introduction of selective digestive tract decontamination in an intensive care unit. *Antimicrob Agents Chemother* 57:3224-9.
79. Strenger V, Gschliesser T, Grisold A, Zarfel G, Feierl G, Masoud L, Hoenigl M, Resch B, Muller W, Urlsberger B. 2011. Orally administered colistin leads to colistin-resistant intestinal flora and fails to prevent faecal colonisation with extended-spectrum beta-lactamase-producing enterobacteria in hospitalised newborns. *Int J Antimicrob Agents* 37:67-9.
80. Bergen PJ, Li J, Nation RL, Turnidge JD, Coulthard K, Milne RW. 2008. Comparison of once-, twice- and thrice-daily dosing of colistin on antibacterial effect and emergence of resistance: studies with *Pseudomonas aeruginosa* in an *in vitro* pharmacodynamic model. *J Antimicrob Chemother* 61:636-42.
81. Cannatelli A, Giani T, Aiezza N, Di Pilato V, Principe L, Luzzaro F, Galeotti CL, Rossolini GM. 2017. An allelic variant of the PmrB sensor kinase responsible for colistin resistance in an *Escherichia coli* strain of clinical origin. *Sci Rep* 7:5071.
82. Olaitan AO, Morand S, Rolain JM. 2016. Emergence of colistin-resistant bacteria in humans without colistin usage: a new worry and cause for vigilance. *Int J Antimicrob Agents* 47:1-3.
83. Dobias J, Poirel L, Nordmann P. 2017. Cross-resistance to human cationic antimicrobial peptides and to polymyxins mediated by the plasmid-encoded MCR-1? *Clin Microbiol Infect Dis* 23:676.
84. Clinical and Laboratory Standards Institute. 2012. Methods for dilution of antimicrobial susceptibility tests for bacteria that grow aerobically. Approved standard. 9th Ed Doc M07-A9.
85. Landman D, Salamera J, Quale J. 2013. Irreproducible and uninterpretable polymyxin B MICs for *Enterobacter cloacae* and *Enterobacter aerogenes*. *J Clin Microbiol* 51:4106-11.
86. Bergen PJ, Li J, Nation RL. 2011. Dosing of colistin-back to basic PK/PD. *Curr Opin Pharmacol* 11:464-9.
87. Yau W, Owen RJ, Poudyal A, Bell JM, Turnidge JD, Yu HH, Nation RL, Li J. 2009. Colistin hetero-resistance in multidrug-resistant *Acinetobacter baumannii* clinical isolates from the Western Pacific region in the SENTRY antimicrobial surveillance programme. *J Infect* 58:138-44.
88. Li J, Rayner CR, Nation RL, Owen RJ, Spelman D, Tan KE, Liolios L. 2006. Heteroresistance to colistin in multidrug-resistant *Acinetobacter baumannii*. *Antimicrob Agents Chemother* 50:2946-50.
89. Hindler JA, Humphries RM. 2013. Colistin MIC variability by method for contemporary clinical isolates of multidrug-resistant Gram-negative bacilli. *J Clin Microbiol* 51:1678-84.
90. Behera B, Mathur P, Das A, Kapil A, Gupta B, Bhoi S, Farooque K, Sharma V, Misra MC. 2010. Evaluation of susceptibility testing methods for polymyxin. *Int J Infect Dis* 14:e596-601.
91. Lo-Ten-Foe JR, de Smet AM, Diederens BM, Kluytmans JA, van Keulen PH. 2007. Comparative evaluation of the VITEK 2, disk diffusion, Etest, broth microdilution, and agar dilution susceptibility testing methods for colistin in clinical isolates, including

- heteroresistant *Enterobacter cloacae* and *Acinetobacter baumannii* strains. Antimicrob Agents Chemother.
92. Hogardt M, Schmoldt S, Gotzfried M, Adler K, Heesemann J. 2004. Pitfalls of polymyxin antimicrobial susceptibility testing of *Pseudomonas aeruginosa* isolated from cystic fibrosis patients. J Antimicrob Chemother 54:1057-61.
  93. Moskowitz SM, Garber E, Chen Y, Clock SA, Tabibi S, Miller AK, Doctor M, Saiman L. 2010. Colistin susceptibility testing: evaluation of reliability for cystic fibrosis isolates of *Pseudomonas aeruginosa* and *Stenotrophomonas maltophilia*. J Antimicrob Chemother 65:1416-23.
  94. Tan TY, Ng SY. 2007. Comparison of Etest, Vitek and agar dilution for susceptibility testing of colistin. Clin Microbiol Infect 13:541-4.
  95. Maalej SM, Meziou MR, Rhimi FM, Hammami A. 2011. Comparison of disc diffusion, Etest and agar dilution for susceptibility testing of colistin against *Enterobacteriaceae*. Lett Appl Microbiol 53:546-51.
  96. EUCAST. 2018. Breakpoints tables for interpretation of MICs and zone diameters, Version 8.0. EUCAST; 2018.
  97. Jouy E, Haenni M, Le Devendec L, Le Roux A, Châtre P, Madec J-Y, Kempf I. 2017. Improvement in routine detection of colistin resistance in *E. coli* isolated in veterinary diagnostic laboratories. J Microbiol Methods 132:125-27.
  98. Arroyo LA, Garcia-Curiel A, Pachon-Ibanez ME, Llanos AC, Ruiz M, Pachon J, Aznar J. 2005. Reliability of the E-test method for detection of colistin resistance in clinical isolates of *Acinetobacter baumannii*. J Clin Microbiol 43:903-5.
  99. Matuschek E, Åhman J, Webster C, Kahlmeter G. 2017. Antimicrobial susceptibility testing of colistin - evaluation of seven commercial MIC products against standard broth microdilution for *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Acinetobacter spp.* Clin Microbiol Infect Dis. 24:865-70
  100. Jorgensen JH, Ferraro MJ. 2009. Antimicrobial susceptibility testing: a review of general principles and contemporary practices. Clin Infect Dis 49:1749-55.
  101. Chew KL, La M-V, Lin RTP, Teo JWP. 2017. Colistin and Polymyxin B Susceptibility testing for carbapenem-resistant and *mcr*-positive *Enterobacteriaceae*: Comparison of Sensititre, MicroScan, Vitek 2, and Etest with broth microdilution. J Clin Microbiol 55:2609-16.
  102. Carretto E, Brovarone F, Russello G, Nardini P, El-Bouseary MM, Aboklaish AF, Walsh TR, Tyrrell JM. 2018. Clinical validation of SensiTest colistin, a broth microdilution-based method to evaluate colistin MICs. J Clin Microbiol 56.
  103. Vourli S, Dafopoulou K, Vrioni G, Tsakris A, Pournaras S. 2017. Evaluation of two automated systems for colistin susceptibility testing of carbapenem-resistant *Acinetobacter baumannii* clinical isolates. J Antimicrob Chemother 72:2528-30.
  104. Lee SY, Shin JH, Lee K, Joo MY, Park KH, Shin MG, Suh SP, Ryang DW, Kim SH. 2013. Comparison of the Vitek 2, MicroScan, and Etest methods with the agar dilution method in assessing colistin susceptibility of bloodstream isolates of *Acinetobacter* species from a Korean university hospital. J Clin Microbiol 51:1924-6.

105. Perez LR. 2015. Evaluation of polymyxin susceptibility profile among KPC-producing *Klebsiella pneumoniae* using Etest and MicroScan WalkAway automated system. *APMIS* 123:951-4.
106. Clinical and Laboratory Standards Institute. 2014. Performance standards for antimicrobial susceptibility testing; 24th informational supplement. CLSI document M100-S24. Clinical and Laboratory Standards Institute, Wayne, PA.
107. Garonzik SM, Li J, Thamlikitkul V, Paterson DL, Shoham S, Jacob J, Silveira FP, Forrest A, Nation RL. 2011. Population pharmacokinetics of colistin methanesulfonate and formed colistin in critically ill patients from a multicenter study provide dosing suggestions for various categories of patients. *Antimicrob Agents Chemother* 55:3824-94.
108. Jang CW, Magnuson T. 2013. A novel selection marker for efficient DNA cloning and recombineering in *E. coli*. *PLoS One* 8:e57075.
109. Flandrois JP, Perriere G, Gouy M. 2016. leBIBIQBPP: a set of databases and a webtool for automatic phylogenetic analysis of prokaryotic sequences. *BMC Bioinformatics* 16:251.
110. Diancourt L, Passet V, Verhoef J, Grimont PA, Brisse S. 2005. Multilocus sequence typing of *Klebsiella pneumoniae* nosocomial isolates. *J Clin Microbiol* 43:4178-82.
111. Monaco M, Giani T, Raffone M, Arena F, Garcia-Fernandez A, Pollini S, Grundmann H, Pantosti A, Rossolini GM. 2014. Colistin resistance superimposed to endemic carbapenem-resistant *Klebsiella pneumoniae*: a rapidly evolving problem in Italy, November 2013 to April 2014. *Euro Surveill* 19.
112. Giani T, Arena F, Vaggelli G, Conte V, Chiarelli A, Henrici De Angelis L, Fornaini R, Grazzini M, Niccolini F, Pecile P, Rossolini GM. 2015. Large nosocomial outbreak of colistin-resistant, carbapenemase-producing *Klebsiella pneumoniae* traced to clonal expansion of an *mgrB* deletion mutant. *J Clin Microbiol* 53:3341-4.
113. Froelich JM, Tran K, Wall D. 2006. A *pmrA* constitutive mutant sensitizes *Escherichia coli* to deoxycholic acid. *J Bacteriol* 188:1180-3.
114. Skov RL, Monnet DL. 2016. Plasmid-mediated colistin resistance (*mcr-1* gene): three months later, the story unfolds. *Euro Surveill* 21.
115. Haenni M, Poirel L, Kieffer N, Chatre P, Saras E, Metayer V, Dumoulin R, Nordmann P, Madec JY. 2016. Co-occurrence of extended spectrum beta lactamase and MCR-1 encoding genes on plasmids. *Lancet Infect Dis* 16:281-2.
116. Barrow K, Kwon DH. 2009. Alterations in two-component regulatory systems of *phoPQ* and *pmrAB* are associated with polymyxin B resistance in clinical isolates of *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother* 53:5150-4.
117. Adams MD, Nickel GC, Bajaksouzian S, Lavender H, Murthy AR, Jacobs MR, Bonomo RA. 2009. Resistance to colistin in *Acinetobacter baumannii* associated with mutations in the PmrAB two-component system. *Antimicrob Agents Chemother* 53:3628-34.
118. Beceiro A, Llobet E, Aranda J, Bengoechea JA, Doumith M, Hornsey M, Dhanji H, Chart H, Bou G, Livermore DM, Woodford N. 2011. Phosphoethanolamine modification of lipid A in colistin-resistant variants of *Acinetobacter baumannii* mediated by the PmrAB two-component regulatory system. *Antimicrob Agents Chemother* 55:3370-9.



119. Gunthard H, Pennekamp A. 1996. Clinical significance of extraintestinal *Hafnia alvei* isolates from 61 patients and review of the literature. *Clin Infect Dis* 22:1040-5.
120. Savini V, Catavittello C, Talia M, Balbinot A, Febbo F, Pompilio A, Di Bonaventura G, Piccolomini R, D'Antonio D. 2009. Isolation of colistin-resistant *Hafnia alvei*. *J Med Microbiol* 58:278-80.
121. Abbott SL, Moler S, Green N, Tran RK, Wainwright K, Janda JM. 2011. Clinical and laboratory diagnostic characteristics and cytotoxic potential of *Hafnia alvei* and *Hafnia paralvei* strains. *J Clin Microbiol* 49:3122-6.
122. Huys G, Cnockaert M, Abbott SL, Janda JM, Vandamme P. 2009. *Hafnia paralvei* sp. nov., formerly known as *Hafnia alvei* hybridization group 2. *Int J Syst Evol Microbiol* 60:1725-8.
123. Capone A, Giannella M, Fortini D, Giordano A, Meledandri M, Ballardini M, Venditti M, Bordi E, Capozzi D, Balice MP, Tarasi A, Parisi G, Lappa A, Carattoli A, Petrosillo N. 2012. High rate of colistin resistance among patients with carbapenem-resistant *Klebsiella pneumoniae* infection accounts for an excess of mortality. *Clin Microbiol Infect* 19:e23-30.
124. Lagacé-Wiens P, Walkty A, Karlowsky JA. 2014. Ceftazidime–avibactam: an evidence-based review of its pharmacology and potential use in the treatment of Gram-negative bacterial infections. *Core Evid* 9:13-25.
125. Wenzler E, Deraedt MF, Harrington AT, Danizger LH. 2017. Synergistic activity of ceftazidime-avibactam and aztreonam against serine and metallo- $\beta$ -lactamase-producing gram-negative pathogens. *Diagn Microbiol Infect Dis* 88:352-4.
126. Davido B, Fellous L, Lawrence C, Maxime V, Rottman M, Dinh A. 2017. Ceftazidime-avibactam and aztreonam an interesting strategy to overcome  $\beta$ -lactam resistance conferred by metallo- $\beta$ -lactamases in *Enterobacteriaceae* and *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother* 61.
127. Kumar A, Ellis P, Arabi Y, Roberts D, Light B, Parrillo JE, Dodek P, Wood G, Simon D, Peters C, Ahsan M, Chateau D. 2009. Initiation of inappropriate antimicrobial therapy results in a fivefold reduction of survival in human septic shock. *Chest* 136:1237-48.
128. Garnacho-Montero J, Ortiz-Leyba C, Herrera-Melero I, Aldabo-Pallas T, Cayuela-Dominguez A, Marquez-Vacaro JA, Carbajal-Guerrero J, Garcia-Garmendia JL. 2008. Mortality and morbidity attributable to inadequate empirical antimicrobial therapy in patients admitted to the ICU with sepsis: a matched cohort study. *J Antimicrob Chemother* 61:436-41.
129. Bardet L, Le Page S, Leangapichart T, Rolain J-M. 2017. LBJMR medium: a new polyvalent culture medium for isolating and selecting vancomycin and colistin-resistant bacteria. *BMC Microbiol* 17:220.
130. Abdul Momin MHF, Bean DC, Hendriksen RS, Haenni M, Phee LM, Wareham DW. 2017. CHROMagar COL-APSE: a selective bacterial culture medium for the isolation and differentiation of colistin-resistant Gram-negative pathogens. *J Med Microbiol* 66:1554-61.
131. Lee JY, Ko KS. 2014. Mutations and expression of PmrAB and PhoPQ related with colistin resistance in *Pseudomonas aeruginosa* clinical isolates. *Diagn Microbiol Infect Dis* 78:271-6.

132. Choi MJ, Ko KS. 2014. Mutant prevention concentrations of colistin for *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* clinical isolates. *J Antimicrob Chemother* 69:275-7.
133. Cannatelli A, Di Pilato V, Giani T, Arena F, Ambretti S, Gaibani P, D'Andrea MM, Rossolini GM. 2014. *In vivo* evolution to colistin resistance by PmrB sensor kinase mutation in KPC-producing *Klebsiella pneumoniae* is associated with low-dosage colistin treatment. *Antimicrob Agents Chemother* 58:4399-403.
134. Poirel L, Pitout JDD, Calvo L, Rodriguez-Martinez J-M, Church D, Nordmann P. 2006. *In vivo* selection of fluoroquinolone-resistant *Escherichia coli* isolates expressing plasmid-mediated quinolone resistance and expanded-spectrum beta-lactamase. *Antimicrob Agents Chemother* 50:1525-7.
135. Pena I, Picazo JJ, Rodriguez-Avial C, Rodriguez-Avial I. 2014. Carbapenemase-producing *Enterobacteriaceae* in a tertiary hospital in Madrid, Spain: high percentage of colistin resistance among VIM-1-producing *Klebsiella pneumoniae* ST11 isolates. *Int J Antimicrob Agents* 43:460-4.
136. Denervaud-Tendon V, Poirel L, Connolly LE, Krause KM, Nordmann P. 2017. Plazomicin activity against polymyxin-resistant *Enterobacteriaceae*, including MCR-1-producing isolates. *J Antimicrob Chemother* 72:2787-91.
137. Dobias J, Déneraud-Tendon V, Poirel L, Nordmann P. 2017. Activity of the novel siderophore cephalosporin cefiderocol against multidrug-resistant Gram-negative pathogens. *Eur J Clin Microbiol Infect Dis* 36:2319-27.
138. Bae S, Kim M-C, Park S-J, Kim HS, Sung H, Kim M-N, Kim S-H, Lee S-O, Choi S-H, Woo JH, Kim YS, Chong YP. 2016. *In vitro* synergistic activity of antimicrobial agents in combination against clinical isolates of colistin-resistant *Acinetobacter baumannii*. *Antimicrob Agents Chemother* 60:6774-9.
139. Oliva A, Cipolla A, Vullo V, Venditti M, Mastroianni CM, Falcone M. 2017. Clinical and *in vitro* efficacy of colistin plus vancomycin and rifampin against colistin-resistant *Acinetobacter baumannii* causing ventilator-associated pneumonia. *New Microbiol* 40:205-7.

# ***ANNEXES***

**Annexe A.** Amorces utilisées pour la recherche des mécanismes de résistance à la colistine chez les bacilles Gram négatif

Gène	Amorce	Séquence 5'→3'	Taille amplicon (pb)	
<b>RECHERCHE DE RESISTANCES PLASMIDIQUES</b>				
<i>mcr-1 + mcr-2<sup>a</sup></i>	Mcr-1/2-F	5'-TAT CGC TAT GTG CTA AAG CC-3'	716	
	Mcr-1/2-R	5'-TCT TGG TAT TTG GCG GTA TC-3'		
<i>mcr-1</i>	Mcr-1-F	5'-TGC AGC ATA CTT CTG TGT GG-3'	1 527	
	Mcr-1-R	5'-TAT CGG TTG CCA TTG GCG TG-3'		
	Mcr-1-extF	5'-TGC CGT AAT TAT CCC ACC GT-3'		1740
	Mcr-1-extR	5'-ATA ACA AAC CCC ACC CCA CC-3'		
<i>mcr-2</i>	Mcr-2-F	5'-ATG ACA TCA CAT CAC TCT TGG-3'	1 617	
	Mcr-2-R	5'-TTA CTG GAT AAA TGC CGC GC-3'		
	Mcr-2-extF	5'-TGG TAC AGC CCC TTT ATT-3'		1781
	Mcr-2-extR	5'-GCT TGA GAT TGG GTT ATG A-3'		
<i>mcr-3</i>	Mcr-3-F	5'-TTG GCA CTG TAT TTT GCA TTT-3'	542	
	Mcr-3-R	5'-TTA ACG AAA TTG GCT GGA ACA-3'		
	Mcr-3-all-F	5'-TTA TGC TGA ACT GGC GTG GA-3'		1 506
	Mcr-3-all-R	5'-TTT TGA CGT CCC AGA TAC CC-3'		
<i>mcr-4</i>	Mcr-4-F	5'-ATT GGG ATA GTC GCC TTT TT-3'	488	
	Mcr-4-R	5'-TTA CAG CCA GAA TCA TTA TCA-3'		
	Mcr-4-extF	5'-ATC TGT TAA GTT TGT TGG TGA C-3'		1820
	Mcr-4-extR	5'-TGA GAG CTA AAT GTA ACA ATAGA-3'		
<i>mcr-5</i>	Mcr-5-F	5'-ACA ACC ACA ATG ACG ACG TC-3'	1 132	
	Mcr-5-R	5'-CTG GAA ATG AAC TTG GCA GC-3'		
	Mcr-5-extF	5'-GCC TGT ATG CAA ACT CAT GC-3'		1508
	Mcr-5-extR	5'-CGC TGC TAG GGG TGT CT-3'		
<b>RECHERCHE DE RESISTANCES CHROMOSOMIQUES</b>				
<b><i>E. coli</i></b>				
<i>pmrAB</i>	Eco- <i>pmrA</i> extF	5'-GGA TTA ACT GGC GTT GAG AC-3'	1883	
	Eco- <i>pmrA</i> extR	5'-TGG CGC AGC GAT ATT GGT CG-3'		
	Eco- <i>pmrB</i> extF	5'-AAT CTG CGC GAC AAA GTG GG-3'		
	Eco- <i>pmrB</i> extR	5'-AAC TAC CGT GTT CAG CGT GC-3'		
<i>phoPQ</i>	Eco- <i>phoP</i> extF	5'-TCG AGC TAT CAC GAT GGT TG-3'	2 468	
	Eco- <i>phoP</i> extR	5'-CGA AAC TGA CGC TAT AAC CG-3'		
	Eco- <i>phoQ</i> extF	5'-GAA GTG ATT ACC ACC GTT CG-3'		
	Eco- <i>phoQ</i> extR	5'-CGT TTC TGC CAG TGA CGT TC-3'		
<i>mgrB</i>	Eco- <i>premgrB</i> F	5'-TCC ATA CCA GTG CTA TCA GC-3'	407	
	Eco- <i>mgrB</i> extR	5'-TAG AGG CGC TAT TCT ACC AC-3'		
<b><i>K. pneumoniae</i></b>				
<i>PmrAB</i>	Kpn- <i>pmrA</i> extF	5'-CAT TTC CGC GCA CTG TCT GC-3'	2 048	
	Kpn- <i>pmrA</i> extR	5'-CAG GTT TCA GTT GCA AAC AG-3'		
	Kpn- <i>pmrB</i> extF	5'-ACC TAC GCG AAA AGA TTG GC-3'		
	Kpn- <i>pmrB</i> extR	5'-GTT TGC TTA ATC TCG CCA GG-3'		
<i>phoPQ</i>	Kpn- <i>phoP</i> extF	5'-GAG CTT CAG ACT ACT ATC GA-3'	2 248	
	Kpn- <i>phoP</i> extR	5'-GGG AAG ATA TGC CGC AAC AG-3'		
	Kpn- <i>phoQ</i> extF	5'-ATA CCC ACA GGA CGT CAT CA-3'		
	Kpn- <i>phoQ</i> extR	5'-CAG GTG TCT GAC AGG GAT TA-3'		
<i>mgrB</i>	Kpn- <i>premgrB</i> F	5'-TTT ACG CAG CAG AGG GCA AC-3'	573	
	Kpn- <i>mgrB</i> extR	5'-AAG GCG TTC ATT CTA CCA CC-3'		

<i>crrAB</i>	Kpn- <i>crrA</i> extF Kpn- <i>crrA</i> extR Kpn- <i>crrB</i> extF Kpn- <i>crrB</i> extR	5'-GTG AGG CCA TCA AAT TCT CG-3' 5'-GGA ACC GAG TAT TGC AAT GG-3' 5'-GCA TCC GAG GAT TAG GCT AC-3' 5'-AAG TCC CAA AAG AGG CAA AC-3'	2 211
<b><i>K. oxytoca</i></b>			
<i>pmrAB</i>	Kox- <i>pmrA</i> extF Kox- <i>pmrA</i> extR Kox- <i>pmrB</i> extF Kox- <i>pmrB</i> extR	5'-GCA AGC GTA TTC GCA GGA TA-3' 5'-CTG ATG AGC TGA CAA ACG GC-3' 5'-CAA CGA CAT TTA CAG CTG GG-3' 5'-CTT TAC TGA GGA TAG CGC CA-3'	1 978
<i>phoPQ</i>	Kox- <i>phoP</i> extF Kox- <i>phoP</i> extR Kox- <i>phoQ</i> extF Kox- <i>phoQ</i> extR	5'-CCA AGA GAC CGA GGT ACA AA-3' 5'-GAG TGA CAA CAC CAG CAC TA-3' 5'-CCA TAC CAT CGA TGT GCT GA-3' 5'-GCA GGT GTC TGT TAG GGA TT-3'	2 333
<i>mgrB</i>	Kox- <i>premgrB</i> F Kox- <i>mgrB</i> extR	5'-GCG TCC ATT CTG GCA TGA AC-3' 5'-AGG CGT TTA TTC TAC CAC CC-3'	332
<b><i>S. enterica</i></b>			
<i>pmrAB</i>	Salm- <i>pmrA</i> extF Salm- <i>pmrA</i> extR Salm- <i>pmrB</i> extF Salm- <i>pmrB</i> extR	5'-CAA ACG ACG TAT TAC CAG GC-3' 5'-CAT CGC TCT TCG CTG AAA AC-3' 5'-CCA ACA CTC TGG AAG TGC AT-3' 5'-GTA GTG TGC TGA TTG TCA GC-3'	1 868
<b><i>P. aeruginosa</i></b>			
<i>pmrAB</i>	Paer- <i>pmrA</i> extF Paer- <i>pmrA</i> extR Paer- <i>pmrB</i> extF Paer- <i>pmrB</i> extR	5'-TGC AGG ACC TCT ACG AAC TG-3' 5'-CAG GTT GAG GCT CGA CAG CA-3' 5'-CAA GCT CGG CAA CCA GTT GA-3' 5'-TCG TAG TAA CGG ATC ATC TT-3'	2 419
<i>phoPQ</i>	Paer- <i>phoP</i> extF Paer- <i>phoP</i> extR Paer- <i>phoQ</i> extF Paer- <i>phoQ</i> extR	5'-CAA GAA TGC CTC GAT CGA AG-3' 5'-TCG AAC ACG AAG AAC TCC TC-3' 5'-CTT CAA GCC GAT CGA TAC GG-3' 5'-CTG AAC ATC CGC TCG AAT CC-3'	2354
<i>parRS</i>	Paer- <i>parR</i> extF Paer- <i>parR</i> extR Paer- <i>parS</i> extF Paer- <i>parS</i> extR	5'-GCA TGC ATT CTA GTA GAG GG-3' 5'-CAT GGT CGA CCA CGA AGA TC-3' 5'-CAA GGG CTA CCT GTT CAA CC-3' 5'-GGC TTT GTC AGC AAT CTG TG-3'	2 213
<b><i>A. baumannii</i></b>			
<i>pmrAB</i>	Aba- <i>pmrA</i> extF Aba- <i>pmrA</i> extR Aba- <i>pmrB</i> extF Aba- <i>pmrB</i> extR	5'-TCA TCA ACC CTC AAC TGG AC-3' 5'-CGA CTT CTT GAA GTG CAA CC-3' 5'-CCA TTT AAG AGC GAA GCT GG-3' 5'-GCC ATA ACT TAT GGA CAG GC-3'	2 209
<i>lpxA</i>	Aba- <i>lpxA</i> extF Aba- <i>lpxA</i> extR	5'-CTG AGT TGG TAA TGC AGA AGC-3' 5'-ACC TCG AGC ATT GTA CCA AC-3'	1 092
<i>lpxB</i>	Aba- <i>lpxB</i> extF Aba- <i>lpxB</i> extR	5'-GAT AGT GAT GTG CTA GGG CT-3' 5'-TAC GAG CTG ATT GTA CGT GC-3'	1 338
<i>lpxC</i>	Aba- <i>lpxC</i> extF Aba- <i>lpxC</i> extR	5'-GAT GAA GAT GAC GTT CCT GC-3' 5'-GCT TAG CCA AGC TTT ACT ACG-3'	1 287
<i>lpxD</i>	Aba- <i>lpxD</i> extF Aba- <i>lpxD</i> extR	5'-GTT AAG CAA GCT GCT GAG CA-3' 5'-CTT CAG TCA CAC GAT CAA CC-3'	1 334

**Annexe B.** Mutations chromosomiques responsables de l'acquisition de résistance à la colistine chez les bacilles Gram négatifs.

Espèce bactérienne	Protéine (taille normale)	Domaine (positions en aa)	Mutation <sup>b</sup>	Références		
<b>ENTEROBACTERIES</b>						
<i>K. pneumoniae</i>	PmrA (223 aa)	REC (1-112)	S42N	[1]		
			G53C	[1,2]		
			G53S	[2]		
			Trans_reg_C (145-216)	-	-	
	PmrB (365 aa)	TM (13-35)		ΔR14	[3]	
				L17Q	[2]	
		HAMP (90-142)		L82R	[4]	
				S85R	[1]	
				T140P	[1]	
		HisKA (143-203)		T157P	[3,5,6]	
			-	S208N	[3]	
			ΔY209	[3]		
			HATPase_c (250-358)	R256G	[5]	
	PhoP (223 aa)	REC (1-112)		V3F	[5]	
				L26Q	[1]	
				S86L	[5]	
				Trans_Reg_c (145-220)	D191Y	[7]
	PhoQ (488 aa)	Senseur PhoQ (10-189)		Δ14-18	[8]	
				R16C	[2]	
				A21S	[9]	
				L26P	[5]	
				L96P	[1]	
				D150G	[5]	
				S174N	[3]	
				HAMP (195-263)	V258F	[5]
				HisKA (267-330)	Y268S	[8]
				-	L348Q	[1]
			HATPase_c (375-482)	G385S	[1]	
		D434N	[10]			
MgrB (47 aa)	-		K3*	[2]		
			L9*	[1]		
			I13*	[1]		
			A14S	[1]		
			W20R	[2]		
			L24H	[11]		
			V26*	[1]		
			M27K	[2]		
			C28F	[1]		
			C28Y	[1,5,10,11]		
			C28*	[1,2]		
	Q30*	[1,2]				
	D31N	[1]				

		Q33*	[2]
		F35I	[1]
		G37S	[11]
		C39Y	[2]
		N42Y/K43I	[2]
		I45T	[2]
		W47R	[2]
		W47*	[2]
		*48Y	[5]
	CrrB (353 aa)	-	Q10L [10,12]
		TM (12-34)	Y31H [12]
		HAMP (81-135)	F84S [13]
			L94M [10]
		HisKA (136-200)	W140R [12]
			N141I [12]
			N141Y [13]
			P151L [13]
			P151S [12]
			G183V [13]
			S195N [12]
	LpxM	V30G	[9]
	YciM	V43G	[9]
<i>E. coli</i>	PmrA (222 aa)	REC (1-112)	R81S [14]
		Trans_reg_C (145-216)	- -
	PmrB (363 aa)	-	Δ7-12 [15]
			L10P [16]
		TM1 (15-37)	- -
			Δ88 [17]
		TM2 (69-91)	C84Y [18]
			V88E [17]
			R89P [17]
		HAMP (92-144)	R93P [17]
			P94L [8]
			L110* [17]
			V125E [8]
			Δ133-136 [8]
		HisKA (145-205)	D149Y [18]
			T156K [15]
			A159V [8,15]
			V161G [14]
		HATPases_c (252-360)	- -
	PhoP (223 aa)	REC (1-112)	- -
		Trans_reg_C (145-220)	- -
	PhoQ (486 aa)	Senseur PhoQ (10-189)	- -
		HAMP (195-263)	- -
		HisKA (267-330)	- -
		HATPases_c (374-480)	E375K [19]

<i>S. enterica</i>	PmrA (222 aa)	REC (1-112)	G15R	[20]		
			G53E	[20]		
			G53R	[20]		
			R81C	[20]		
			R81H	[20]		
		Trans_reg_C (145-216)	-			
	PmrB (356 aa)	TM (13-35)		Δ11-14	[21]	
				L14F	[20]	
				L14S	[20]	
				M15L	[21]	
				L22P	[20]	
				S29R	[20]	
		HAMP (89-141)			T92A	[20]
					P94Q	[20]
					E121A	[20]
					S124P	[20]
					N130Y	[20]
		HisKA (142-202)			T147P	[20]
					R155P	[20]
					T156M	[20]
T156P					[20]	
V161G	[20]					
V161L	[20]					
V161M	[20]					
E166K	[20]					
HATPase_c (249-356)			G206R	[20]		
			G206W	[20]		
			S305R	[20]		

#### **BACILLES GRAM NEGATIF NON FERMENTANTS**

<i>P. aeruginosa</i>	PmrA (221 aa)	REC (1-112)	-	-	
		Trans_reg_C (145-216)	L157Q	[22]	
	PmrB (477 aa)	-		L14P	[23]
				V15I	[8]
				TM1 (15-37)	-
	PD (38-160)			ΔD45	[23,24]
				A54V	[23]
				A67T	[8]
	TM2 (161-183)			L167P	[15,19]
	HAMP (186-238)			G188D	[23]
				F237L	[3]
	HisKA (239-304)			L243Q	[23]
				A247T	[25]
				A248V	[23]
				S257N	[23]
M292I				[23]	
			M292T	[26]	



		HATPase_c (348-459)	-	-
	PhoQ (448 aa)	-	R6C	[27]
		TM1 (7-29)	-	-
		-	ΔV57-Q332	[27]
		PD (30-166)	N104I	[3]
			K123Q	[8,22]
			K123E	[3]
			Q133E	[3]
			A143V	[22]
			V152*	[25]
		TM2 (167-189)	V184G	[3]
		-	A207R	[3]
			R214H	[3]
			H223R	[25]
		HisKA (238-300)	V260G	[8]
		HATPase_c (343-448)	ΔL364-G365	[27]
			I421*	[27]
			Fr en I421	[27]
			D433*	[27]
			R444C	[27]
	ParR (235 aa)	REC (7-117)	L18I	[3]
			N24S	[3]
			S24N	[3]
			M59I	[28]
		Trans_reg_C (152-228)	E156K	[28]
	ParS (428 aa)	TM1 (5-27)	L14Q	[28]
		PD (28-131)	V101M	[28]
		TM2 (132-154)	L137P	[28]
		HAMP (155-207)	-	-
		HisKA (208-273)	Q232E	[3]
		HATPase_c (318-428)	G361R	[3]
			H398R	[29]
	ColS	-	A106V	[30]
	CprS	-	R241C	[30]
<i>A. baumannii</i>	PmrA (224 aa)	REC (2-112)	E8D	[31,32]
			M12I	[33]
			M12R	[8]
			P102H	[34]
		-	S119T	[33]
		Trans_reg_C (150-221)	-	-
	PmrB (444 aa)	TM1 (10-29)	T13N	[34]
			S14L	[35]
			S17R	[31]
			Fr en F26	[36]
		PD (30-141)	Δ32-35	
			D64V	[33]
			A80V	[33]

		L87F	[35]
		Y116H	[31]
		I121F	[37]
	TM2 (142-164)	M145K	[35]
		ΔL160	[33]
	-	P170L	[33,38]
		P170Q	[33]
		A183T	[37]
		A184V	[37]
		P190S	[37]
		T192I	[37]
		L178F	[33]
	HisKA (218-280)	A226V	[33]
		A227V	[34–36]
		Q228P	[37]
		R231L	[33]
		T232I	[31]
		P233S	[3,33–36,38]
		P233T	[34]
		T235I	[8,33]
		N256I	[33]
		A262P	[34]
		R263C	[33]
		R263H	[8]
		R263L	[31]
		R263P	[33]
		Q277H	[33]
	-	G315D	[33]
	HATPase_c (326-437)	N353Y	[35]
		P377L	[33]
		F387Y	[35]
		V390G	[8]
		S403F	[35]
		A444V	[8]
LpxA (262 aa)	-	Fr en I25	[39]
	-	G68D	[39]
	-	Q72K	[39]
	-	Fr en H121	[39]
	-	Fr en D130	[39]
	-	H159D	[39]
	-	Q234*	[39]
LpxC (276 aa)	-	P30L	[39]
	-	Fr en D45	[39]
	-	Fr en T285	[39]
LpxD (356 aa)	-	Fr en K317	[39]

<sup>a</sup>Domaines prédits par le logiciel SMART en utilisant les séquences protéiques de *Escherichia coli* MG1655, de *Klebsiella pneumoniae* MGH 78578, de *Salmonella enterica* LT2, de *P. aeruginosa* PA01, et de *A. baumannii* AB0057.

aa, acide aminé; REC, Domaine receveur homologue CheY; Trans\_reg\_C, Protéine régulatrice de la transcription, domaine C-terminal; TM, Domaine transmembranaire; TM1, Premier domaine transmembranaire; TM2, Second domaine transmembranaire; PD, Domaine périplasmique; HAMP, Histidines kinase, adénylyl cyclases, méthyl-binding protéines, et phosphatases; HisKA, Domaine phosphoaccepteur Histidine kinase A; HTAPase\_c, HATPases histidine kinase-like; Senseur PhoQ, système de transduction du signal par phosphorylation.

<sup>b</sup>Δ, délétion; Fr, décalage du cadre de lecture; \*, codon stop

### **Références :**

- [1] Olaitan AO, et al. 2014. Worldwide emergence of colistin resistance in *Klebsiella pneumoniae* from healthy humans and patients in Lao PDR, Thailand, Israel, Nigeria and France owing to inactivation of the PhoP/PhoQ regulator *mgrB*: an epidemiological and molecular study. *Int J Antimicrob Agents*.
- [2] Nordmann P, et al. 2016. Rapid detection of polymyxin resistance in *Enterobacteriaceae*. *Emerg Infect Dis*.
- [3] Choi MJ, et al. 2014. Mutant prevention concentrations of colistin for *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* clinical isolates. *J Antimicrob Chemother*.
- [4] Cannatelli A, et al. 2014. *In vivo* evolution to colistin resistance by PmrB sensor kinase mutation in KPC-producing *Klebsiella pneumoniae* is associated with low-dosage colistin treatment. *Antimicrob Agents Chemother*.
- [5] Cheng YH, et al. 2015. Colistin resistance mechanisms in *Klebsiella pneumoniae* strains from Taiwan. *Antimicrob Agents Chemother*.
- [6] Jayol A, et al. 2014. Resistance to colistin associated with a single amino acid change in protein PmrB among *Klebsiella pneumoniae* isolates of worldwide origin. *Antimicrob Agents Chemother*.
- [7] Jayol A, et al. 2015. Heteroresistance to colistin in *Klebsiella pneumoniae* associated with alterations in the PhoPQ regulatory system. *Antimicrob Agents Chemother* 2015.
- [8] Lee J-Y, et al. 2015. Preservation of acquired colistin resistance in Gram-negative bacteria. *Antimicrob Agents Chemother*.
- [9] Halaby T, et al. 2016. Genomic characterization of colistin heteroresistance in *Klebsiella pneumoniae* during a nosocomial outbreak. *Antimicrob Agents Chemother*.
- [10] Wright MS, et al. 2014. Genomic and transcriptomic analyses of colistin-resistant clinical isolates of *Klebsiella pneumoniae* reveal multiple pathways of resistance. *Antimicrob Agents Chemother*.
- [11] Cannatelli A, et al. 2014. MgrB inactivation is a common mechanism of colistin resistance in KPC-producing *Klebsiella pneumoniae* of clinical origin. *Antimicrob Agents Chemother*.
- [12] Cheng YH, et al. 2016. Amino acid substitutions of CrrB responsible for resistance to colistin through CrrC in *Klebsiella pneumoniae*. *Antimicrob Agents Chemother*.
- [13] Jayol A, et al. 2017. High level resistance to colistin mediated by various mutations in the *crrB* gene among carbapenemase-producing *Klebsiella pneumoniae*. *Antimicrob Agents Chemother*.
- [14] Quesada A, et al. 2015. Polymorphism of genes encoding PmrAB in colistin-resistant strains of *Escherichia coli* and *Salmonella enterica* isolated from poultry and swine. *J Antimicrob Chemother*.
- [15] Olaitan AO, et al. 2016. Emergence of colistin-resistant bacteria in humans without colistin usage: a new worry and cause for vigilance. *Int J Antimicrob Agents*.

- [16] Cannatelli A, et al. 2017. An allelic variant of the PmrB sensor kinase responsible for colistin resistance in an *Escherichia coli* strain of clinical origin. *Sci Rep*.
- [17] Hjort K, et al. 2016. Unstable tandem gene amplification generates heteroresistance (variation in resistance within a population) to colistin in *Salmonella enterica*. *Mol Microbiol*.
- [18] Phan M-D, et al. 2017. Modifications in the *pmrB* gene are the primary mechanism for the development of chromosomally encoded resistance to polymyxins in uropathogenic *Escherichia coli*. *J Antimicrob Chemother*.
- [19] Olaitan AO, et al. 2015. Clonal transmission of a colistin-resistant *Escherichia coli* from a domesticated pig to a human in Laos. *J Antimicrob Chemother*.
- [20] Sun S, et al. 2009. Genetic analysis of colistin resistance in *Salmonella enterica* serovar *Typhimurium*. *Antimicrob Agents Chemother*.
- [21] Olaitan AO, et al. 2015. Acquisition of extended-spectrum cephalosporin- and colistin-resistant *Salmonella enterica* subsp. *enterica* serotype Newport by pilgrims during Hajj. *Int J Antimicrob Agents*.
- [22] Lee JY, et al. 2014. Mutations and expression of PmrAB and PhoPQ related with colistin resistance in *Pseudomonas aeruginosa* clinical isolates. *Diagn Microbiol Infect Dis*.
- [23] Moskowitz SM, et al. 2011. PmrB mutations promote polymyxin resistance of *Pseudomonas aeruginosa* isolated from colistin-treated cystic fibrosis patients. *Antimicrob Agents Chemother*.
- [24] Schurek KN, et al. 2009. Involvement of *pmrAB* and *phoPQ* in polymyxin B adaptation and inducible resistance in non-cystic fibrosis clinical isolates of *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother*.
- [25] Barrow K, et al. 2009. Alterations in two-component regulatory systems of *phoPQ* and *pmrAB* are associated with polymyxin B resistance in clinical isolates of *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother*.
- [26] Abraham N, et al. 2009. A single amino acid substitution in PmrB is associated with polymyxin B resistance in clinical isolate of *Pseudomonas aeruginosa*. *FEMS Microbiol Lett*.
- [27] Miller AK, et al. 2011. PhoQ mutations promote lipid A modification and polymyxin resistance of *Pseudomonas aeruginosa* found in colistin-treated cystic fibrosis patients. *Antimicrob Agents Chemother*.
- [28] Muller C, et al. 2011. A two-component regulatory system interconnects resistance to polymyxins, aminoglycosides, fluoroquinolones, and beta-lactams in *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother*.
- [29] Lee JY, et al. 2014. Development of colistin resistance in *pmrA*-, *phoP*-, *parR*- and *cprR*-inactivated mutants of *Pseudomonas aeruginosa*. *J Antimicrob Chemother*.
- [30] Gutu AD, et al. 2013. Polymyxin resistance of *Pseudomonas aeruginosa phoQ* mutants is dependent on additional two-component regulatory systems. *Antimicrob Agents Chemother*.
- [31] Lesho E, et al. 2013. Emergence of colistin-resistance in extremely drug-resistant *Acinetobacter baumannii* containing a novel *pmrCAB* operon during colistin therapy of wound infections. *J Infect Dis*.
- [32] Rolain JM, et al. 2013. Real-time sequencing to decipher the molecular mechanism of resistance of a clinical pan-drug-resistant *Acinetobacter baumannii* isolate from Marseille, France. *Antimicrob Agents Chemother*.
- [33] Arroyo LA, et al. 2011. The *pmrCAB* operon mediates polymyxin resistance in *Acinetobacter baumannii* ATCC 17978 and clinical isolates through phosphoethanolamine modification of lipid A. *Antimicrob Agents Chemother*.
- [34] Adams MD, et al. 2009. Resistance to colistin in *Acinetobacter baumannii* associated with mutations in the PmrAB two-component system. *Antimicrob Agents Chemother*.

- [35] Beceiro A, et al. 2011. Phosphoethanolamine modification of lipid A in colistin-resistant variants of *Acinetobacter baumannii* mediated by the PmrAB two-component regulatory system. *Antimicrob Agents Chemother*.
- [36] Kim Y, et al. 2014. *In vivo* emergence of colistin resistance in *Acinetobacter baumannii* clinical isolates of sequence type 357 during colistin treatment. *Diagn Microbiol Infect Dis*.
- [37] Park YK, et al. 2011. Correlation between overexpression and amino acid substitution of the PmrAB locus and colistin resistance in *Acinetobacter baumannii*. *Int J Antimicrob Agents*.
- [38] Pournaras S, et al. 2014. Growth retardation, reduced invasiveness, and impaired colistin-mediated cell death associated with colistin resistance development in *Acinetobacter baumannii*. *Antimicrob Agents Chemother*.
- [39] Moffatt JH, et al. 2010. Colistin resistance in *Acinetobacter baumannii* is mediated by complete loss of lipopolysaccharide production. *Antimicrob Agents Chemother*.



# Polymyxins: Antibacterial Activity, Susceptibility Testing, and Resistance Mechanisms Encoded by Plasmids or Chromosomes

Laurent Poirel,<sup>a,b,c</sup> Aurélie Jayol,<sup>a,b,c</sup> Patrice Nordmann<sup>a,b,c,d</sup>

Emerging Antibiotic Resistance Unit, Medical and Molecular Microbiology, Department of Medicine, University of Fribourg, Fribourg, Switzerland<sup>a</sup>; French INSERM European Unit, University of Fribourg (LEA-IAME), Fribourg, Switzerland<sup>b</sup>; National Reference Center for Emerging Antibiotic Resistance, Fribourg, Switzerland<sup>c</sup>; University of Lausanne and University Hospital Center, Lausanne, Switzerland<sup>d</sup>

<b>SUMMARY</b> .....	<b>558</b>
<b>INTRODUCTION</b> .....	<b>558</b>
Structure .....	<b>559</b>
Mechanism of Action .....	<b>559</b>
Spectrum of Activity .....	<b>560</b>
Pharmacodynamics .....	<b>560</b>
<b>USE OF COLISTIN IN HUMAN AND VETERINARY MEDICINE</b> .....	<b>560</b>
Use in Human Medicine .....	<b>560</b>
Commercial formulations .....	<b>561</b>
Routes of administration .....	<b>561</b>
Pharmacokinetics .....	<b>561</b>
Dosing regimen .....	<b>562</b>
(i) Patients with normal renal function .....	<b>562</b>
(ii) Patients with renal insufficiency .....	<b>563</b>
Toxicity .....	<b>564</b>
Use in Veterinary Medicine .....	<b>564</b>
<b>METHODS FOR SUSCEPTIBILITY TESTING</b> .....	<b>565</b>
Dilution Methods .....	<b>565</b>
Broth dilution methods .....	<b>565</b>
(i) Broth microdilution method .....	<b>565</b>
(ii) Broth macrodilution method (or tube dilution method) .....	<b>565</b>
Agar dilution method .....	<b>566</b>
Routine Susceptibility Testing Methods .....	<b>566</b>
Nonautomatic systems .....	<b>566</b>
(i) DD test (Kirby-Bauer procedure) .....	<b>566</b>
(ii) Etest strips .....	<b>567</b>
(iii) UMIC system .....	<b>567</b>
Automatic systems .....	<b>567</b>
(i) Vitek 2 system .....	<b>567</b>
(ii) Phoenix automated microbiology system .....	<b>567</b>
(iii) MicroScan system .....	<b>568</b>
(iv) Sensititre system .....	<b>568</b>
Impact of Materials on MIC Determination .....	<b>568</b>
Impact of medium .....	<b>568</b>
Impact of powder composition .....	<b>568</b>
Impact of the composition and treatment of plates .....	<b>568</b>
Presence or absence of P-80 .....	<b>569</b>
Impacts of Subcultures and Storage on MICs .....	<b>569</b>
Impact of subcultures .....	<b>569</b>
Impact of storage .....	<b>569</b>
Interpretive Criteria .....	<b>569</b>
Quality Controls .....	<b>569</b>
Correlation between MICs of Colistin and Polymyxin B .....	<b>570</b>
Qualitative Detection Techniques .....	<b>570</b>

(continued)

Published 8 March 2017

**Citation** Poirel L, Jayol A, Nordmann P. 2017. Polymyxins: antibacterial activity, susceptibility testing, and resistance mechanisms encoded by plasmids or chromosomes. *Clin Microbiol Rev* 30:557–596. <https://doi.org/10.1128/CMR.00064-16>.

**Copyright** © 2017 American Society for Microbiology. All Rights Reserved.

Address correspondence to Laurent Poirel, laurent.poirel@unifr.ch.

Rapid detection of heterogeneous populations among colistin-resistant Gram-negative bacteria by use of capillary electrophoresis .....	570
Rapid detection of colistin-resistant <i>A. baumannii</i> isolates by use of the Micromax assay.....	570
Rapid detection of colistin-resistant <i>Enterobacteriaceae</i> isolates by use of the Rapid Polymyxin NP test.....	571
Selective medium .....	571
Genotypic Methods.....	571
<b>RESISTANCE MECHANISMS IN ENTEROBACTERIACEAE .....</b>	<b>572</b>
Intrinsic Resistance Mechanisms in <i>Proteus mirabilis</i> and <i>Serratia marcescens</i> .....	572
Mechanisms Responsible for Acquired Resistance in <i>Enterobacteriaceae</i> .....	572
Genes encoding LPS-modifying enzymes.....	573
(i) The <i>pmrC</i> gene .....	573
(ii) The <i>pmrHFIJKLM</i> operon and the <i>pmrE</i> gene .....	573
(iii) The <i>pmrA</i> and <i>pmrB</i> genes, which encode the PmrAB two-component system..	573
(iv) The <i>phoP</i> and <i>phoQ</i> genes, which encode the PhoPQ two-component system..	574
Regulators of the PmrAB and PhoPQ Two-Component Systems .....	574
The <i>mgrB</i> gene.....	574
The <i>crrAB</i> operon .....	574
The Intrinsic Regulator RamA.....	578
Plasmid-Mediated Resistance to Polymyxins .....	578
Other Mechanisms Contributing to Polymyxin Resistance in <i>Enterobacteriaceae</i> .....	580
Hyperproduction of CPS.....	580
Role of porins .....	583
Role of efflux pumps .....	583
Mechanisms of Polymyxin Resistance in <i>Pseudomonas aeruginosa</i> and <i>Acinetobacter baumannii</i> .....	583
<i>Pseudomonas aeruginosa</i> .....	583
<i>Acinetobacter baumannii</i> .....	583
<b>EPIDEMIOLOGY OF RESISTANCE TO POLYMYXINS .....</b>	<b>584</b>
General Epidemiology of Resistance to Polymyxins .....	584
North America.....	584
South America.....	584
Europe .....	584
Middle East.....	587
Africa.....	587
Asia.....	587
Risk Factors.....	587
Specific Epidemiology of the Plasmid-Mediated <i>mcr-1</i> Resistance Gene.....	588
<b>CONCLUSIONS .....</b>	<b>588</b>
<b>ACKNOWLEDGMENTS.....</b>	<b>589</b>
<b>REFERENCES .....</b>	<b>589</b>
<b>AUTHOR BIOS.....</b>	<b>596</b>

**SUMMARY** Polymyxins are well-established antibiotics that have recently regained significant interest as a consequence of the increasing incidence of infections due to multidrug-resistant Gram-negative bacteria. Colistin and polymyxin B are being seriously reconsidered as last-resort antibiotics in many areas where multidrug resistance is observed in clinical medicine. In parallel, the heavy use of polymyxins in veterinary medicine is currently being reconsidered due to increased reports of polymyxin-resistant bacteria. Susceptibility testing is challenging with polymyxins, and currently available techniques are presented here. Genotypic and phenotypic methods that provide relevant information for diagnostic laboratories are presented. This review also presents recent works in relation to recently identified mechanisms of polymyxin resistance, including chromosomally encoded resistance traits as well as the recently identified plasmid-encoded polymyxin resistance determinant MCR-1. Epidemiological features summarizing the current knowledge in that field are presented.

**KEYWORDS** Gram-negative bacteria, MCR-1, lipopolysaccharide, polymyxins, toxicity

## INTRODUCTION

Colistin (also known as polymyxin E) is a polypeptide antibiotic that was originally isolated in 1947 from the soil bacterium *Paenibacillus polymyxa* subsp. *colistinus* (1). Colistin and polymyxin B belong to the class of polymyxins, which is one of the primary classes of antibiotics with activity against most Gram-negative bacteria.

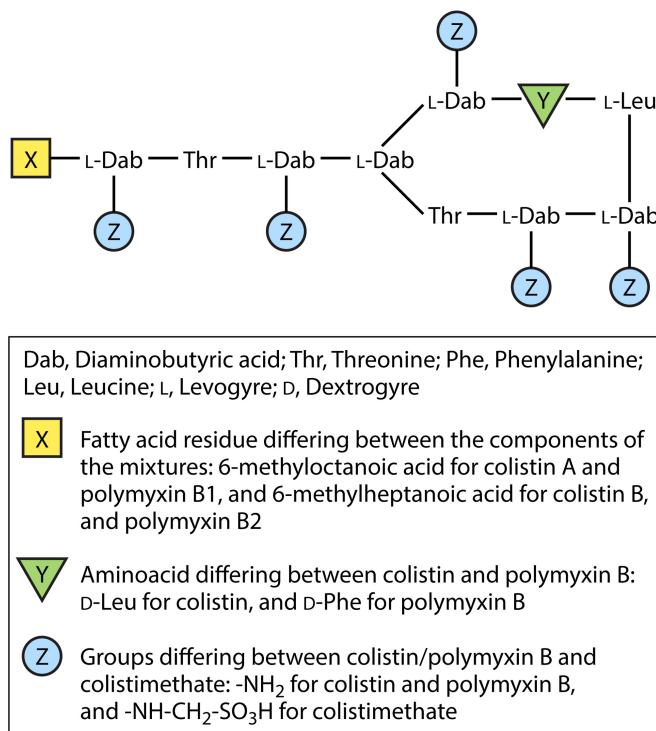


FIG 1 Structures of colistin A and B, colistimethate A and B, and polymyxin B1 and B2.

**Structure**

The chemical structure of polymyxins is similar to that of cationic antimicrobial peptides (CAMPs) (defensins and gramicidins), which represent the first line of defense against bacterial colonization in eukaryotic cells (2). Polymyxins are cationic polypeptides that consist of a cyclic heptapeptide possessing a tripeptide side chain acylated at the N terminus by a fatty acid tail (3, 4) (Fig. 1). The inherent toxicity of colistin may be explained by the hydrophobic properties of the N-terminal fatty acyl segment, which also accounts significantly for its antimicrobial activity, and also by positions 6 and 7, which are very important (5, 6).

Colistin and polymyxin B differ by only a single amino acid in the peptide ring, with a phenylalanine in polymyxin B and a leucine in colistin (Fig. 1) (7). Polymyxin B is administered directly as an active antibiotic, whereas colistin is administered as an inactive prodrug, colistin methanesulfonate (also known as colistimethate [CMS]) (Fig. 1) (7).

The terms “colistin” and “colistimethate” are not interchangeable, since they correspond to different forms of colistin available for clinical use (4). Indeed, colistimethate sodium is a polyanionic inactive prodrug that is less toxic than colistin sulfate (Fig. 1) (4, 8). Colistimethate is formed by the reaction of colistin with formaldehyde and sodium bisulfite (9). This prodrug is transformed in aqueous media, and also *in vivo* in biological fluids, and is converted into colistin and several inactive methanesulfonated compounds (10, 11).

**Mechanism of Action**

The target of polymyxins is the outer membrane of Gram-negative bacteria. Because of an electrostatic interaction occurring between the  $\alpha,\gamma$ -diaminobutyric acid (Dab) residue of the positively charged polymyxin on one side and the phosphate groups of the negatively charged lipid A membrane on the other side, divalent cations (Ca<sup>2+</sup> and Mg<sup>2+</sup>) are displaced from the negatively charged phosphate groups of membrane lipids (12). The lipopolysaccharide (LPS) is therefore destabilized, consequently increasing the permeability of the bacterial membrane, leading to leakage of the cytoplasmic



content and ultimately causing cell death (4, 13). Note that even though the LPS is the initial target, the exact mode of action of polymyxins still remains unclear.

Another antibacterial mechanism is the endotoxin effect. The endotoxin of Gram-negative pathogens corresponds to the lipid A portion of the LPS; polymyxins have the ability to bind to and neutralize this LPS molecule released during cell lysis (14).

Finally, another mode of action of the polymyxins is the inhibition of vital respiratory enzymes (inhibition of type II NADH-quinone oxidoreductases [NDH-2]) in the bacterial inner membrane (15).

### Spectrum of Activity

Polymyxins have a narrow antibacterial spectrum, mainly against common Gram-negative bacteria. They are active against most members of the *Enterobacteriaceae* family, including *Escherichia coli*, *Enterobacter* spp., *Klebsiella* spp., *Citrobacter* spp., *Salmonella* spp., and *Shigella* spp. Polymyxins also have significant activity against common nonfermentative Gram-negative bacteria, including *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Stenotrophomonas maltophilia* (13).

Conversely, some species are naturally resistant to polymyxins, including *Proteus* spp., *Morganella morganii*, *Providencia* spp., *Serratia marcescens*, *Pseudomonas mallei*, *Burkholderia cepacia*, *Chromobacterium* spp., *Edwardsiella* spp., *Brucella*, *Legionella*, *Campylobacter*, and *Vibrio cholerae*. Polymyxins are not active against Gram-negative cocci (*Neisseria* spp.), Gram-positive bacteria, and anaerobic bacteria (13).

### Pharmacodynamics

The antibacterial effect of colistin is concentration dependent (4, 16–18). The pharmacokinetic-pharmacodynamic (PK-PD) index that best predicts the antibacterial activity against *A. baumannii* and *P. aeruginosa* is the ratio of the area under the concentration-time curve for free drug from 0 to 24 h to the MIC ( $fAUC_{0-24}/MIC$ ), with this index being superior to the maximum concentration of drug in serum ( $C_{max}$ )/MIC relationship, suggesting that time-averaged exposure to colistin is more important than the achievement of high peak concentrations (19–21). An average steady-state plasma colistin concentration of 2  $\mu\text{g/ml}$  has been suggested as a reasonable target value for isolates with MICs of  $\leq 1 \mu\text{g/ml}$ , maximizing the antimicrobial activity while minimizing the risk of nephrotoxicity (22). An inadequate AUC/MIC ratio likely leads to treatment failure. The colistin antibacterial effect is extremely rapid, occurring as early as 5 min after exposure (17, 18, 23, 24).

A postantibiotic effect was observed against *Klebsiella pneumoniae*, *P. aeruginosa*, and *A. baumannii* (25). However, it is important to highlight that polymyxins have minimal postantibiotic effects at clinically relevant concentrations. Despite the major initial killing rate observed against colistin-susceptible strains exposed to colistin alone, regrowth has been reported for *A. baumannii* (17) and *K. pneumoniae* (18) in static time-kill studies. Colistin heteroresistance, a phenomenon corresponding to the emergence of a colistin-resistant subpopulation (that can grow in the presence of  $\geq 4 \mu\text{g/ml}$  of colistin) within a susceptible population (i.e., with a MIC of  $\leq 2 \mu\text{g/ml}$ ), has been observed for *A. baumannii* (26, 27), *K. pneumoniae* (18, 28), and *P. aeruginosa* (23).

## USE OF COLISTIN IN HUMAN AND VETERINARY MEDICINE

### Use in Human Medicine

After its discovery in 1947, colistin was used in Japan and Europe during the 1950s (29). Then, after its approval by the U.S. FDA in 1959, colistimethate (CMS), the inactive prodrug of colistin, replaced colistin for parenteral administration (29). Colistin and CMS have been used widely for decades for treatment of infections caused by Gram-negative bacteria. However, in the 1970s, because of their toxicity, especially nephrotoxicity (30), their use was reconsidered. They were then replaced by novel, more active and less toxic antibiotics, such as aminoglycosides, quinolones, and  $\beta$ -lactams. For 20 years, the use of colistin was restricted to ophthalmic and topical uses. Systemic or nebulized colistin was used only for cystic fibrosis patients.

However, the increasing prevalence of multidrug-resistant (MDR) Gram-negative bacteria (31), particularly *K. pneumoniae*, *A. baumannii*, and *P. aeruginosa*, has forced physicians to reintroduce systemic polymyxin as a valuable therapeutic option (4, 13, 32).

Considering the paucity of novel antibiotics, colistin is currently often the only effective antibiotic agent against MDR organisms, particularly carbapenemase-producing bacteria.

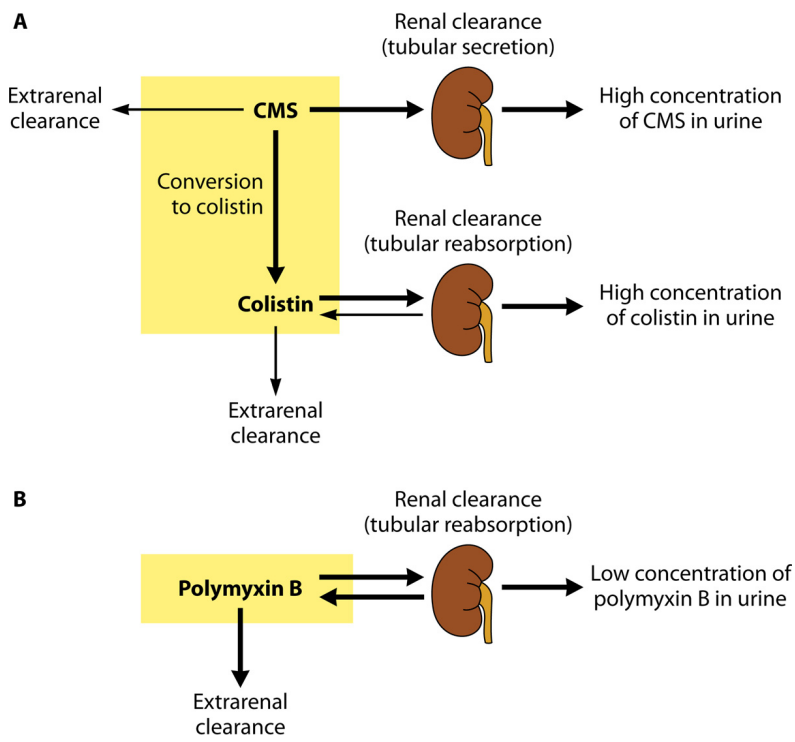
**Commercial formulations.** There are more than 30 polymyxin molecules, among which there are five main chemical compounds (polymyxins A to E), each containing multiple components. Although colistin (polymyxin E) and polymyxin B are both used in clinical practice (33), colistin is the most widely used polymyxin (23). The two most common commercially available parenteral formulations of the colistin prodrug, CMS, are Colomycin (Forest Laboratories UK Limited, Dartford, United Kingdom), primarily employed in Europe, and Coly-Mycin M Parenteral (Monarch Pharmaceuticals, Inc., Bristol, TN), primarily employed in the United States (34). Unfortunately, the vials of both formulations contain different dry powder quantities, and the two products are differently labeled, with Colomycin being labeled in international units (IU) of CMS and Coly-Mycin M Parenteral being labeled in milligrams of CMS or colistin base activity (CBA) (34). The conversion is as follows: 1 million units (MU) CMS = 80 mg CMS = 30 mg CBA (35). To add to the confusion, some other brands corresponding to generic products are now available (36). The multiplicity of terms used to express contents of vials and dose regimens unfortunately creates confusion and does not allow any meaningful comparison of data collected from studies performed in different parts of the world.

**Routes of administration.** Colistin sulfate can be administered orally as tablets and syrup for selective digestive tract decontamination (no absorption) and topically for the treatment of bacterial skin infections (13). CMS, the less toxic prodrug, has different administration routes, i.e., parenteral (including intravenous) and intramuscular, but intrathecal or intraventricular administration is also possible (13). The intramuscular injection is rarely used in clinical practice because it may be very painful locally, and also because its absorption is variable (33). Both colistin sulfate and CMS can be delivered through inhalation by aerosol therapy, but there is a higher frequency of bronchoconstriction with colistin sulfate (33). Delivery of CMS by inhalation and by the intrathecal and intraventricular routes allows much higher concentrations in lung fluid and cerebrospinal fluid, respectively, than those seen with systemic administration. Moreover, those routes of administration lead to negligible plasma exposure and are less toxic (in particularly less nephrotoxic) (22).

In aqueous solutions, colistimethate sodium is transformed into colistin; therefore, it should be administered shortly after reconstitution to avoid the toxicity associated with colistin (37).

**Pharmacokinetics.** Because of their discovery and their introduction into clinical use more than 50 years ago, polymyxins were never subjected to the drug development approval process currently required by international drug regulatory authorities. Consequently, the PK and PD data on the rational use of polymyxins (maximizing antibacterial activity and minimizing toxicity and development of resistance) were not available until recently. The fact that, until recently, plasma concentrations of CMS and formed colistin could not be differentiated because of a lack of suitable techniques was another obstacle limiting progress in this area. The recent development of chromatographic methods allowing quantitative assessment of each compound separately significantly contributed to the renewed interest in prescribing colistin and colistimethate (38, 39). It was clearly demonstrated that the observed antimicrobial activity results from the action of colistin itself, which is generated *in vivo* when CMS is given. For accurate PK information, a prerequisite is to quantify separately the inactive prodrug (CMS) and the active entity (colistin) (34).

After its parenteral administration, a large proportion of CMS is eliminated mainly through the kidneys by glomerular filtration and tubular secretion (Fig. 2A) (11).



**FIG 2** Overview of the pharmacokinetic pathways for colistimethate (CMS) and colistin (A) and for polymyxin B (B). The thicknesses of the arrows indicate the relative magnitudes of the respective clearance pathways when kidney function is normal. CMS includes fully and all partially methanesulfonated derivatives of colistin. After administration of CMS, extensive renal excretion of the prodrug occurs, with some of the excreted CMS being converted to colistin within the urinary tract. (The figure is based in part on data from reference 7.)

Because in a healthy individual the clearance of CMS by the kidneys is much higher than its conversion to colistin, no more than 20 to 25% of a CMS dose is hydrolyzed *in vivo* into an active colistin entity (7). Consequently, the colistin concentrations resulting from the original CMS administration are low. In contrast to CMS, colistin is eliminated predominantly by a nonrenal way because of its extensive renal tubular reabsorption (Fig. 2A) (11, 40). Although colistin is poorly excreted in urine, the urinary concentration of colistin may be relatively high after administration of CMS due to the conversion of CMS (highly excreted by the kidneys) into colistin within the urinary tract (7).

In contrast to colistin, polymyxin B is administered directly in its active antibacterial form. As for colistin formed from CMS, polymyxin B is subject to very extensive renal tubular reabsorption and is thus eliminated mainly by a nonrenal clearance mechanism(s) (Fig. 2B) (7).

**Dosing regimen.** Due to renewed interest in its use in the context of infections caused by multidrug-resistant bacteria, and considering the increasing rates of resistance to colistin currently observed, CMS has to be administered carefully. In particular, the regimens allowing maximal antibacterial activity and minimal development of resistance have to be defined accurately, since the regimens need to minimize adverse effects (23). A study analyzing product data characteristics of intravenous CMS revealed a lack of uniformity between manufacturers, with quite broad variations in term of indications, dose regimens (3 to 12 MU/day), and PK (36). Moreover, dosing regimens given by manufacturers are often discordant with the dosing regimens recommended by the recent literature (21, 34, 41).

**(i) Patients with normal renal function.** The currently used dosage regimens of CMS generate suboptimal exposure to colistin in many critically ill patients, in particular in renally competent patients. Two studies reported low plasma colistin  $C_{max}$  values

following administration of 174 mg to 250 mg (2 to 3 MU) of CMS every 8 or 12 h, with steady-state levels of 1.15 to 5.14  $\mu\text{g/ml}$  or 0.68 to 4.65  $\mu\text{g/ml}$ , respectively (42). Moreover, a significant delay in obtaining steady-state plasma concentrations of formed colistin was reported for CMS treatment started without administration of a loading dose (43). In the latter study, concentrations of colistin in the plasma were reported to be below the MIC breakpoint (2  $\mu\text{g/ml}$ ), which is a main drawback considering that a delayed initiation of appropriate antibiotic therapy has been shown to be associated with increased mortality rates, in particular for critically ill patients (44). In addition, low colistin concentrations may induce the amplification of colistin-resistant subpopulations (18, 45). Interestingly, on consideration of the current dose range product recommendations for CMS, it was confirmed that its administration at the upper limit to patients with normal renal function resulted in low and potentially suboptimal plasma colistin concentrations, especially when the MIC for the infecting bacterial strain was in the upper range (2  $\mu\text{g/ml}$ ) or if the infection was associated with a high bacterial inoculum (21). That study also revealed that steady-state plasma colistin concentrations are highly variable, with up to a 10-fold range achieved across patients at a given creatinine clearance (21).

In contrast, there is a relatively low interpatient variability (3.3-fold) across a wide range of creatinine clearance values following administration of polymyxin B (46). Considering that polymyxin B is not given as a prodrug, it is easier to rapidly achieve a desired plasma concentration of polymyxin B (46).

There is no consensus about dosing regimens, even though recently published dosing suggestions seem to be widely accepted (19). Compared to those suggested by the manufacturers, the regimens in recent studies support the administration of a loading dose and of higher doses of CMS in order to achieve adequate colistin concentrations leading to a better therapeutic effect (21, 41, 47). The dosing regimen currently recommended by the recent literature (for patients with good renal function) is a loading dose of 4.5 MU of CMS followed by maintenance doses of 4.5 MU twice daily (48–50). A colistin-containing combination therapy has to be considered if the infecting pathogen shows an MIC of colistin above 1  $\mu\text{g/ml}$ , if there is a high inoculum, or in dealing with deep-seated infections (e.g., in lungs). One therefore has to consider adding antibiotics to colistin regimens, especially for patients with relatively normal renal function (21, 22).

Data about the pharmacokinetics, effectiveness, and safety of polymyxins were recently reviewed by the European Medicines Agency (EMA). There have been recommended changes in terms of product information in order to ensure the safer use of polymyxins (51). According to the EMA, polymyxins should be reserved for the treatment of serious infections due to aerobic Gram-negative pathogens with limited treatment options (51). Also, they should be given with another suitable antibiotic when possible. The recommended dose for CMS in adults is 9 MU daily in 2 or 3 divided doses as a slow intravenous infusion. For dealing with critically ill patients, a loading dose of 9 MU should be given. For patients with renal impairment, doses should obviously be reduced, with consideration of the creatinine clearance.

Because the efficacy and toxicity of colistin are dose dependent, it is crucial that optimal dose regimens be used to maximize the antimicrobial activity and to minimize adverse effects and the development of resistance. This is especially important for critically ill patients, as they are most at risk for high morbidity and mortality (52).

**(ii) Patients with renal insufficiency.** A study showed that colistin levels were elevated in patients with renal insufficiency, presumably due to decreased elimination of the antibiotic generating a higher rate of conversion of CMS to colistin (43). Development of nephrotoxicity is consequently higher in patients with renal insufficiency than in patients with normal renal function (53).

Dalino et al. (54) suggested a new dose adjustment for high-dose colistin therapy for patients with renal insufficiency. For patients with creatinine clearance of 20 to 50 ml/min, they recommend a loading dose of 9 MU and maintenance doses of 4.5 MU

every 24 h. For patients with creatinine clearance of <20 ml/min, they recommend a loading dose of 9 MU and maintenance doses of 4.5 MU every 48 h (21, 55).

**Toxicity.** Rates of toxicity following intravenous administration of CMS are considered lower today than those observed in previous studies, and it has to be mentioned that the criteria for defining toxicity have also been updated (56). The lower toxicity may be related to the fact that there are fewer chemical impurities in CMS but also to the fact that monitoring in intensive care units (ICUs) is better nowadays and the coadministration of other nephrotoxic drugs is significantly avoided (33).

Colistin has a narrow therapeutic window, and major adverse effects related to its parenteral use are neurotoxicity and nephrotoxicity. Neurotoxicity is dose dependent and reversible (55) and may cause peripheral and facial paresthesia, weakness, dizziness/vertigo, visual disturbances, confusion, ataxia, and neuromuscular blockade, even leading to respiratory failure or apnea (56). The most common neurotoxicological effect is paresthesia (occurring in 27% of patients), and there is no report of neuromuscular blockade or apnea in the recent literature (56). Nephrotoxicity is the most common and concerning adverse effect, especially with the newly recommended high-dose regimen. Similarly to neurotoxicity, nephrotoxicity is dose dependent. The risk of colistin-associated nephrotoxicity increases with plasma colistin concentrations above 2.5 to 3  $\mu\text{g/ml}$ , as revealed by recent PK-PD analyses (57). Other risk factors for nephrotoxicity include coadministration of other drugs that are also nephrotoxic (anti-inflammatory drugs, vancomycin, or aminoglycoside antibiotics) and patient-related factors (advanced age, male sex, hypoalbuminemia, hyperbilirubinemia, preexisting chronic kidney disease, and severity of illness) (33, 56). Nephrotoxicity is reported to be a rapid-onset effect, with most cases occurring within the first week of treatment, and is mostly reversible (33, 55). Rates of nephrotoxicity in recent studies ranged from 6% to 55% (33). The large range of nephrotoxicity rates may be explained partially by different definitions of renal failure, the dosing regimens used, the concomitant administration of nephrotoxic drugs, and the use of colistin monitoring to adapt dosing regimens. The RIFLE (risk–injury–failure–loss–end-stage renal disease) classification is used to determine colistin-associated nephrotoxicity (58).

Two recent comparative studies involving large numbers of patients showed that the nephrotoxicity rates were lower for polymyxin B than for CMS/colistin (59, 60).

### Use in Veterinary Medicine

As opposed to the case in human medicine, in veterinary medicine colistin has been used extensively for decades for the treatment and prevention of infectious diseases. The majority of polymyxin consumption corresponds to orally administered forms, with different formulations (premix, powder, or oral solutions). The main usage is related to enterobacterial infections, and in particular to gastrointestinal infections caused by *E. coli* in poultry and pigs within intensive husbandry systems (61). Apart from this common usage for treating infections caused by *Enterobacteriaceae*, another usage corresponds to growth promotion, which is a common practice worldwide. Furthermore, the fact that only a thin line exists between oral metaphylactic therapy, preventive starter rations, and growth promotion adds to the problem. In 2011, polymyxins were the fifth most sold class of antimicrobials (7%) for treating food-producing animals in Europe (61).

Despite this extensive use in veterinary medicine, the resistance rate to colistin in *E. coli* strains recovered from healthy animals remains <1% in many European countries (62). However, resistance to colistin has increasingly been reported (10%) among porcine-pathogenic *E. coli* strains in Belgium (63), and the emergence of resistance has been described for cattle (64). Moreover, some recent data revealed the possibility of horizontal transmission from farm animals to humans in Asia (65). Given the increasing need to retain the efficacy of colistin to treat MDR infections in humans, the potential for spreading colistin-resistant isolates from animals to humans, and the recent identification of colistin-resistant *Enterobacteriaceae* organisms harboring a plasmid-borne colistin resistance determinant in animals and food products (see below), the use of colistin in veterinary medicine is being reevaluated. As a very recent example, the

formal Ministry of Agriculture of China decided to ban colistin as a feed additive for animals (66). Also, the European Medicines Agency provided a position paper in June 2016, in which updated advice on the use of colistin products in animals within the European Union is provided (67).

## METHODS FOR SUSCEPTIBILITY TESTING

Despite such a long term of clinical use (decades), the optimal method for polymyxin susceptibility testing still remains undefined. However, the recent emergence of MDR Gram-negative bacteria and the subsequent increased use of colistin prompted the scientific community to develop rapid and reliable methods to determine the susceptibility of isolates to polymyxins, as this is now an urgent need in clinical laboratories. Polymyxin susceptibility testing is now a major challenge, as human infections with colistin-resistant Gram-negative bacteria are associated with higher patient mortality (68). The difficulties in testing susceptibility to polymyxins are diverse, including poor diffusion of polymyxins into agar, the inherent cationic properties of polymyxins, the occurrence of heteroresistance to polymyxins in many species, and the lack of a reliable reference method that may allow reliable comparisons of commercial tests (69, 70).

### Dilution Methods

The aim of dilution methods is to determine the MIC, corresponding to the lowest concentration of polymyxin that inhibits visible bacterial growth after an incubation of 16 to 24 h at  $35 \pm 2^\circ\text{C}$ .

**Broth dilution methods.** Broth dilution is a technique in which a bacterial suspension at a predetermined concentration is tested against various concentrations of antimicrobial agent in a liquid medium with a predetermined formulation. Two types of broth dilution methods are available: (i) the broth macrodilution method, performed with a minimum volume of 2 ml in standard test tubes; and (ii) the broth microdilution (BMD) method, performed with a volume of 0.05 to 0.1 ml in microtitration trays.

**(i) Broth microdilution method.** BMD is the reference susceptibility test method. It is currently the only method recommended by the Clinical and Laboratory Standards Institute (CLSI) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (71, 72) for polymyxin antimicrobial susceptibility testing.

According to CLSI recommendations, BMD is performed with cation-adjusted Mueller-Hinton broth (CA-MHB), a range of 2-fold dilutions of polymyxins (ranging from 0.12 to 512  $\mu\text{g/ml}$ ), and a final bacterial inoculum of  $5 \times 10^5$  CFU/ml in each well (73). BMD is considered to be the optimal method and is currently recommended for susceptibility testing in the recent document proposed by the joint CLSI-EUCAST Polymyxin Breakpoints Working Group ([http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST\\_files/General\\_documents/Recommendations\\_for\\_MIC\\_determination\\_of\\_colistin\\_March\\_2016.pdf](http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/General_documents/Recommendations_for_MIC_determination_of_colistin_March_2016.pdf)).

However, BMD is quite laborious, and manual preparation (if the technique used is not an automated one) of antibiotic solutions may lead to significant errors. It is therefore not adaptable for most clinical microbiology laboratories. Furthermore, non-reproducible and noninterpretable MIC results have been reported due to the presence of skip wells (i.e., wells that exhibit no growth, whereas growth is observed in wells with higher antibiotic concentrations) for *Enterobacter* species (69), *P. aeruginosa* (72), and *A. baumannii* (73). This phenomenon might be caused by heteroresistant subpopulations for *Enterobacter* spp. (69). In parallel, "skip well" isolates of *P. aeruginosa* have been found to have increased expression of the *pmrAB*, *phoQ*, and *arn* genes related to changes in the LPS structure, reducing the potential binding sites of polymyxins (74).

Nevertheless, BMD currently remains the reference method for determination of MICs because of its reproducibility, reliability, and possibility of automation.

**(ii) Broth macrodilution method (or tube dilution method).** The growth medium (CA-MHB), the inoculum bacterial suspension, the preparation of 2-fold dilutions of polymyxins, the incubation conditions, and the reading of the plate are identical to those for the broth microdilution method. The only difference is the volume of growth



medium and the use of test tubes instead of trays. When evaluated against BMD results, the results of the broth macrodilution method showed the highest agreement (83%) compared to other available methods, and no false susceptibility was observed (70).

**Agar dilution method.** Agar dilution is another reference method that relies on various concentrations of polymyxin molecules in Mueller-Hinton agar (usually 2-fold serial dilutions), followed by the seeding of a defined bacterial inoculum onto the agar plate. In accordance with the CLSI recommendations, the polymyxin powder is dissolved in sterile water and added to molten MH agar to provide 2-fold dilutions (usually ranging from 0.12  $\mu\text{g/ml}$  to 512  $\mu\text{g/ml}$ ) (70, 71). A bacterial inoculum corresponding to a 0.5 McFarland standard (approximately  $10^8$  CFU/ml) is prepared, and then 10-fold dilutions are performed. One microliter of this dilution is spotted manually or with an automated system, and each spot consequently inoculates  $10^4$  CFU of bacteria.

Agar dilution may theoretically avoid the adsorption of colistin to the plates, but no study has measured the colistin concentration in agar dilution plates to confirm this hypothesis. Numerous studies have demonstrated a strong correlation between agar dilution and BMD (70, 75, 76), with the exception of results obtained with *P. aeruginosa* and *S. maltophilia* isolates from cystic fibrosis patients (77, 78). One advantage of the agar dilution method is the ability to test multiple strains on the same plate and the possibility to semiautomate the method. However, the agar dilution method also presents some disadvantages, as it is very laborious if not automated and the plates (not available from commercial sources) must be used within a week of preparation.

Many studies have employed the agar dilution method as a standard; however, BMD remains the primary reference method for polymyxin MIC testing. In a recent document proposed by the joint CLSI-EUCAST Polymyxin Breakpoints Working Group, it is stated that agar dilution is not recommended for susceptibility testing ([http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST\\_files/General\\_documents/Recommendations\\_for\\_MIC\\_determination\\_of\\_colistin\\_March\\_2016.pdf](http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/General_documents/Recommendations_for_MIC_determination_of_colistin_March_2016.pdf)).

### Routine Susceptibility Testing Methods

**Nonautomatic systems. (i) DD test (Kirby-Bauer procedure).** The disk diffusion (DD) test refers to the diffusion of a given concentration of polymyxin from disks into MH agar that has been seeded with a defined bacterial inoculum. According to the CLSI and EUCAST guidelines, the disk diffusion test is performed by applying a bacterial inoculum corresponding to a 0.5 McFarland standard (approximately  $10^8$  CFU/ml) suspended in 0.85% NaCl onto the entire surface of an MH agar plate by use of a sterile cotton swab. Paper disks impregnated with polymyxin are placed on the inoculated agar surface. Following the CLSI guidelines, the contents of colistin and polymyxin B on the paper disks are 10  $\mu\text{g}$  and 300 U, respectively (72), while following the EUCAST recommendations, the colistin content is 50  $\mu\text{g}$  (71). The growth inhibition zone diameter around the disk is measured after incubation for 16 to 24 h at  $35 \pm 2^\circ\text{C}$ . The diameter of the inhibition zone is proportional to the bacterial susceptibility to polymyxins and inversely correlates with the MIC of the bacterial strain.

The DD test is easy and cheap and does not require specific equipment. These advantages explain why this method is commonly used as a primary test method to screen large numbers of isolates. However, the poor and slow diffusion of polymyxins through agar gives small zones of inhibition and limits the predictive accuracy of the DD test. In fact, many studies showed that the DD test is a nonreliable method for measuring susceptibility to colistin for Gram-negative rods, giving an unacceptable and very high rate of false susceptibility (up to 35%) compared to that with dilution methods (76, 78–80). A higher concentration of colistin in the disk (50  $\mu\text{g}$  as recommended by EUCAST versus 10  $\mu\text{g}$  as recommended by CLSI) does not improve the reliability of the test (80). Susceptible results should therefore be confirmed by dilution tests. On the other hand, no false resistance results are found with this method (80).

This method is not reliable and should be abandoned. For human medicine, EUCAST recommends that precise MIC determination be mandatory before clinical use and no longer provides disk breakpoints (71). For veterinary medicine, EUCAST recommends

precise determination of the MIC each time that the diameter of the inhibition zone is between 15 and 18 mm for a given strain.

(ii) **Etest strips.** Etests are thin plastic test strips impregnated with increasing antibiotic concentrations. MICs are read with the concentration scale marked on the upper surface. According to the manufacturer's recommendations, this method is performed by applying a bacterial inoculum of approximately  $10^8$  CFU/ml (turbidimetry of a 0.5 McFarland standard) suspended in 0.85% NaCl onto the entire surface of an MH agar plate by use of a sterile cotton swab. Etest strips containing a colistin concentration gradient (ranging from 0.016 to 256  $\mu\text{g/ml}$ ) are placed on the inoculated agar surface, and the MIC is determined after incubation for 16 to 24 h at  $35 \pm 2^\circ\text{C}$ . The MIC value is defined by the intersection of the lower part of the ellipse-shaped growth inhibition area with the test strip. When the intersection occurs around the MIC endpoint, the highest MIC intersection is recorded (75). When small colonies grow within the zone of inhibition, the strain must be considered heteroresistant to colistin, and the highest MIC intersection is recorded (75, 81).

Several studies, notably including few resistant isolates, found an excellent correlation between the Etest and reference techniques (75, 76, 80, 82). However, studies including larger numbers of resistant isolates reported high rates of false susceptibility (up to 32%) for Gram-negative rods compared to those with dilution methods (69, 70, 78, 83). The Etest method may fail to detect resistance to colistin even when isolates exhibit high MICs by dilution methods (70, 83). In addition, there are discrepancies between MICs measured by Etest and MICs measured by dilution methods (70, 82). It has been reported that the Etest strip method underestimates the level of resistance of polymyxin-resistant strains ( $\text{MIC} \geq 4 \mu\text{g/ml}$ ) and overestimates the MIC values for susceptible strains ( $\text{MIC} < 4 \mu\text{g/ml}$ ) (70).

This method is easy to perform but is relatively expensive and does not reliably detect colistin-resistant isolates. As for the disk diffusion test, susceptibility results obtained by Etest require a 24-h delay.

(iii) **UMIC system.** The UMIC system (Biocentric) consists of broth microdilution unitary panels in which the wells contain prediluted lyophilized colistin at concentrations ranging from 0.06 to 64  $\mu\text{g/ml}$ . The inoculation is performed manually, and the required incubation time ranges from 18 to 24 h. The performance of this system has not yet been evaluated.

**Automatic systems.** Use of instruments may allow susceptibility testing to be performed in a shorter period than that required for manual methods, as the sensitive optical detection systems of current instruments measure subtle changes in bacterial growth. To date, four automated instruments capable of measuring susceptibility to polymyxins are available. Two of them generate overall rapid (4 to 16 h) susceptibility test results (Vitek 2 and Phoenix), while the others (MicroScan and Sensititre) are overnight systems. These systems are associated with computer software to interpret susceptibility results.

(i) **Vitek 2 system.** The Vitek 2 system (bioMérieux) uses plastic reagent cards that contain microliter quantities of antibiotics and test media in wells (84). It tests colistin concentrations ranging from 0.5 to 16  $\mu\text{g/ml}$  and monitors turbidimetry to determine bacterial growth during a period of 4 to 10 h. Compared to dilution methods, the Vitek 2 system displays a low sensitivity for detecting colistin-resistant Gram-negative isolates (83) and is not reliable for detecting heteroresistant subpopulations (76).

(ii) **Phoenix automated microbiology system.** The BD Phoenix automated microbiology system (BD Diagnostics) has a large incubator reader. Panels test colistin concentrations ranging from 0.5 to 4  $\mu\text{g/ml}$ , and the inoculation is manual or automatic (84). MIC results are generated in 6 to 16 h. No study has evaluated the performance of Phoenix for detection of colistin resistance among Gram-negative bacteria. The only published study evaluating polymyxin susceptibility by using the Phoenix system unfortunately did not include colistin-resistant strains (85). Nevertheless, we recently evaluated the accuracy of this system by testing 100 enterobacterial isolates (60 colistin-resistant and 40 colistin-susceptible isolates) and found a high rate (15%) of



false-susceptible results. We observed a low sensitivity for detecting colistin heteroresistance in *K. pneumoniae* and *Enterobacter cloacae* isolates (our unpublished data) but a good sensitivity for detecting plasmid-mediated colistin resistance.

**(iii) MicroScan system.** The MicroScan system (Beckman Coulter Diagnostics) uses microdilution trays with colistin concentrations of 2 and 4  $\mu\text{g}/\text{ml}$ . The trays are inoculated manually and incubated in the instrument for 16 to 20 h (84). Compared to dilution methods, the categorical agreement of the MicroScan system is 87% for *Acinetobacter* isolates (86), and the sensitivity is 88% for detection of polymyxin B resistance in *K. pneumoniae* isolates (87).

**(iv) Sensititre system.** The Sensititre system (Thermo Fisher Scientific) is an automated incubation and reading system (84). The tests are standard broth microdilution panels containing prediluted ranges of lyophilized colistin within the wells (0.12 to 128  $\mu\text{g}/\text{ml}$ ). Inoculation may be performed by using a Sensititre autoinoculator. Growth is measured after an incubation of 18 to 24 h. A single study has evaluated the Sensititre method, and a 96% categorical agreement with BMD was found, with no false susceptibility results reported (70).

### Impact of Materials on MIC Determination

**Impact of medium.** Polymyxin resistance is regulated by the two-component systems PhoP/PhoQ and PmrA/PmrB (88), which respond to cation (calcium, iron, and magnesium) concentrations and pH variations. These systems are involved in the LPS modifications leading to polymyxin resistance.

There is a high variability of cation concentrations in MH medium depending on the commercial brand, and calcium and magnesium concentrations measured for each brand tested are far below the recommendations of the CLSI (89). This is why the CLSI recommends cation-adjusted MH or supplementation of the culture medium with cations (72, 73).

Iso-Sensitest agar is a well-defined medium with stabilized mineral content that was developed to overcome problems associated with traditional media used for antimicrobial susceptibility tests. Comparison of the agar dilution and Etest methods on MH and Iso-Sensitest agar (76) revealed a lack of detection of the resistant subpopulation of heteroresistant *E. cloacae* isolates for the Etest performed on MH agar, while Iso-Sensitest agar was more sensitive for detecting the resistant subpopulation with both methods (76).

However, a cation-dependent inhibition of antimicrobial activity has been reported for polymyxin antibiotics (90, 91). In fact, it is suspected that the colistin antimicrobial activity might be overestimated if tested using conventional cation-adjusted MH as recommended by the CLSI. Note that the calcium concentration recommended by the CLSI for determining colistin susceptibility *in vitro* is 2-fold higher than the concentration found in human interstitial space fluid *in vivo* (92). A recent study revealed that the MIC of colistin might be misestimated if tested with conventional cation-adjusted growth media (overestimation for *P. aeruginosa* and *A. baumannii* and underestimation for *E. coli*) (92). The use of cation-adjusted or non-cation-adjusted medium therefore remains questionable, and a consensus is still needed.

**Impact of powder composition.** MIC testing is performed using commercially available polymyxin B and colistin sulfate powders. The variability in the relative proportions of the mixture components between powder batches and manufacturers is a potential source of variability of the results (93, 94). In parallel, MICs obtained using BMD with purified forms of the major compounds of polymyxin B were within a  $\log_2$  dilution of the MICs obtained using the U.S. Pharmacopoeia polymyxin B sulfate powder mixture (95). These data suggest that the powder composition may not have an impact on polymyxin susceptibility testing. Note that CMS, as a prodrug, cannot be used for susceptibility testing, as it yields erroneously high MIC values (96).

**Impact of the composition and treatment of plates.** Due to their cationic properties, polymyxins adhere to the negative charges of the microtiter trays commonly used for BMD. Karvanen et al. (97) measured the colistin concentrations following incubation

in polypropylene, polystyrene, and glass tubes. The adsorption was proportionally higher at lower concentrations of the drug. Consequently, the results of colistin BMD measurements significantly differ if tests are conducted in microtiter plates with different coated wells (98). The amount of colistin adsorbed to the plate surface therefore depends on various factors, such as the coating applied to the plate, and is not consistent from well to well (K. Sei, presented at the January 2012 Meeting of the CLSI Subcommittee on Antimicrobial Susceptibility Testing, Tempe, AZ, 22 to 24 January 2012). Since the nature and treatment of plastics are not addressed in the CLSI recommendations, significant variability is observed between laboratories performing the reference BMD method.

**Presence or absence of P-80.** Polysorbate 80 (P-80 or Tween 80) is a surfactant used for the preparation of BMD panels used for susceptibility testing (99). This surfactant has been recommended by the CLSI to prevent or at least mitigate binding of lipoglycopeptides to plastics (72, 73). The presence of 0.002% P-80 mitigates colistin adsorption to polystyrene microplates (Sei, presented at the January 2012 Meeting of the CLSI Subcommittee on Antimicrobial Susceptibility Testing). When P-80 is added to a final concentration of 0.002% in the well, the polymyxin MICs are 4- to 8-fold lower than those obtained without P-80 among isolates with low MICs by BMD testing (70, 99).

It is noteworthy that the effect of P-80 on bacterial viability has not been well evaluated. Also, another concern is that P-80 may act synergistically with polymyxins, consequently giving artificially lower MICs (100). Also, in *P. aeruginosa*, P-80 increases cell permeability and lyses spheroblasts (101). On the other hand, polymyxins destabilize the outer membrane, allowing P-80 to access the inner membrane and induce cell lysis. Therefore, isolates resistant to polymyxins would not be affected significantly by P-80. Therefore, only isolates with polymyxin MICs of  $\leq 1 \mu\text{g/ml}$  might be affected (94).

In January 2014, the CLSI subcommittee decided to pursue a recommendation of polymyxin BMD testing without P-80. However, if a susceptibility breakpoint of  $\leq 1 \mu\text{g/ml}$  is chosen, the ability to detect susceptible isolates without using P-80 may be compromised (94). Since the use of P-80 is still questionable, a solution might be to determine MICs in glass plates, as colistin fixation on glass is less extensive (94). However, glass plates are fragile and expensive.

### Impacts of Subcultures and Storage on MICs

**Impact of subcultures.** A study by Li et al. (26) revealed a loss of colistin resistance when resistant isolates were subcultured without selective pressure. For instance, about 98% of a colistin-resistant *A. baumannii* population lost the resistance phenotype after a single passage in a colistin-free medium.

**Impact of storage.** A loss of colistin resistance was also observed after 6 to 8 months of storage at  $-70^\circ\text{C}$  (70). Among 25 isolates that initially tested resistant by a dilution method, five (20%) tested susceptible by the same method after freezing. The availability of easy, rapid, and inexpensive techniques allowing screening of colistin resistance on fresh cultures in routine laboratories is consequently a real clinical need.

### Interpretive Criteria

There is a lack of consensus between the two organizations setting up breakpoints for polymyxins, namely, the CLSI in the United States (72) and EUCAST in Europe (71). The zone diameter and MIC interpretive criteria given by those two organizations for colistin and polymyxin B are shown in Table 1. However, recent data related to PK suggest that the current breakpoints might be too high (21).

### Quality Controls

Quality control organisms are required during susceptibility testing in order to ensure accuracy and standardization of the procedures. Quality control can be assessed using the *E. coli* ATCC 25922 (NCTC 12241; CIP 76.24) and *P. aeruginosa* ATCC 27853

**TABLE 1** Colistin and polymyxin B breakpoints according to CLSI and EUCAST in 2014

Criteria and bacterial group	Colistin						Polymyxin B					
	Disk content (µg)	Zone diam interpretative criteria (mm) <sup>a</sup>		MIC interpretative criteria (µg/ml) <sup>a</sup>			Disk content (IU)	Zone diam interpretative criteria (mm) <sup>a</sup>		MIC interpretative criteria (µg/ml) <sup>a</sup>		
		Sensitive (S)	Resistant (R)	S	Intermediate (I)	R		S	R	S	I	R
CLSI criteria												
<i>Enterobacteriaceae</i>		—	—	—	—	—	300	— <sup>b</sup>	— <sup>b</sup>	—	—	—
<i>Acinetobacter</i> spp.		—	—	≤2	—	≥4	—	— <sup>b</sup>	— <sup>b</sup>	≤2	—	≥4
<i>Pseudomonas</i> spp.	10	≥11	≤10	≤2	4	≥8	300	≥12	≤11	≤2	4	≥8
EUCAST criteria												
<i>Enterobacteriaceae</i>	50	≥18 <sup>b,c</sup>	<15 <sup>b,c</sup>	≤2	—	>2	—	— <sup>b</sup>	— <sup>b</sup>	-	—	-
<i>Acinetobacter</i> spp.		— <sup>c</sup>	— <sup>c</sup>	≤2	—	>2	—	— <sup>b</sup>	— <sup>b</sup>	-	—	-
<i>Pseudomonas</i> spp.		— <sup>c</sup>	— <sup>c</sup>	≤4	—	>4	—	— <sup>b</sup>	— <sup>b</sup>	—	—	—

<sup>a</sup>—, not determined or absent.

<sup>b</sup>Zone diameter interpretative criteria for *Enterobacteriaceae* given by EUCAST are only for veterinary medicine; MIC determination is required for diameters ranging from 15 to 18 mm.

<sup>c</sup>No zone diameter interpretative criteria for human medicine; the MIC must be determined before use.

(NCTC 12903; CIP 76110) reference strains. The disk diffusion and MIC quality control ranges for these strains determined by the CLSI are shown in Table 2 (72).

**Correlation between MICs of Colistin and Polymyxin B**

Despite the high similarity of the molecular structures of colistin and polymyxin B, a recent study including 15,377 Gram-negative bacteria revealed differences between the MICs of colistin and polymyxin B (102). MIC values determined by the Sensititre system were 2-fold higher for polymyxin B than for colistin for 55% and 53% of *Klebsiella* species isolates (*n* = 4,177) and *E. coli* isolates (*n* = 6,311), respectively. However, a categorical agreement of >99% was obtained for enterobacterial strains when breakpoints of ≤2/≥4 for both colistin and polymyxin B were applied. That study showed a high level of agreement between MICs of colistin and polymyxin B for *P. aeruginosa* and *Acinetobacter* spp.

**Qualitative Detection Techniques**

**Rapid detection of heterogeneous populations among colistin-resistant Gram-negative bacteria by use of capillary electrophoresis.** Sautrey et al. (103) proposed a capillary electrophoresis method for rapid detection of heterogeneous populations of colistin-resistant strains. However, further development is required for such applications to be used in clinical laboratories on a daily basis.

**Rapid detection of colistin-resistant *A. baumannii* isolates by use of the Micromax assay.** The Micromax assay is based on the detection of released nucleotides, indicating cell wall damage, in the presence of colistin (104). After incubation with 0.5 µg/ml of colistin, strains are considered resistant to colistin if ≤11% of bacteria present cell wall damage. Bacteria are incubated for 90 min in Mueller-Hinton broth to achieve exponential growth and then incubated for 60 min with colistin at concentrations of 0 and 0.5 µg/ml, respectively. Bacteria embedded in agarose are incubated with a lysis solution removing only weakened cell walls. The released fragmented DNA may be stained with the fluorochrome SYBR gold (Molecular Probes, Eugene, OR) and visualized by fluorescence microscopy (45 min to 60 min of technical processing and scoring under the microscope). This method is faster than the routine automatic microdilution

**TABLE 2** Zone diameter and MIC quality control ranges for polymyxins according to CLSI guidelines

Strain	Zone diam (mm) range		MIC (µg/ml) range	
	Colistin	Polymyxin B	Colistin	Polymyxin B
<i>E. coli</i> ATCC 25922	11–17	13–19	0.25–2	0.25–2
<i>P. aeruginosa</i> ATCC 27853	11–17	14–18	0.5–4	0.5–2

procedure (3 h 30 min versus 6 to 8 h) and is accurate for detecting colistin resistance in *A. baumannii* (100% sensitivity and 96% specificity). Another advantage is that it can be automated. However, the manual task and the cost of the materials (fluorochrome and epifluorescence microscope) are disadvantages for its routine use.

**Rapid detection of colistin-resistant *Enterobacteriaceae* isolates by use of the Rapid Polymyxin NP test.** We developed the Rapid Polymyxin NP test, which is based on the detection of bacterial growth in the presence of a defined polymyxin concentration (105). Detection is based on detection of glucose metabolism upon bacterial growth. Glucose metabolism induces the formation of acid, leading to a color change of the red phenol used as a pH indicator. The test is performed with a final concentration of bacteria of ca.  $10^8$  CFU/ml in each well (or tube), and the final concentration of polymyxin is 3.75  $\mu$ g/ml. Visual inspection of the tray is made after 10 min and then every hour for 2 h. The test is considered positive (indicating polymyxin resistance) if the isolate grows in the presence of colistin (color change from orange to yellow), while it is considered negative (indicating polymyxin susceptibility) if the isolate does not grow in the presence of polymyxin (no color change). This test is rapid (less than 2 h) and easy to perform.

By testing a total of 200 enterobacterial isolates exhibiting either resistance (intrinsic or acquired, or various) or susceptibility to polymyxins, the specificity and sensitivity of this test were evaluated at 99.3% and 95.4%, respectively, compared to BMD as the reference method (105). Note that the Rapid Polymyxin NP test identified the isolates exhibiting a heteroresistance phenotype as well as those producing the plasmid-mediated MCR-1 determinant (see below).

For the Rapid Polymyxin NP test, the adequate culture media for culturing the bacteria prior to the test were Mueller-Hinton agar, Luria-Bertani agar, Columbia agar plus 5% sheep blood, chocolate agar, UriSelect 4 agar, and eosin methylene blue agar.

The Rapid Polymyxin NP test may also detect colistin-resistant *Enterobacteriaceae* directly from blood cultures (106). Results are obtained within 4 h.

**Selective medium.** So far, no selective medium allowing screening for any type of polymyxin-resistant Gram-negative isolates (with intrinsic, chromosomally encoded, or plasmid-mediated polymyxin resistance) has been available. Neither commercial nor in-house screening culture media had been designed that might permit screening of patients possibly colonized by polymyxin-resistant isolates. Therefore, we developed SuperPolymyxin, a selective culture medium for detection of any type of polymyxin-resistant Gram-negative organism (107). The SuperPolymyxin medium prevents swarming of *Proteus* spp. (intrinsically resistant to polymyxins) and also the growth of Gram-positive bacteria and fungi, by addition of daptomycin and amphotericin B, respectively. Its base corresponds to the eosin methylene blue medium (Levine's medium) (108) selective for Gram-negative bacteria, which differentiates lactose fermenters (black colonies) from nonfermenters (colorless or light lavender colonies). In addition, differentiation of lactose fermenters is also possible to some extent. The SuperPolymyxin medium contains a colistin concentration (3.5  $\mu$ g/ml) that allows clear categorization between polymyxin-resistant and -susceptible isolates. The sensitivity and specificity of this medium have been found to be 100% (107).

### Genotypic Methods

Although the mechanisms underlying resistance to polymyxins have not all been elucidated, acquisition of colistin resistance in Gram-negative bacteria has been attributed to lipopolysaccharide (LPS) modifications via diverse routes, including (i) the addition of cationic groups to the LPS reducing the overall negative charge of the LPS and consequently preventing the fixation of polymyxins; (ii) loss of the LPS and, consequently, loss of the polymyxin target; (iii) the overproduction of capsule polysaccharide (CPS) hiding polymyxin binding sites; and (iv) the release of CPS trapping polymyxins. Specific modifications of outer membrane porins and overexpression of efflux pump systems have also been described (88).

Several molecular mechanisms have been associated with colistin resistance in Gram-negative bacteria, such as alterations in the PmrA/PmrB, PhoP/PhoQ, ParR/ParS, ColR/ColS, and CprR/CprS two-component systems and alterations in the *mgrB* gene, which encodes a negative regulator of PhoPQ. Mutations leading to the addition of cationic groups on lipid A result in a less anionic lipid A and, consequently, to less fixation of polymyxins (88).

Similarly, alterations in the *lpxA*, *lpxC*, and *lpxD* genes of *A. baumannii* result in inactivation of lipid A biosynthesis, leading to a complete loss of LPS and, consequently, to a loss of the polymyxin target (109).

The mechanisms of polymyxin resistance can be identified by sequencing those specific genes. However, molecular techniques cannot be envisioned in the near future considering that (i) many chromosomally encoded mechanisms of resistance remain to be identified, (ii) it is difficult to extrapolate whether some substitutions identified in proteins known to be involved in LPS biosynthesis lead to resistance, and (iii) the levels of expression of the corresponding genes may vary and consequently influence the level of resistance to polymyxins.

There is an exception that corresponds to the recent identification of the plasmid-borne *mcr-1/mcr-2* genes, whose products confer resistance to polymyxins (see below). According to the current knowledge on the topic, identification of these genes may be considered a signature of resistance or reduced susceptibility to polymyxins. This is why identifying the gene makes sense in this case, since qualitative genetic results may be translated directly into a nonsusceptibility phenotype. Screening of both *mcr-1* and *mcr-2* can be performed by using a standard PCR protocol using the primers MCR-1/2-Fw (5'-TAT CGC TAT GTG CTA AAG CC-3') and MCR-1/2-Rv (5'-TCT TGG TAT TTG GCG GTA TC-3'), giving rise to a 715-bp amplicon. Also, a SYBR green-based real-time PCR assay that provides a simple, specific, sensitive, and rapid molecular tool for detection of *mcr-1*-positive isolates was recently published (110). That technique was validated on human and animal isolates and may be applied to extensive surveillance studies.

Porin mutations and overexpression of efflux pump systems may also be involved in colistin resistance (88), and it is very likely that phenotypic resistance to polymyxins in clinical isolates often results from combined resistance mechanisms (e.g., defects in outer membrane proteins combined with structural modification of the LPS). Phenotypic methods, such as the Rapid Polymyxin NP test, are consequently very relevant for determining the subsequent therapeutic decision, since they actually concretely determine the susceptibility or lack thereof of isolates, in contrast to genotypic methods, which detect only potential resistance and require sequencing of multiple genes.

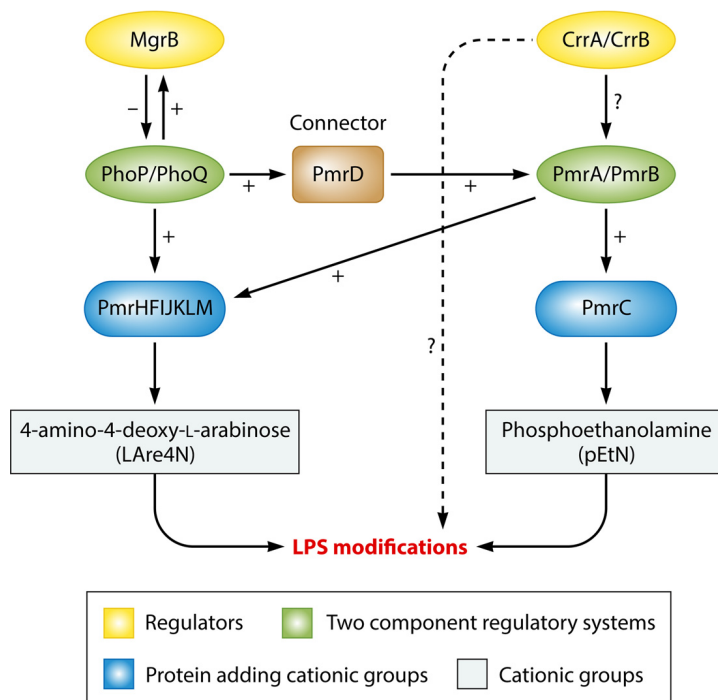
## RESISTANCE MECHANISMS IN ENTEROBACTERIACEAE

### Intrinsic Resistance Mechanisms in *Proteus mirabilis* and *Serratia marcescens*

In *P. mirabilis* and *S. marcescens*, naturally occurring resistance to polymyxins is linked to the constitutive expression of the *arnBCADTEF* operon and/or the *eptB* gene, causing addition of phosphoethanolamine (pEtN) and/or 4-amino-4-deoxy-L-arabinose (L-Ara4N) cationic groups to the LPS. This modification increases the charge of the LPS, which is the initial target of the polymyxins, and therefore decreases polymyxin binding, giving rise to intrinsic resistance of these species (111–113).

### Mechanisms Responsible for Acquired Resistance in *Enterobacteriaceae*

Acquired resistance to polymyxins has been identified in several genera of the *Enterobacteriaceae*, such as *Klebsiella*, *Escherichia*, *Enterobacter*, and *Salmonella*. Colistin resistance mechanisms remain unknown for some bacterial species, but several molecular mechanisms have been identified. The most common is modification of the LPS via cationic substitution, similar to that observed in bacteria with intrinsic resistance to polymyxins. A single transferable mechanism of resistance has been identified so far (see below), with most of the resistance mechanisms being encoded chromosomally.



**FIG 3** Regulation pathways of LPS modifications in *Klebsiella pneumoniae*.

Similar to what is observed in strains that are naturally resistant to colistin, addition of cationic groups (L-Ara4N and pEtN) to the LPS is responsible for acquisition of colistin resistance in *Enterobacteriaceae*. A large panel of genes and operons are involved in qualitative modification of the LPS (Fig. 3), including genes and operons coding for enzymes that are directly involved in LPS modifications (genes responsible for synthesis of cationic groups and/or their addition to the LPS), i.e., the *pmrC* gene, the *pmrE* gene, and the *pmrHFUJKLM* operon; regulatory genes, such as those encoding proteins involved in the PmrAB and PhoPQ two-component systems; and the regulators of these two-component systems, i.e., the *mgrB* gene, which negatively regulates the PhoPQ system, and the newly described *crrAB* two-component regulatory system, which regulates the PmrAB system.

**Genes encoding LPS-modifying enzymes.** (i) **The *pmrC* gene.** The *pmrCAB* operon codes for three proteins, namely, the phosphoethanolamine (pEtN) phosphotransferase PmrC, the response regulator PmrA (also called BasR), and the sensor kinase protein PmrB (also called BasS) (114). The phosphoethanolamine phosphotransferase PmrC adds a pEtN group to the LPS (Fig. 3) (114).

(ii) **The *pmrHFUJKLM* operon and the *pmrE* gene.** The *pmrHFUJKLM* operon (also called the *arnBCADTEF* or *pbgPE* operon) codes for a total of seven proteins (115). The *pmrE* gene and the *pmrHFUJKLM* operon are responsible for the synthesis of the L-aminoarabinose group (L-Ara4N) and its fixation to lipid A (Fig. 3) (115).

(iii) **The *pmrA* and *pmrB* genes, which encode the PmrAB two-component system.** Environmental stimuli, such as macrophage phagosomes, ferric (Fe<sup>3+</sup>) iron, aluminum (Al<sup>3+</sup>), and low pH (e.g., pH 5.5), mediate activation of PmrB through its periplasmic domain (114). The PmrAB and PhoPQ two-component systems are normally activated when bacteria are phagocytized into macrophages, allowing bacterial survival (114).

PmrB is a protein with tyrosine kinase activity that activates PmrA by phosphorylation. PmrA in turn activates the transcription of the *pmrCAB* operon, the *pmrHFUJKLM* operon, and the *pmrE* gene involved in LPS modification (pEtN and L-Ara4N addition) (Fig. 3) (114).

Specific mutations within the *pmrA* and *pmrB* genes have been described as being responsible for acquired colistin resistance in *K. pneumoniae* (105, 116–120), *Enterobac-*



*ter aerogenes* (121), and *Salmonella enterica* (122, 123) (Table 3). These mutations are responsible for constitutive activation of the PmrAB two-component system, leading to upregulation of the *pmrCAB* operon, the *pmrHFJKLM* operon, and the *pmrE* gene, and thus to the synthesis of pEtN and L-Ara4N and their transfer to lipid A (Fig. 3).

Some polymorphism in the *pmrAB* genes of colistin-resistant *E. coli* has been reported (124, 125), but the involvement of these mutations in the colistin resistance phenotype has not formally been demonstrated, since no complementation or site-directed mutagenesis has been performed.

**(iv) The *phoP* and *phoQ* genes, which encode the PhoPQ two-component system.**

The *phoPQ* operon codes for two proteins, namely, the regulator protein PhoP and the sensor protein kinase PhoQ. Environmental stimuli, such as macrophage phagosomes, low magnesium ( $Mg^{2+}$ ), and low pH (e.g., pH 5.5), mediate activation of PhoQ through its periplasmic domain (114). The PhoPQ two-component system allows the expression of genes that code for magnesium transport, enzymes that modify the LPS to allow resistance to cationic antimicrobial peptides, and enzymes that decrease the cell stress caused by acidic pH or some virulence factors (126, 127). The PhoPQ two-component system therefore allows bacterial survival under conditions of low magnesium or acidic pH or in the presence of cationic antimicrobial peptides.

PhoQ is a protein with tyrosine kinase activity that activates PhoP by phosphorylation. PhoP in turn activates the transcription of the *pmrHFJKLM* operon, involved in the addition of L-Ara4N to the LPS (Fig. 3) (126, 127). PhoP can also activate the PmrA protein, either directly or indirectly via the PmrD connector protein, causing the addition of pEtN to the LPS.

Several mutations in the *phoP* and *phoQ* genes are responsible for acquired resistance to polymyxins in *K. pneumoniae* (81, 105, 117, 118, 120, 128) (Table 3). One mutation potentially involved in colistin resistance in *E. coli* has also been described (65). These mutations are responsible for constitutive activation of the PhoPQ two-component system, leading to upregulation of the *pmrHFJKLM* operon and thus to the synthesis of L-Ara4N and its transfer to lipid A (Fig. 3).

### Regulators of the PmrAB and PhoPQ Two-Component Systems

**The *mgrB* gene.** MgrB (also called YobG) is a small transmembrane protein of 47 amino acids (129). Upon activation of PhoP, the *mgrB* gene is upregulated. The MgrB protein in turn represses the expression of the PhoQ-encoding gene, leading to negative regulation of the PhoPQ two-component system (Fig. 3) (129). Inactivation of the *mgrB* gene (the negative regulator of the PhoPQ two-component system) leads to overexpression of the *phoPQ* operon, thus causing *pmrHFJKLM* operon activation, leading to the production of L-Ara4N responsible for the acquisition of colistin resistance.

Several missense mutations resulting in amino acid substitutions and nonsense mutations and therefore leading to a truncated MgrB protein may be responsible for acquired resistance to colistin in *K. pneumoniae* (Table 3). Other alterations, such as insertions or deletions of small nucleotide sequences in the *mgrB* gene, or even some complete deletions of the *mgrB* locus, have been reported (120, 130, 131). Insertional inactivation caused by diverse insertion sequences (IS), belonging to several families and inserted at different locations within the *mgrB* gene, is often responsible for acquired resistance to colistin in *K. pneumoniae* (105, 117, 120, 130–132) and *Klebsiella oxytoca* (133, 134). Recently, the transposition of genes encoding extended-spectrum  $\beta$ -lactamases (ESBLs) or carbapenemases, leading to disruption of the chromosomal *mgrB* gene, was reported as a source of resistance to colistin (135, 136). Notably, selective pressure with  $\beta$ -lactams leading to the acquisition of  $\beta$ -lactamase genes may therefore be responsible for coselection of colistin resistance. Despite the high homology observed among *mgrB* gene sequences of *Enterobacteriaceae* organisms (129), disruption of this gene has so far not been found to be responsible for acquired resistance to colistin in genera other than *Klebsiella*.

**The *crrAB* operon.** The *crrAB* (colistin resistance regulation) operon codes for two proteins, namely, the regulatory protein CrrA and the sensor protein kinase CrrB. The

**TABLE 3** Chromosomal mutations and amino acid deletions responsible for acquired colistin resistance in *Klebsiella pneumoniae*, *Enterobacter aerogenes*, *Escherichia coli*, *Salmonella enterica*, *P. aeruginosa*, and *A. baumannii* isolates

Bacterial group and species	Protein (normal length [aa])	Domain involved (residues) <sup>a,b</sup>	Amino acid change <sup>d</sup>	Reference(s)
<i>Enterobacteriaceae</i>				
<i>K. pneumoniae</i>	PmrA (223)	REC (1–112)	S42N	120
			G53C	105, 120
			G53S	105
	PmrB (365)	Trans_reg_C (145–216) TM (13–35) HAMP (90–142) HisKA (143–203)	ΔR14	118
			L17Q	105
			L82R	116
			S85R	120
			T140P	120
			T157P	117–119
			S208N	118
			ΔY209	118
			R256G	117
			PhoP (223)	HATPase_c (250–358) REC (1–112)
	L26Q	120		
	S86L	117		
	PhoQ (488)	Trans_reg_C (145–220) PhoQ sensor (10–189) HAMP (195–263) HisKA (267–330)	D191Y	81
			R16C	105
			L26P	117
			L96P	120
			D150G	117
			S174N	118
			V258F	117
			L348Q	120
			G385S	120
			MgrB (47)	HATPase_c (375–482)
	K3*	105		
	L9*	120		
	I13*	120		
	A14S	120		
	W20R	105		
	L24H	130		
	V26*	120		
	M27K	105		
	C28F	120		
	C28Y	117, 120, 128, 130		
	C28*	105, 120		
	Q30*	105, 120		
	D31N	120		
	Q33*	105		
	F35I	120		
	G37S	130		
	C39Y	105		
N42Y/K43I	105			
I45T	105			
CrrB (353)	TM (12–34) HAMP (81–135) HisKA (136–200)	W47R	105	
		W47*	105	
		*48Y	117	
		Q10L	128, 137	
		Y31H	137	
		L94 M	128	
		W140R	137	
		N141I	137	
		P151S	137	
		S195N	137	
<i>E. aerogenes</i>	PmrA	REC (1–112) Trans_reg_C (145–216)	G53S	121
			R81S <sup>c</sup>	125
<i>E. coli</i>	PmrB (363)	TM1 (15–37) TM2 (69–91)	Δ7–12 <sup>c</sup>	124

(Continued on next page)



**TABLE 3** (Continued)

Bacterial group and species	Protein (normal length [aa])	Domain involved (residues) <sup>a,b</sup>	Amino acid change <sup>d</sup>	Reference(s)		
<i>S. enterica</i>		HAMP (92–144)				
		HisKA (145–205)	T156K <sup>c</sup>	124		
			A159V <sup>c</sup>	124		
				V161G <sup>c</sup>	125	
	PhoP (223)		HATPase_c (252–360)			
			PhoQ_sensor (10–189)			
			HAMP (195–263)			
	PhoQ (486)		HisKA (267–330)			
			REC (1–112)			
			Trans_reg_C (145–220)			
	PmrA (222)		PhoQ_sensor (10–189)			
			HAMP (195–263)			
			HisKA (267–330)			
	PmrB (356)		HATPase_c (374–480)	E375K <sup>c</sup>	65	
			REC (1–112)	G15R <sup>c</sup>	123	
				G53E <sup>c</sup>	123	
			G53R <sup>c</sup>	123		
			R81C <sup>c</sup>	123		
			R81H <sup>c</sup>	123		
			Trans_reg_C (145–216)			
			TM (13–35)	Δ11–14 <sup>c</sup>	122	
				L14F <sup>c</sup>	123	
				L14S <sup>c</sup>	123	
				M15L <sup>c</sup>	122	
				L22P <sup>c</sup>	123	
				S29R <sup>c</sup>	123	
			HAMP (89–141)		T92A <sup>c</sup>	123
					P94Q <sup>c</sup>	123
					E121A <sup>c</sup>	123
					S124P <sup>c</sup>	123
HisKA (142–202)				N130Y <sup>c</sup>	123	
				T147P <sup>c</sup>	123	
				R155P <sup>c</sup>	123	
				T156M <sup>c</sup>	123	
				T156P <sup>c</sup>	123	
				V161G <sup>c</sup>	123	
				V161L <sup>c</sup>	123	
				V161M <sup>c</sup>	123	
				E166K <sup>c</sup>	123	
		M186I <sup>c</sup>	123			
	HATPase_c (249–356)		G206R <sup>c</sup>	123		
			G206W <sup>c</sup>	123		
		S305R <sup>c</sup>	123			
Nonfermentative bacilli	<i>P. aeruginosa</i>	REC (1–112)				
		Trans_reg_C (145–216)	L157Q	166		
	PmrB (477)		L14P	167		
		TM1 (15–37)				
		PD (38–160)	ΔD45	74, 167		
			A54V	167		
		TM2 (161–183)	L167P	166		
		HAMP (186–238)	G188D	167		
			F237L	118		
		HisKA (239–304)	L243Q	167		
			A247T	168		
			A248V	167		
			S257N	167		
			M292I	167		
			M292T	169		
	PhoQ (448)	HATPase_c (348–459)				
		TM1 (7–29)	R6C	170		

(Continued on next page)

**TABLE 3** (Continued)

Bacterial group and species	Protein (normal length [aa])	Domain involved (residues) <sup>a,b</sup>	Amino acid change <sup>d</sup>	Reference(s)			
<i>A. baumannii</i>	ParR (235)	PD (30–166)	ΔV57–Q332	170			
			N104I	118			
			K123Q	166			
			K123E	118			
			Q133E	118			
			A143V	166			
			V152*	168			
			TM2 (167–189)	V184G	118		
				A207R	118		
				R214H	118		
				H223R	168		
				V260G	163, 247		
	HisKA (238–300)	163, 247					
	ParS (428)	HATPase_c (343–448)	ΔL364–G365	170			
			I421*	170			
			Fr at I421	170			
			D433*	170			
			R444C	170			
			REC (7–117)	L18I	118		
				N24S	118		
				S24N	118		
				M59I	171		
			ColS	Trans_reg_C (152–228)	E156K	171	
	L14Q	171					
	PD (28–131)	V101 M			171		
		L137P			171		
	TM2 (132–154)	HAMP (155–207)			118		
		HisKA (208–273)			118		
	HATPase_c (318–428)	G361R			118		
		H398R			247		
	CprS	PmrA (224)	A106V	172			
			R241C	172			
			REC (2–112)	E8D	177, 180		
				M12I	174		
				P102H	173		
				S119T	174		
			PmrB (444)	Trans_reg_C (150–221)	TM1 (10–29)	T13N	173
						S14L	175
						S17R	177
					PD (30–141)	Fr at F26	176
	ΔA32–E35	174					
	D64V	174					
A80V	174						
L87F	175						
Y116H	177						
TM2 (142–164)	I121F	178					
	M145K	175					
	ΔL160	174					
	P170L	174, 179					
	P170Q	174					
	A183T	178					
	A184V	178					
	P190S	178					
	T192I	178					
HisKA (218–280)	L208F	174					
	A226V	174					
	A227V	173, 175, 176					
	Q228P	178					
	R231L	174					
	T232I	177					
	P233S	118, 173–176, 179					
	P233T	173					
	T235I	174					
	N256I	174					

(Continued on next page)

TABLE 3 (Continued)

Bacterial group and species	Protein (normal length [aa])	Domain involved (residues) <sup>a,b</sup>	Amino acid change <sup>d</sup>	Reference(s)
			A262P	173
			R263C	174
			R263L	177
			R263P	174
			Q277H	174
			G315D	174
		HATPase_c (326–437)	N353Y	175
			P377L	174
			F387Y	175
			S403F	175
	LpxA (262)		Fr at I25	109
			G68D	109
			Q72K	109
			Fr at H121	109
			Fr at D130	109
			H159D	109
			Q234*	109
	LpxC (276)		P30L	109
			Fr at D45	109
			Fr at T285	109
	LpxD (356)		Fr at K317	109

<sup>a</sup>Domains predicted in SMART by using protein sequences of *Escherichia coli* K-12 substrain MG1655, *Klebsiella pneumoniae* subsp. *pneumoniae* MGH 78578, *Salmonella enterica* serovar Typhimurium LT2, *P. aeruginosa* PAO1, and *A. baumannii* AB0057. REC, CheY-homologous receiver domain; Trans\_reg\_C, transcriptional regulatory protein, C-terminal domain; TM, transmembrane domain; TM1, first transmembrane domain; TM2, second transmembrane domain; PD, periplasmic domain; HAMP, histidine kinases, adenyl cyclases, methyl-binding proteins, and phosphatases; HisKA, histidine kinase A (phosphoacceptor) domain; HATPase\_c, histidine kinase-like ATPases; PhoQ sensor, phosphorelay signal transduction system.

<sup>b</sup>A periplasmic domain (PD) was not predicted in SMART but was assumed to be between TM1 and TM2.

<sup>c</sup>The involvement of the mutation in the colistin resistance profile was determined by *in silico* analysis.

<sup>d</sup>Δ, deletion; Fr, frameshift; \*, stop codon.

physiological role of the *crrAB* operon is still unknown. However, inactivation of the *crrB* gene leads to overexpression of the *pmrAB* operon, thus causing activation of the *pmrHFIJKLM* operon and of the *pmrC* and *pmrE* genes, consequently leading to the production of L-Ara4N and pEtN, both of which are responsible for the acquisition of colistin resistance (Fig. 3) (128). CrrB inactivation may also modify lipid A through activation of a glycosyltransferase-like protein (128).

Six amino acid substitutions in the CrrB protein have been identified as being responsible for acquired resistance to polymyxins in *K. pneumoniae* (Table 3) (128, 137).

### The Intrinsic Regulator RamA

The intrinsic regulator RamA of *K. pneumoniae* is known to play a significant role in the overall response to antimicrobials. It regulates genes that are linked to permeability barriers and therefore may be involved in reduced susceptibility to antibiotics. It was recently shown that increased levels of this regulator caused LPS alterations and consequently reduced susceptibility to polymyxins (138).

### Plasmid-Mediated Resistance to Polymyxins

The plasmid-mediated *mcr-1* gene, responsible for horizontal transfer of colistin resistance, was described first for *E. coli* and *K. pneumoniae* isolates recovered in China between 2011 and 2014 (139). The encoded MCR-1 protein is a member of the phosphoethanolamine transferase enzyme family, as its acquisition results in the addition of phosphoethanolamine to lipid A, and consequently in a more cationic LPS, similarly to the chromosomal mutations mentioned above.

Overall, production of MCR-1 in *E. coli* leads to 4- to 8-fold increases of the MICs of polymyxins. Therefore, without additional resistance mechanisms, production of MCR-1 is enough to confer resistance to colistin in *E. coli* and other enterobacterial species, such as

*K. pneumoniae* (our unpublished data). Note that despite the fact that polymyxins actually share the same mechanism of action as that of the cationic antimicrobial peptides (CAMPs) cathelicidin LL-37,  $\alpha$ -defensin 5 (HD5), and  $\beta$ -defensin 3 (HDB3), which are normal components of the immune system, coresistance to CAMPs and polymyxins has not been observed (J. Dobias, L. Poirel, and P. Nordmann, submitted for publication).

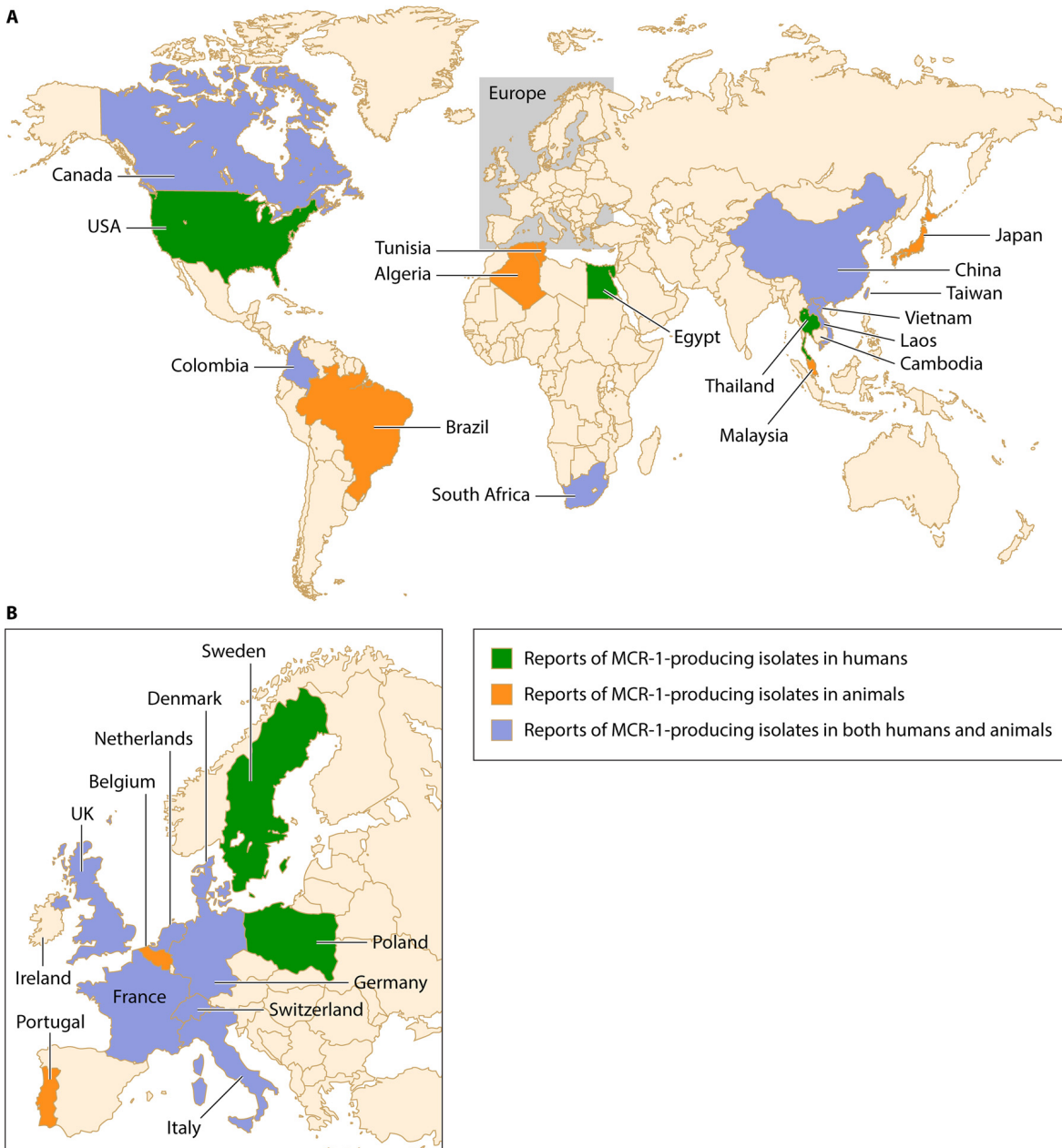
Apart from resistance to polymyxin antibiotics, production of MCR-1 was shown to confer resistance to lysozyme (140). The structure of MCR-1 was recently solved at a 1.32-Å resolution, revealing that its active site is similar to that of related phosphoethanolamine transferases (141). Threonine 285 was identified as the putative nucleophile for catalysis, as it was phosphorylated in the catalytic domain of MCR-1 (cMCR-1). Four zinc ions were identified in the active site of cMCR-1, which is thus a metalloenzyme. The binding sites for the lipid A and phosphatidylethanolamine substrates were not apparent in the cMCR-1 structure, likely indicating that they were present in the membrane domain.

Following these initial findings, the *mcr-1* gene was reported worldwide and beyond China, on all continents (Fig. 4; Table 4). The earliest *mcr-1*-positive strain was collected from chickens in China 3 decades ago (142), when colistin first started to be used in food-producing animals. The *mcr-1* gene has been found in various genera of the *Enterobacteriaceae* (*Escherichia*, *Klebsiella*, *Enterobacter*, *Cronobacter*, *Salmonella*, *Shigella*, and *Kluyvera*) isolated from the environment, vegetable and meat foods, animals, and human beings (Fig. 4; Table 4). Note that the occurrence of MCR-1-producing *E. coli* in the environment in Switzerland but also in Asian imported vegetables in the same country highlights the likely wide occurrence of that resistance trait in many different environments (143).

The hypothesis that animals, particularly pigs and cattle, might be a main source of MCR-1 producers is very strong. Indeed, several features are in accordance with such a hypothesis, including the high selective pressure in veterinary practice and the wide occurrence of that resistance trait in isolates recovered from animals (144).

The genetics of acquisition of the *mcr-1* gene has been investigated extensively. This gene was found in plasmids possessing various backbones (IncI2, IncHI2, IncP, IncX4, IncFI, and IncFIB) and of various sizes (58 to 251 kb). Upstream of the *mcr-1* gene, the IS*ApI1* insertion sequence element is inconstantly identified (Table 4) (139). Thanh et al. (145) described an *mcr-1* gene disrupted by a 22-bp duplication in a *Shigella sonnei* isolate. This isolate was colistin susceptible, but under selective pressure with colistin, one copy of the 22-bp tandem repeat could be deleted, restoring the open reading frame of *mcr-1* and leading to colistin resistance. This deactivated version of the colistin resistance gene *mcr-1* suggests a fitness cost for the active *mcr-1* gene. Some but not all plasmids bearing the *mcr-1* gene carry other antimicrobial resistance genes encoding resistance to clinically relevant antibiotics for human medicine, such as  $\beta$ -lactams, aminoglycosides, quinolones, fosfomycin, sulfonamides, and tetracyclines. The location of the *mcr-1* gene on multidrug resistance plasmids is worrying because the use of antimicrobials other than polymyxins can participate in the coselection of isolates carrying *mcr-1* and in their spread. More worryingly, the plasmid-mediated *mcr-1* gene has been identified in highly drug-resistant *Enterobacteriaceae* isolates harboring plasmids encoding carbapenemase genes (*bla*<sub>NDM-1</sub>, *bla*<sub>NDM-5</sub>, *bla*<sub>NDM-9</sub>, *bla*<sub>OXA-48</sub>, *bla*<sub>KPC-2</sub>, and *bla*<sub>VIM-1</sub>) (146–152). Note that the *mcr-1* gene was recently identified on the chromosome of an *E. coli* strain in Switzerland, suggesting that this resistance gene might be integrated and therefore stabilized in the genome in some isolates (153).

Further investigations are required to better understand the process of acquisition of the *mcr-1* gene; however, we recently showed that it was located within a 2,600-bp genetic structure, defined as the “*mcr-1* cassette,” that might have been mobilized by transposition (154). The cassette was found to carry its own promoter sequences driving the expression of *mcr-1*. In addition, it was shown that several isolates may possess the *mcr-1* gene located in a composite transposon structure made of two copies of IS*ApI1* (155). However, that structure has not been identified systematically, and therefore further investigations are still required to better understand the process



**FIG 4** Reports of MCR-1-producing isolates in humans, animals, and both humans and animals.

of acquisition of that gene from an unknown progenitor in plasmids replicating in *Enterobacteriaceae*.

MCR-1-producing enterobacterial isolates have often been identified as colonizers in either humans or animals. Nevertheless, there are some reported cases of infections, including two patients with bacteremia in Switzerland (156).

A functional variant of *mcr-1* (Q3L) encoding MCR-1.2 was detected in KPC-3-producing *K. pneumoniae* in Italy (157), likely sharing the same activity as MCR-1.

In addition, the plasmid-mediated colistin resistance gene *mcr-2* was identified in *E. coli* strains recovered from piglets in Belgium (158). It shared 77% nucleotide identity with *mcr-1* and was carried on an IncX4 plasmid.

**Other Mechanisms Contributing to Polymyxin Resistance in *Enterobacteriaceae***

**Hyperproduction of CPS.** A study showed that the capsule polysaccharide (CPS) acts as a protective barrier against polymyxins in *K. pneumoniae* (159). The upregulation

**TABLE 4** Worldwide reports of *Enterobacteriaceae* isolates harboring a plasmid-mediated *mcr-1* gene

Species	Country of isolation	Country of origin or traveled region	Sample origin	Period (yr)	Plasmid features <sup>a</sup>				Reference(s)
					Inc type	Size (kb)	Mobile element	Other antimicrobial resistance(s)	
<i>E. coli</i>	China	China	Chickens	1980–2014	—	—	—	—	142
	China	China	Human (fecal carriage)	Before 2011	—	—	—	—	218, 219
	China	China	Chickens, pigs, and humans (infections)	2011–2014	Incl2	64	IS <i>Apl1</i>	No	139
	China	China	—	2011–2014	InclH2	251	IS <i>Apl1</i>	Resistance to cefotaxime ( <i>bla</i> <sub>CTX-M-14</sub> ), aminoglycosides, florfenicol, oleaquinolox, cotrimoxazole, fosfomycin ( <i>fosA3</i> ), and ciprofloxacin ( <i>qnrA</i> )	220
	China	China	Chicken meat	2014	Incl2	65	—	—	146
	China	China	Human (blood)	2014–2015	—	—	—	—	147
	China	China	Human (fecal carriage)	2015	—	—	—	—	221
	China	USA	Human (fecal carriage)	2016	InclF1	33	—	Resistance to β-lactams ( <i>bla</i> <sub>CTX-M-15</sub> and <i>bla</i> <sub>TEM-1</sub> )	148
	Laos	Laos	Human (fecal carriage) and pigs	2012	—	—	—	—	222
	Thailand	Thailand	Human (fecal carriage)	2012	—	—	—	—	223
	Vietnam	Vietnam	Pigs (fecal carriage)	2014–2015	—	—	IS <i>Apl1</i>	Resistance to cefotaxime ( <i>bla</i> <sub>CTX-M-55</sub> ), trimethoprim ( <i>dhfrA12</i> ), tetracycline ( <i>tetA</i> ), aminoglycosides [ <i>aadA3</i> , <i>aph(3')-I</i> ], phenicol ( <i>cmiA1</i> ), quinolones ( <i>qnrS1</i> , <i>qnrA</i> ), lincosamides [ <i>inu(P)</i> ], and sulfonamides ( <i>sul2</i> , <i>sul3</i> )	224
	Cambodia	Cambodia	Human (feces)	2012	—	—	IS <i>Apl1</i>	—	224
	Malaysia	Malaysia	Water, chickens, pigs	2013	—	—	IS <i>Apl1</i> or not	—	225
	Japan	Japan	Cattle (mastitis)	2008–2013	Incl2	60–61	—	—	226
	France	France	Veal calves (feces)	2005–2014	InclH2	—	—	Resistance to cefotaxime ( <i>bla</i> <sub>CTX-M-1</sub> ), sulfonamides, and tetracyclines	227
	France	France	Broiler, turkeys, pigs	2007–2014	—	—	—	—	228
	Italy	Italy	Human (urine, surgical wound)	2013–2015	—	—	—	—	229
	Italy	Italy	Human (rectal swabs)	2015	NT	35	—	—	230
	United Kingdom	United Kingdom or Egypt	Human (blood, stools)	2012–2015	InclH2, Incl2	—	IS <i>Apl1</i> or not	—	231
	Switzerland	—	River water	2012	—	—	—	—	143
	Switzerland	Thailand and Vietnam	Vegetables	2014	—	—	—	—	143
	Switzerland	Switzerland	Human (urinary tract infection)	2015	—	60	—	Resistance to chloramphenicol, florfenicol, and cotrimoxazole	149
	Switzerland	Switzerland	Human (blood)	2016	InclFIB	30 and 80	—	—	156
Belgium	Belgium	Calves and piglets (feces)	2011–2012	InclP	80	Truncated IS <i>Apl1</i>	Resistance to trimethoprim ( <i>dhfrA1</i> ), tetracycline ( <i>tetA</i> ), aminoglycosides [ <i>aadA1</i> , <i>aph(3)-lb/strB1</i> ], and sulfonamides ( <i>sulI</i> )	223	
Netherlands	Europe	Chicken meat	2009–2014	—	—	—	—	232	
Netherlands	Netherlands	Chickens, veal calves, turkeys	2010–2015	—	—	—	—	233	
Netherlands	Netherlands	Retail meat (mostly chicken and turkey)	2009–2016	—	—	—	—	233	
Netherlands	Netherlands	Human (blood)	2011	—	—	—	—	233	

(Continued on following page)

TABLE 4 (Continued)

Species	Country of isolation	Country of origin or traveled region	Sample origin	Period (yr)	Plasmid features <sup>a</sup>			Reference(s)
					Inc type	Size (kb)	Mobile element	
	Netherlands	Travel in Tunisia, South America, China, Southeast Asia	Human (fecal carriage)	2012–2013	—	—	—	234
	Netherlands	Germany	Veal valves, broilers, and turkey	2010–2015	IncH2 or IncX4	—	ISApI1	235
	Germany	Pligs and human (wound infection)	Chicken meat	2010–2015	IncH2 or IncX4	—	ISApI1 or not	150
	Denmark	Europe	Chicken meat	2012–2014	IncI2 or IncX4	—	—	236
	Denmark	Denmark	Human (bloodstream infection)	2015	IncI2	—	—	236
	Canada	Unknown	Beef meat	2010	IncH2A	—	—	151
	Canada	Lived in Egypt for 5 years	Human (gastrostomy site and rectum)	2011	IncI2	—	—	151
	USA	USA	Human (urine)	2016	IncF	225	ISApI1	237
	Algeria	Algeria	Chickens	2015	—	—	—	222
	Tunisia	Tunisia/France	Chickens	2015	IncH2	—	—	238
	Egypt	Egypt	Human (sputum)	2015	—	>90	—	239
	South Africa	South Africa	Human	2014–2015	IncI2	65, 70	ISApI1 or not	154
					IncH2	150	—	154
					IncX4	30	—	154
					IncI2	62	ISApI1	240
					IncX4	62	—	241
					—	—	—	147
<i>K. pneumoniae</i>					—	—	—	139
					IncI2	64	ISApI1	224
					—	—	—	242
					IncFI	70	—	242
					IncFI	65	—	242
					IncI2	65	ISApI1	152
					IncH2	—	ISApI1	218, 225, 243
					IncI2	58	—	226
					IncX4	—	—	244
					IncP	—	—	244
					IncP	—	—	244
					IncH2, IncX4	—	No	231
					IncH2, IncI2	—	ISApI1 or not	231
					IncX4	30	—	235
					IncX4	30	—	235
					IncX4	30	—	235
					IncH2	—	No	231
					IncI2	—	—	145
					IncI2	57	—	216

<sup>a</sup>NT, nontypeable; —, unknown.



of capsular biosynthesis genes indeed reduces the interactions of polymyxins with the bacterial surface, leading to polymyxin resistance.

*K. pneumoniae* is able to release anionic capsular polysaccharides from its surface (160). This release leads to the trapping of cationic antimicrobial peptides, such as polymyxins, thus decreasing the amount of antibiotic reaching the bacterial surface. The CPS is connected to the bacterial surface through an ionic interaction with the LPS, and this interaction is stabilized by divalent cations (161). As a consequence, the release of CPS in the presence of polymyxins is likely due to perturbation of the cation-dependent bridges between the molecules of LPS.

**Role of porins.** It has been shown that a periplasmic protein (Ydel) regulated by the PhoPQ and PmrAB two-component systems can interact with the OmpD porin to increase bacterial resistance to polymyxins in *Salmonella enterica* (162).

**Role of efflux pumps.** The role of efflux in colistin resistance is not well understood, but several studies suggested the involvement of efflux pumps in colistin resistance. Mutations in *kpnEF* and *acrAB*, encoding components of efflux pumps, may actually lead to a 2-fold decrease of the MIC of colistin and increase bacterial survival in the presence of a low concentration of polymyxins (163, 164). Addition to the test medium of low doses of the efflux pump inhibitor carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) decreased the MICs for resistant strains (128- to 512-fold reductions) and partially or completely inhibited the regrowth of resistant subpopulations (165). However, this observation should be considered with caution owing to the nonspecific effect of CCCP on efflux systems, with a likely wider impact on bacterial metabolism.

### **Mechanisms of Polymyxin Resistance in *Pseudomonas aeruginosa* and *Acinetobacter baumannii***

***Pseudomonas aeruginosa.*** The colistin resistance in *P. aeruginosa* is mediated by five two-component systems that regulate LPS modifications. As for the *Enterobacteriaceae*, alterations in the PmrAB (74, 118, 166–169) and PhoPQ (118, 166, 168, 170) two-component systems have been shown to be responsible for acquired resistance to colistin. Mutations in these two-component systems cause constitutive alterations and consequently activate transcription of the *pmrHFJKLM* operon and the subsequent addition of L-Ara4N to the LPS, finally leading to colistin resistance. Notably, unlike what is observed in *K. pneumoniae*, the colistin resistance mediated by PhoPQ modifications does not depend on the PmrAB system.

Three other two-component systems have been proved to contribute to colistin resistance in *P. aeruginosa*, namely, ParRS, ColRS, and CprRS. The ParRS (polymyxin adaptive resistance) two-component system is involved in adaptive resistance to polymyxins (118, 166, 171). Mutations in this system cause constitutive expression of the *pmrHFJKLM* operon and thus lead to the addition of L-Ara4N to the LPS, leading to colistin resistance. Additionally, mutations in the ColRS and CprRS two-component regulatory systems may also contribute to polymyxin resistance, since the association of mutations in the *phoQ* gene and mutations in the *colS* or *cprS* gene confers a high level of colistin resistance (172). The action of the ColRS and CprRS systems may occur through the activation of the *phoQ* gene and/or through other genes that have not yet been identified.

***Acinetobacter baumannii.*** The main mechanism of colistin resistance in *A. baumannii* corresponds to the addition of cationic groups to the LPS (qualitative modification of the LPS); nevertheless, acquired resistance to colistin may also be the consequence of a complete loss of LPS production (quantitative modification of the LPS).

The addition of cationic groups in *A. baumannii* is mediated by mutations in PmrAB (118, 173–180). Mutations in the *pmrA* and *pmrB* genes have been shown to cause colistin resistance through upregulation of the *pmrCAB* operon, leading to pEtN synthesis but not to L-Ara4N synthesis (unlike in *Enterobacteriaceae*).

The second mechanism of colistin resistance in *A. baumannii* corresponds to the complete loss of LPS caused by alterations of the lipid A biosynthesis genes, namely, the *lpxA*, *lpxC*, and *lpxD* genes. Mutations identified in those genes were either



substitutions, truncations, frameshifts (109), or insertional inactivation by the insertion sequence IS*Aba11* (181).

## EPIDEMIOLOGY OF RESISTANCE TO POLYMYXINS

### General Epidemiology of Resistance to Polymyxins

Although polymyxins currently retain significant *in vitro* activity against most Gram-negative organisms, resistance to these antibiotics is increasingly being reported among clinical isolates (29, 182).

The SENTRY antimicrobial surveillance program carried out a worldwide survey in 2009 and reported low rates of resistance to polymyxins among Gram-negative pathogens (*Acinetobacter* spp., *P. aeruginosa*, *E. coli*, and *Klebsiella* spp.) (<0.1% to 1.5%) (183). However, a rising trend was observed in a 2006-2009 study period focusing on *K. pneumoniae* isolates (resistance rates of 1.2% in 2006 and 1.8% in 2009), probably because of the extensive and/or inadequate usage of polymyxins worldwide for treating infections with MDR Gram-negative bacteria.

Colistin resistance in *K. pneumoniae* represents a growing public health concern, since this bacterial species is one of the main pathogens of nosocomial infection and has gathered a wide range of resistance mechanisms to broad-spectrum antibiotics over the years. Table 5 shows the populations studied (mainly carbapenem-resistant *K. pneumoniae* [CR-KP] clinical isolates), along with the methods that have been used to determine the rate of colistin resistance, since some methods are now known to underestimate the level of colistin resistance and therefore may significantly bias the proposed rates. The occurrence of colistin resistance in *K. pneumoniae* has been reported in surveillance studies and clinical case reports worldwide (Table 5) (183). Many studies report an increase of the resistance rate among multidrug-resistant *K. pneumoniae* isolates, particularly among CR-KP isolates, with high colistin resistance rates reported (Table 5). More worryingly, multiple outbreaks with carbapenem- and colistin-resistant isolates have been reported in North America and Europe (Table 6; Fig. 5).

**North America.** Multicenter surveys showed low rates of resistance among *K. pneumoniae* isolates in Canada (2.9%) (184) and the United States (4%) (185) (Table 5). However, the colistin resistance rate was higher (6.7 to 18%) among carbapenemase-producing isolates (185-187). In addition, outbreaks with colistin-resistant, KPC-producing *K. pneumoniae*, mostly attributed to the international epidemic clone type ST258, have been reported in the United States (188, 189) and Mexico (190) (Table 6; Fig. 5).

**South America.** The results from the SENTRY antimicrobial surveillance program showed a moderate resistance rate in Latin America in 2009 (3%) (183); however, the emergence of colistin-resistant *K. pneumoniae* isolates has been reported in Argentina (191), Colombia (119), and Brazil (87).

**Europe.** Multiple outbreaks of both carbapenem- and colistin-resistant *K. pneumoniae* isolates have been reported in Europe (Table 6; Fig. 5). Outbreaks with KPC-producing *K. pneumoniae* isolates attributed to the international epidemic clone type ST258 have been reported in the Netherlands (192), Hungary (193), Greece (194, 195), and Italy (196, 197).

In addition to the two outbreaks attributed to the ST258 clone (196, 197), a large nosocomial outbreak of colistin-resistant and KPC-producing ST512 *K. pneumoniae* isolates was reported in Italy (198) (Table 6; Fig. 5). More worryingly, colistin resistance was recently reported at a high level (>20%) among carbapenemase-producing isolates in ICUs of two Italian hospitals (199, 200), with an even higher rate (36.1%) in hospitals in Rome (68) (Table 6). Moreover, a national study reported a countrywide level of colistin resistance among KPC-producing *K. pneumoniae* isolates, with 43% of isolates being resistant to colistin (201) (Table 5).

In Greece, several outbreaks caused by KPC-producing, colistin-resistant *K. pneumoniae* isolates have been reported (194, 195) (Table 6; Fig. 5). Studies performed in two Greek hospitals reported a huge increase in colistin resistance within a few years (<3.5% incidence before 2010 and >20% incidence after 2010) (202, 203).

**TABLE 5** Studies reporting prevalences of colistin resistance among *K. pneumoniae* clinical isolates<sup>d</sup>

Study type and period	Country	Setting	Test method	Total no. of isolates	Carbapenemase producers (no. [%])	Polymyxin-resistant isolates (no. [%])	Reference(s)
Studies reporting colistin resistance rates among overall clinical isolates							
2006–2009	Worldwide	Worldwide surveillance	BMD	9,774	NA	(1.5)	183
2007–2008	Canada	National	BMD	515	NA	15 (2.9)	184
2013–2014	United States	Multicenter	BMD	1,205	NA	(4)	185
2006–2007	South Korea	Multicenter (9 hospitals)	BMD	221 <sup>a</sup>	15 (13.3)	24 (10.9) <sup>b</sup>	206
2004–2005	Singapore	Single center	Agar dilution	16	NA	1 (6)	207
Studies reporting colistin resistance rates among CR-KP isolates							
2008–2011	Canada	Multicenter	Etest	30	30 (100)	2 (6.7)	184, 186
2013–2014	United States	Multicenter	BMD	69	69 (100)	(18) <sup>c</sup>	185
2003–2004	United States	Multicenter	BMD or agar dilution	96	96 (100)	(9)	187
2010–2013	Greece	Single center (only ICU)	Vitek2, Etest	92	92 (100)	20 (21.7)	202
2010	Greece	Single center	Vitek2, Etest	120	120 (100)	25 (20.8)	203
2014	Italy	Single center (only ICU)	Vitek2, Etest	214	214 (100)	47 (21.9)	199
2013	Italy	Single center (only ICU)	BMD	25	24 (100)	6 (24.0)	200
2013–2014	Italy	National	BMD	178	178 (100)	76 (43)	201
2010–2011	Italy	Multicenter (9 hospitals)	Vitek2, BMD	97	97 (100)	35 (36.1)	68
2010–2012	Spain	Hospital	Agar dilution	79	79 (100)	18 (22.8)	204
2014	France	National	BMD	561	561 (100)	35 (6.2)	205
2012–2013	Turkey	Single center	Vitek2, Etest	37	36 (98)	(2.7)	245
2006–2007	Israel	Single center	?	88	88 (100)	(4.5)	246
2009–2010	China	Single center	Agar dilution	68	68 (100)	3 (4.4)	208
2012	Taiwan	National	Sensititre	247	55 (22.3)	(12.1)	209

<sup>a</sup>Isolated from blood samples.

<sup>b</sup>Number of colistin-resistant isolates according to EUCAST breakpoints.

<sup>c</sup>Eighteen percent colistin resistance among 4 *E. coli* and 69 *K. pneumoniae* isolates harboring carbapenemases.

<sup>d</sup>NA, not applicable.

**TABLE 6** Studies reporting outbreaks of colistin-resistant and carbapenemase-producing isolates<sup>a</sup>

Study period	Country	Setting	Test method	Colistin MIC (µg/ml)	Resistance mechanism	Total no. of cases	Beta-lactamase(s)	Sequence type	Reference
2010	United States	One single hospital (ICU and medical ward)	Agar dilution	>128	ND	5	KPC-2	ST258	188
2009	United States	Two hospitals and a long-term acute care setting in Detroit, MI	Etest	8–64	ND	4	KPC	ND	189
2012–2013	Mexico	One single hospital in Mexico City	BMD	4	ND	15	KPC-2	ST258	190
2013	Netherlands	One hospital and a nursing home	Vitek2, Etest	ND	ND	6	KPC-2, SHV-12	ST258	192
2008–2009	Hungary	Three hospitals in Miskolc	Etest	16–32	ND	8	KPC-2, SHV-12, TEM-1, SHV-11	ST258	193
2008	Greece	ICUs of two distinct hospitals	Etest	12–128	ND	6	KPC-2, SHV-12	ST258	194
2004–2005	Greece	One single hospital in Athens (ICU)	Etest	12–>1,024	ND	13 (multiclonal)	ND	ND	195
2010	Italy	Two hospitals in Catania, Sicily	BMD	8–64	ND	8	KPC-3, SHV-11, TEM-1, OXA-9	ST258	196
2011	Italy	One single hospital in Palermo, Sicily	Etest	3–128	ND	28 (multiclonal) <sup>d</sup>	KPC-3, SHV-11, TEM-1, OXA-9	ST258	197
				3–32	ND	3	KPC-3, SHV-12, TEM-1	ST273	
				4–12	ND	2	KPC-3, SHV-28, TEM-1, CTX-M-15	ST15	
2010–2013	Italy	One single hospital (22 different wards)	Vitek2, Sensititre	4–>16	<i>mgrB</i> Δnt109/119	Multiclonal <sup>b</sup>			198
					Unknown <sup>c</sup>	50	KPC-3	ST512	
					Unknown <sup>c</sup>	5	KPC-3	ST512	
2010–2012	Spain	One hospital	Agar dilution	ND	ND	2	KPC-2	ST101	204
2014	France	Hospital in Picardie	BMD	4–64	Unknown <sup>d</sup>	15	VIM-1 OXA-48, CTX-M-15	ST22 ST11	205

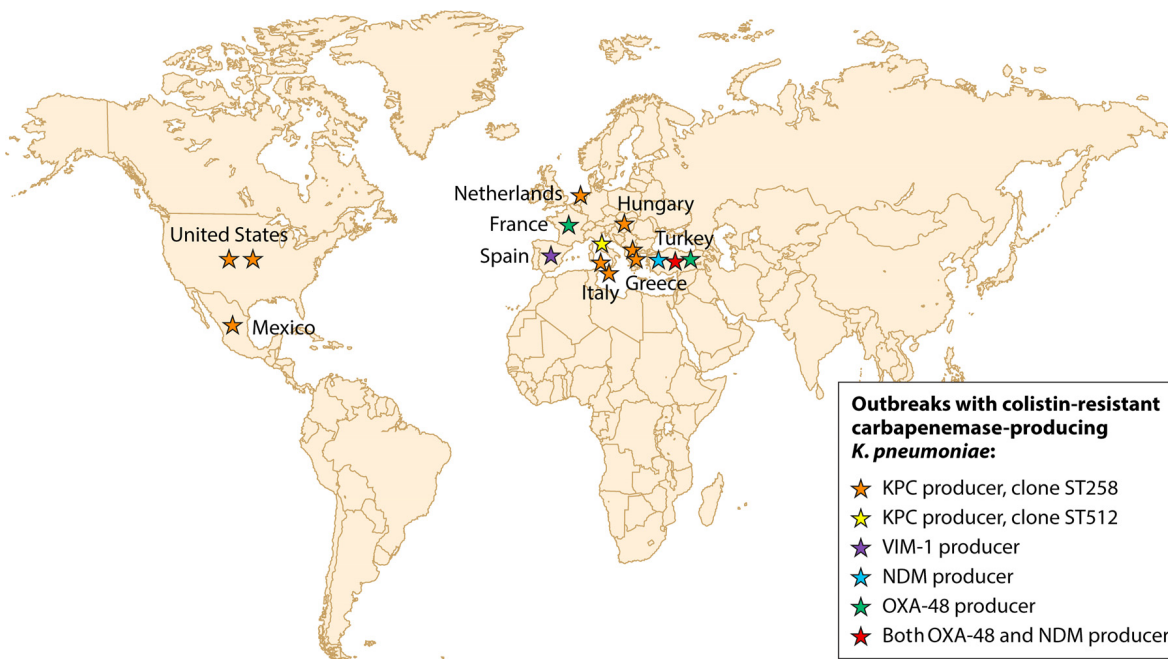
<sup>a</sup>One patient with two colistin-resistant clones (belonging to ST-258 and ST-273).

<sup>b</sup>There was a total of 93 bloodstream infections, but only isolates recovered in 2013 were investigated further.

<sup>c</sup>No mutation in the *mgrB*, *pmrA*, or *pmrB* gene was responsible for colistin resistance.

<sup>d</sup>No mutation in the *mgrB* gene was responsible for colistin resistance.

<sup>e</sup>ND, not determined.



**FIG 5** Outbreaks caused by colistin-resistant, carbapenemase-producing *K. pneumoniae* isolates. Each star indicates a single report.

During a 2-year period (2010 to 2012) in Spain, a study showed an increase of the prevalence of colistin resistance among carbapenemase-producing *K. pneumoniae* isolates, from 13.5 to 31.7%, and an outbreak of colistin-resistant, VIM-1-producing *K. pneumoniae* was reported (204).

In France, a national survey revealed a low rate of colistin resistance (6.2%) among carbapenemase-producing *K. pneumoniae* isolates; nevertheless, an outbreak of OXA-48 carbapenemase-producing and colistin-resistant *K. pneumoniae* isolates was reported (205).

**Middle East.** In Turkey and Israel, low rates of resistance to colistin among CR-KP isolates have been reported (2.7% and 4.5%, respectively). However, multiclonal outbreaks with OXA-48-, NDM-, and both OXA-48- and NDM-producing *K. pneumoniae* are currently ongoing in Turkey (our unpublished data).

**Africa.** A very low colistin resistance rate was reported among *K. pneumoniae* isolates in Tunisia (1.2%), but this rate was probably underestimated because susceptibility to colistin was primarily screened using a DD method generating high false susceptibility rates (205). The emergence of colistin resistance was reported for *K. pneumoniae* isolates recovered in South Africa (81, 119) and Nigeria (120).

**Asia.** Moderate rates of colistin resistance (about 6 to 11%) have been reported for *K. pneumoniae* isolates from South Korea (206) and Singapore (207), and similar resistance rates (4.4 to 12.1%) were found among CR-KP isolates in China (208) and Taiwan (209). Colistin-resistant *K. pneumoniae* isolates have also been reported in Laos and Thailand (120).

**Risk Factors**

The use of colistin was found to be an independent risk factor for the occurrence of resistance in Gram-negative bacteria (210, 211). Important increases of colistin resistance rates among ESBL-producing *K. pneumoniae* isolates (from 0 to 71% and from 11.1 to 75%) were reported after the introduction of selective digestive tract decontamination in two intensive care units (212, 213). Moreover, this decontamination failed to prevent colonization by ESBL-producing *Enterobacteriaceae*, and such a strategy should be abandoned. Note that the inappropriate use of colistin (such as suboptimal dosing or prolonged monotherapy) has been shown to be a source of colistin resistance selection (214, 215). The occurrence of colistin resistance in *P. aeruginosa* was most

effectively prevented by 8-h dosing intervals compared to 12- or 24-h dosing intervals (45).

### Specific Epidemiology of the Plasmid-Mediated *mcr-1* Resistance Gene

Notably, plasmid-borne resistance to polymyxins has been reported for few different enterobacterial species so far, mainly among *E. coli* isolates and rarely for *Salmonella enterica*, *Enterobacter* spp., and *K. pneumoniae*. There are also some scattered reports of MCR-1-producing isolates in other species, such as *Cronobacter sakazakii* (152) and *Kluyvera ascorbata* (216). According to the current literature on the subject, the distribution of MCR-1 appears to be worldwide, covering all continents (217). It remains to be determined if the identification of the *mcr-1* gene worldwide corresponds to subsequent spread from an original source (China?) or to simultaneous gene mobilization events in different parts of the world. Ongoing epidemiological surveys should provide some important clues.

It is actually speculated that the original source of the gene, or at least of its mobilization and emergence, might be the animal world. This speculation is based on the fact that MCR-1-producing *E. coli* isolates have been identified in several animals and animal food products, including chickens and chicken meat, pigs and piglets, cattle, calves, and turkeys, but also in humans (Fig. 4). The corresponding samples were collected from many Asian countries (Cambodia, China, Japan, Laos, Malaysia, Taiwan, Singapore, and Vietnam) but also from Europe (Belgium, Denmark, France, Germany, Portugal, Italy, the Netherlands, Spain, Sweden, Switzerland, and the UK), the Americas (Argentina, Brazil, and Canada), and Africa (Algeria, Egypt, South Africa, and Tunisia) (Table 4). Note that a study conducted in Switzerland identified MCR-1-producing *E. coli* isolates in vegetables imported from Asia (143), and positive isolates were also identified in environmental water samples in Switzerland and Malaysia (Table 4).

The speculation of an animal origin of the *mcr-1* gene is also based on genetic features, since this gene is often associated with the insertion sequence *ISApI1*, identified in *Pasteurella multocida*, which is a common animal pathogen, and also with the *bla*<sub>CMY-2</sub> and *florR* genes, which are often identified in animal enterobacterial isolates (144). Finally, another feature suggesting an animal source of the problem is the heavy usage of polymyxins in veterinary medicine, with usage on many different animal species.

Dating the emergence of MCR-1-positive strains remains quite difficult; however, a Chinese study retrospectively identified positive isolates recovered from chickens during the 1980s (142), and they were discovered as early as 2005 in veal calves in France (144). It therefore seems that the emergence of MCR-positive isolates, at least in animals, is not a recent event. Very likely, there has been some silent dissemination of that resistance mechanism throughout the last few decades, and the current situation shows an ongoing further dissemination rather than an emerging phenomenon.

### CONCLUSIONS

Polymyxins are gaining increasing interest because of the current epidemiological situation, with MDR Gram-negative bacteria spreading worldwide and with a paucity of novel marketed antibiotics. In some areas where infections caused by carbapenem-resistant *Enterobacteriaceae* are now common (such as Greece or Italy), the use of polymyxins (alone or often in combination with other antibiotics) is becoming crucial and may even be considered first-line therapy. The reevaluation of some critical issues in relation to polymyxins (accurate susceptibility testing, defining correct breakpoints, and better appreciating the toxicity issues) now opens new perspectives for its use. Studies that may permit a better evaluation of the PK-PD data, the toxicity level, and appropriate drug combinations are therefore crucial.

The recent identification of plasmid-mediated mechanisms of resistance to polymyxins also modifies the perspective. Indeed, epidemiological studies have to be initiated in order to better evaluate the extent of dissemination of this resistance in human and veterinary medicine and the impact of its occurrence. The perspective of

nosocomial dissemination of MDR Gram-negative organisms possessing resistance determinants to all main antibiotics is frightening, in particular for *K. pneumoniae*, which is one of the main nosocomial pathogens. Whether veterinary medicine is affecting the epidemiological situation by providing selective pressure with polymyxins has to be precisely determined. Whether discontinuing some specific usages of these drugs (prophylaxis or metaphylaxis in animals and decontamination of MDR bacteria in humans) should be considered is therefore an open debate.

Ultimately, reinforcing the detection of polymyxin-resistant isolates must be encouraged. Prospective epidemiological surveys are needed, since the current knowledge on this issue is very scarce. Actually, the recent development of a rapid diagnostic test for detection of polymyxin resistance, along with the development of a screening agar medium, will contribute to facilitating those surveys.

## ACKNOWLEDGMENTS

This work was funded by the University of Fribourg, Switzerland, and by a grant from the ANIWAH ERA-NET project, Switzerland (ANIWAH).

## REFERENCES

- Benedict RG, Langlykke AF. 1947. Antibiotic activity of *Bacillus polymyxa*. *J Bacteriol* 54:24.
- Hancock RE. 1997. Peptide antibiotics. *Lancet* 349:418–422. [https://doi.org/10.1016/S0140-6736\(97\)80051-7](https://doi.org/10.1016/S0140-6736(97)80051-7).
- Falagas ME, Rafailidis PI, Matthaiou DK. 2010. Resistance to polymyxins: mechanisms, frequency and treatment options. *Drug Resist Updat* 13:132–138. <https://doi.org/10.1016/j.drug.2010.05.002>.
- Li J, Nation RL, Turnidge JD, Milne RW, Coulthard K, Rayner CR, Paterson DL. 2006. Colistin: the re-emerging antibiotic for multidrug-resistant Gram-negative bacterial infections. *Lancet Infect Dis* 6:589–601. [https://doi.org/10.1016/S1473-3099\(06\)70580-1](https://doi.org/10.1016/S1473-3099(06)70580-1).
- Brink AJ, Richards GA, Colombo G, Bortolotti F, Colombo P, Jehl F. 2014. Multicomponent antibiotic substances produced by fermentation: implications for regulatory authorities, critically ill patients and generics. *Int J Antimicrob Agents* 43:1–6. <https://doi.org/10.1016/j.ijantimicag.2013.06.013>.
- Gallardo-Godoy A, Muldoon C, Becker B, Elliott AG, Lash LH, Huang JX, Butler MS, Pelington R, Kavanagh AM, Ramu S, Phetsang W, Blaskovich MA, Cooper MA. 2016. Activity and predicted nephrotoxicity of synthetic antibiotics based on polymyxin B. *J Med Chem* 59:1068–1077. <https://doi.org/10.1021/acs.jmedchem.5b01593>.
- Nation RL, Velkov T, Li J. 2014. Colistin and polymyxin B: peas in a pod, or chalk and cheese? *Clin Infect Dis* 59:88–94. <https://doi.org/10.1093/cid/ciu213>.
- Bergen PJ, Li J, Rayner CR, Nation RL. 2006. Colistin methanesulfonate is an inactive prodrug of colistin against *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother* 50:1953–1958. <https://doi.org/10.1128/AAC.00035-06>.
- Barnett M, Bushby SR, Wilkinson S. 1964. Sodium sulphomethyl derivatives of polymyxins. *Br J Pharmacol Chemother* 23:552–574. <https://doi.org/10.1111/j.1476-5381.1964.tb01610.x>.
- Li J, Milne RW, Nation RL, Turnidge JD, Coulthard K. 2003. Stability of colistin and colistin methanesulfonate in aqueous media and plasma as determined by high-performance liquid chromatography. *Antimicrob Agents Chemother* 47:1364–1370. <https://doi.org/10.1128/AAC.47.4.1364-1370.2003>.
- Li J, Milne RW, Nation RL, Turnidge JD, Smeaton TC, Coulthard K. 2004. Pharmacokinetics of colistin methanesulphonate and colistin in rats following an intravenous dose of colistin methanesulphonate. *J Antimicrob Chemother* 53:837–840. <https://doi.org/10.1093/jac/dkh167>.
- Dixon RA, Chopra I. 1986. Leakage of periplasmic proteins from *Escherichia coli* mediated by polymyxin B nonapeptide. *Antimicrob Agents Chemother* 29:781–788. <https://doi.org/10.1128/AAC.29.5.781>.
- Falagas ME, Kasiakou SK. 2005. Colistin: the revival of polymyxins for the management of multidrug-resistant Gram-negative bacterial infections. *Clin Infect Dis* 40:1333–1341. <https://doi.org/10.1086/429323>.
- Li J, Nation RL, Milne RW, Turnidge JD, Coulthard K. 2005. Evaluation of colistin as an agent against multi-resistant Gram-negative bacteria. *Int J Antimicrob Agents* 25:11–25. <https://doi.org/10.1016/j.ijantimicag.2004.10.001>.
- Deris ZZ, Akter J, Sivanesan S, Roberts KD, Thompson PE, Nation RL, Li J, Velkov T. 2013. A secondary mode of action of polymyxins against Gram-negative bacteria involves the inhibition of NADH-quinone oxidoreductase activity. *J Antibiot (Tokyo)* 67:147–151. <https://doi.org/10.1038/ja.2013.111>.
- Bulitta JB, Yang JC, Yohonn L, Ly NS, Brown SV, D'Hondt RE, Jusko WJ, Forrest A, Tsuji BT. 2010. Attenuation of colistin bactericidal activity by high inoculum of *Pseudomonas aeruginosa* characterized by a new mechanism-based population pharmacodynamic model. *Antimicrob Agents Chemother* 54:2051–2062. <https://doi.org/10.1128/AAC.00881-09>.
- Owen RJ, Li J, Nation RL, Spelman D. 2007. In vitro pharmacodynamics of colistin against *Acinetobacter baumannii* clinical isolates. *J Antimicrob Chemother* 59:473–477. <https://doi.org/10.1093/jac/dkl512>.
- Poudyal A, Howden BP, Bell JM, Gao W, Owen RJ, Turnidge JD, Nation RL, Li J. 2008. In vitro pharmacodynamics of colistin against multidrug-resistant *Klebsiella pneumoniae*. *J Antimicrob Chemother* 62:1311–1318. <https://doi.org/10.1093/jac/dkn425>.
- Bergen PJ, Landersdorfer CB, Zhang J, Zhao M, Lee HJ, Nation RL, Li J. 2012. Pharmacokinetics and pharmacodynamics of 'old' polymyxins: what is new? *Diagn Microbiol Infect Dis* 74:213–223. <https://doi.org/10.1016/j.diagmicrobio.2012.07.010>.
- Cheah SE, Wang J, Nguyen VTT, Turnidge JD, Li J, Nation RL. 2015. New pharmacokinetic/pharmacodynamic studies of systemically administered colistin against *Pseudomonas aeruginosa* and *Acinetobacter baumannii* in mouse thigh and lung infection models: smaller response in lung infection. *J Antimicrob Chemother* 70:3291–3297. <https://doi.org/10.1093/jac/dkv267>.
- Garonzik SM, Li J, Thamlikitkul V, Paterson DL, Shoham S, Jacob J, Silveira FP, Forrest A, Nation RL. 2011. Population pharmacokinetics of colistin methanesulfonate and formed colistin in critically ill patients from a multicenter study provide dosing suggestions for various categories of patients. *Antimicrob Agents Chemother* 55:3284–3294. <https://doi.org/10.1128/AAC.01733-10>.
- Landersdorfer CB, Nation RL. 2015. Colistin: how should it be dosed for the critically ill? *Semin Respir Crit Care Med* 36:126–135. <https://doi.org/10.1055/s-0034-1398390>.
- Bergen PJ, Li J, Nation RL. 2011. Dosing of colistin—back to basic PK/PD. *Curr Opin Pharmacol* 11:464–469. <https://doi.org/10.1016/j.coph.2011.07.004>.
- Gunderson BW, Ibrahim KH, Hovde LB, Fromm TL, Reed MD, Rotschafer JC. 2003. Synergistic activity of colistin and ceftazidime against multiantibiotic-resistant *Pseudomonas aeruginosa* in an in vitro pharmacodynamic model. *Antimicrob Agents Chemother* 47:905–909. <https://doi.org/10.1128/AAC.47.3.905-909.2003>.
- John EB, Bennett RD, Blaser MJ. 2015. Polymyxins (polymyxin B and colistin), p 549–555. In Bennett JE, Dolin R, Blaser MJ (ed), *Mandell*,



- Douglas, and Bennett's principles and practice of infectious diseases, 8th ed. Elsevier/Saunders, Philadelphia, PA.
26. Li J, Rayner CR, Nation RL, Owen RJ, Spelman D, Tan KE, Liolios L. 2006. Heteroresistance to colistin in multidrug-resistant *Acinetobacter baumannii*. *Antimicrob Agents Chemother* 50:2946–2950. <https://doi.org/10.1128/AAC.00103-06>.
  27. Yau W, Owen RJ, Poudyal A, Bell JM, Turnidge JD, Yu HH, Nation RL, Li J. 2009. Colistin hetero-resistance in multidrug-resistant *Acinetobacter baumannii* clinical isolates from the Western Pacific region in the SENTRY antimicrobial surveillance programme. *J Infect* 58:138–144. <https://doi.org/10.1016/j.jinf.2008.11.002>.
  28. Meletis G, Tzampaz E, Sianou E, Tzavaras I, Sofianou D. 2011. Colistin heteroresistance in carbapenemase-producing *Klebsiella pneumoniae*. *J Antimicrob Chemother* 66:946–947. <https://doi.org/10.1093/jac/dkr007>.
  29. Bialvaei AZ, Samadi Kafil H. 2015. Colistin, mechanisms and prevalence of resistance. *Curr Med Res Opin* 31:707–721. <https://doi.org/10.1185/03007995.2015.1018989>.
  30. Koch-Weser J, Sidel VW, Federman EB, Kanarek P, Finer DC, Eaton AE. 1970. Adverse effects of sodium colistimethate. Manifestations and specific reaction rates during 317 courses of therapy. *Ann Intern Med* 72:857–868.
  31. Magiorakos AP, Srinivasan A, Carey RB, Carmeli Y, Falagas ME, Giske CG, Harbarth S, Hindler JF, Kahlmeter G, Olsson-Liljequist B, Paterson DL, Rice LB, Stelling J, Struelens MJ, Vatopoulos A, Weber JT, Monnet DL. 2012. Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. *Clin Microbiol Infect* 18:268–281. <https://doi.org/10.1111/j.1469-0691.2011.03570.x>.
  32. Biswas S, Brunel JM, Dubus JC, Reynaud-Gaubert M, Rolain JM. 2012. Colistin: an update on the antibiotic of the 21st century. *Expert Rev Anti Infect Ther* 10:917–934. <https://doi.org/10.1586/eri.12.78>.
  33. Yahav D, Farbman L, Leibovici L, Paul M. 2012. Colistin: new lessons on an old antibiotic. *Clin Microbiol Infect* 18:18–29. <https://doi.org/10.1111/j.1469-0691.2011.03734.x>.
  34. Ortwine JK, Kaye KS, Li J, Pogue JM. 2015. Colistin: understanding and applying recent pharmacokinetic advances. *Pharmacotherapy* 35: 11–16. <https://doi.org/10.1002/phar.1484>.
  35. Nation RL, Li J, Cars O, Couet W, Dudley MN, Kaye KS, Mouton JW, Paterson DL, Tam VH, Theuretzbacher U, Tsuji BT, Turnidge JD. 2014. Consistent global approach on reporting of colistin doses to promote safe and effective use. *Clin Infect Dis* 58:139–141. <https://doi.org/10.1093/cid/cit680>.
  36. Theuretzbacher U. 2014. Product information for parenteral colistin varies substantially across Europe. *J Antimicrob Chemother* 69: 1987–1992. <https://doi.org/10.1093/jac/dku064>.
  37. Wallace SJ, Li J, Rayner CR, Coulthard K, Nation RL. 2008. Stability of colistin methanesulfonate in pharmaceutical products and solutions for administration to patients. *Antimicrob Agents Chemother* 52: 3047–3051. <https://doi.org/10.1128/AAC.00103-08>.
  38. Li J, Milne RW, Nation RL, Turnidge JD, Coulthard K, Johnson DW. 2001. A simple method for the assay of colistin in human plasma, using pre-column derivatization with 9-fluorenylmethyl chloroformate in solid-phase extraction cartridges and reversed-phase high-performance liquid chromatography. *J Chromatogr B Biomed Sci Appl* 761:167–175. [https://doi.org/10.1016/S0378-4347\(01\)00326-7](https://doi.org/10.1016/S0378-4347(01)00326-7).
  39. Li J, Milne RW, Nation RL, Turnidge JD, Coulthard K, Valentine J. 2002. Simple method for assaying colistin methanesulfonate in plasma and urine using high-performance liquid chromatography. *Antimicrob Agents Chemother* 46:3304–3307. <https://doi.org/10.1128/AAC.46.10.3304-3307.2002>.
  40. Li J, Milne RW, Nation RL, Turnidge JD, Smeaton TC, Coulthard K. 2003. Use of high-performance liquid chromatography to study the pharmacokinetics of colistin sulfate in rats following intravenous administration. *Antimicrob Agents Chemother* 47:1766–1770. <https://doi.org/10.1128/AAC.47.5.1766-1770.2003>.
  41. Mohamed AF, Karaiskos I, Plachouras D, Karvanen M, Pontikis K, Jansson B, Papadomichelakis E, Antoniadou A, Giamarellou H, Armaganidis A, Cars O, Friberg LE. 2012. Application of a loading dose of colistin methanesulfonate in critically ill patients: population pharmacokinetics, protein binding, and prediction of bacterial kill. *Antimicrob Agents Chemother* 56:4241–4249. <https://doi.org/10.1128/AAC.06426-11>.
  42. Markou N, Markantonis SL, Dimitrakis E, Panidis D, Boutzouka E, Karatzas S, Rafailidis P, Apostolakis H, Baltopoulos G. 2008. Colistin serum concentrations after intravenous administration in critically ill patients with serious multidrug-resistant, Gram-negative bacilli infections: a prospective, open-label, uncontrolled study. *Clin Ther* 30:143–151. <https://doi.org/10.1016/j.clinthera.2008.01.015>.
  43. Plachouras D, Karvanen M, Friberg LE, Papadomichelakis E, Antoniadou A, Tsangaris I, Karaiskos I, Poulakou G, Kontopidou F, Armaganidis A, Cars O, Giamarellou H. 2009. Population pharmacokinetic analysis of colistin methanesulfonate and colistin after intravenous administration in critically ill patients with infections caused by Gram-negative bacteria. *Antimicrob Agents Chemother* 53:3430–3436. <https://doi.org/10.1128/AAC.01361-08>.
  44. Kumar A, Ellis P, Arabi Y, Roberts D, Light B, Parrillo JE, Dodek P, Wood G, Simon D, Peters C, Ahsan M, Chateau D. 2009. Initiation of inappropriate antimicrobial therapy results in a fivefold reduction of survival in human septic shock. *Chest* 136:1237–1248. <https://doi.org/10.1378/chest.09-0087>.
  45. Bergen PJ, Li J, Nation RL, Turnidge JD, Coulthard K, Milne RW. 2008. Comparison of once-, twice- and thrice-daily dosing of colistin on antibacterial effect and emergence of resistance: studies with *Pseudomonas aeruginosa* in an in vitro pharmacodynamic model. *J Antimicrob Chemother* 61:636–642. <https://doi.org/10.1093/jac/dkm511>.
  46. Sandri AM, Landersdorfer CB, Jacob J, Boniatti MM, Dalarosa MG, Falci DR, Behle TF, Bordinho RC, Wang J, Forrest A, Nation RL, Li J, Zavascki AP. 2013. Population pharmacokinetics of intravenous polymyxin B in critically ill patients: implications for selection of dosage regimens. *Clin Infect Dis* 57:524–531. <https://doi.org/10.1093/cid/cit334>.
  47. Vicari G, Bauer SR, Neuner EA, Lam SW. 2013. Association between colistin dose and microbiologic outcomes in patients with multidrug-resistant Gram-negative bacteremia. *Clin Infect Dis* 56:398–404. <https://doi.org/10.1093/cid/cis909>.
  48. Cheng CY, Sheng WH, Wang JT, Chen YC, Chang SC. 2010. Safety and efficacy of intravenous colistin (colistin methanesulfonate) for severe multidrug-resistant Gram-negative bacterial infections. *Int J Antimicrob Agents* 35:297–300. <https://doi.org/10.1016/j.ijantimicag.2009.11.016>.
  49. Michalopoulos AS, Falagas ME. 2011. Colistin: recent data on pharmacodynamics properties and clinical efficacy in critically ill patients. *Ann Intensive Care* 1:30. <https://doi.org/10.1186/2110-5820-1-30>.
  50. Roberts JA, Lipman J. 2012. Editorial commentary: closing the loop—a colistin clinical study to confirm dosing recommendations from PK/PD modeling. *Clin Infect Dis* 54:1727–1729. <https://doi.org/10.1093/cid/cis311>.
  51. European Medicines Agency. 2014. European Medicines Agency completes review of polymyxin-based medicines: recommendations issued for safe use in patients with serious infections resistant to standard antibiotics. European Medicines Agency, London, United Kingdom.
  52. Roberts JA, Abdul-Aziz MH, Lipman J, Mouton JW, Vinks AA, Felton TW, Hope WW, Farkas A, Neely MN, Schentag JJ, Drusano G, Frey OR, Theuretzbacher U, Kuti JL. 2014. Individualised antibiotic dosing for patients who are critically ill: challenges and potential solutions. *Lancet Infect Dis* 14:498–509. [https://doi.org/10.1016/S1473-3099\(14\)70036-2](https://doi.org/10.1016/S1473-3099(14)70036-2).
  53. Lee YJ, Wi YM, Kwon YJ, Kim SR, Chang SH, Cho S. 2015. Association between colistin dose and development of nephrotoxicity. *Crit Care Med* 43:1187–1193. <https://doi.org/10.1097/CCM.0000000000000931>.
  54. Dalfino L, Puntillo F, Mosca A, Monno R, Spada ML, Coppolecchia S, Miragliotta G, Bruno F, Brienza N. 2012. High-dose, extended-interval colistin administration in critically ill patients: is this the right dosing strategy? A preliminary study. *Clin Infect Dis* 54:1720–1726. <https://doi.org/10.1093/cid/cis286>.
  55. Ordooei Javan A, Shokouhi S, Sahraei Z. 2015. A review on colistin nephrotoxicity. *Eur J Clin Pharmacol* 71:801–810. <https://doi.org/10.1007/s00228-015-1865-4>.
  56. Falagas ME, Kasiakou SK. 2006. Toxicity of polymyxins: a systematic review of the evidence from old and recent studies. *Crit Care* 10:R27. <https://doi.org/10.1186/cc3995>.
  57. Sorli L, Luque S, Grau S, Berenguer N, Segura C, Montero MM, Alvarez-Lerma F, Knobel H, Benito N, Horcajada JP. 2013. Trough colistin plasma level is an independent risk factor for nephrotoxicity: a prospective observational cohort study. *BMC Infect Dis* 13:380. <https://doi.org/10.1186/1471-2334-13-380>.
  58. Bellomo R, Kellum JA, Ronco C. 2007. Defining and classifying acute renal failure: from advocacy to consensus and validation of the RIFLE criteria. *Intensive Care Med* 33:409–413. <https://doi.org/10.1007/s00134-006-0478-x>.
  59. Akajagbor DS, Wilson SL, Shere-Wolfe KD, Dakum P, Charurat ME, Gilliam BL. 2013. Higher incidence of acute kidney injury with intrave-

- nous colistimethate sodium compared with polymyxin B in critically ill patients at a tertiary care medical center. *Clin Infect Dis* 57:1300–1303. <https://doi.org/10.1093/cid/cit453>.
60. Phe K, Lee Y, McDanel PM, Prasad N, Yin T, Figueroa DA, Musick WL, Cottreau JM, Hu M, Tam VH. 2014. In vitro assessment and multicenter cohort study of comparative nephrotoxicity rates associated with colistimethate versus polymyxin B therapy. *Antimicrob Agents Chemother* 58:2740–2746. <https://doi.org/10.1128/AAC.02476-13>.
  61. Catry B, Cavaleri M, Baptiste K, Grave K, Grein K, Holm A, Jukes H, Liebana E, Navas AL, Mackay D, Magiorakos AP, Romo MA, Moulin G, Madero CM, Pomba MC, Powell M, Pyoral S, Rantala M, Ruzauskas M, Sanders P, Teale C, Threlfall EJ, Torneke K, van Duijkeren E, Edo JT. 2015. Use of colistin-containing products within the European Union and European Economic Area (EU/EEA): development of resistance in animals and possible impact on human and animal health. *Int J Antimicrob Agents* 46:297–306. <https://doi.org/10.1016/j.ijantimicag.2015.06.005>.
  62. Kempf I, Fleury MA, Drider D, Bruneau M, Sanders P, Chauvin C, Madec JY, Jouy E. 2013. What do we know about resistance to colistin in *Enterobacteriaceae* in avian and pig production in Europe? *Int J Antimicrob Agents* 42:379–383. <https://doi.org/10.1016/j.ijantimicag.2013.06.012>.
  63. Boyen F, Vangroenweghe F, Butaye P, De Graef E, Castryck F, Heylen P, Vanrobaeys M, Haesebrouck F. 2010. Disk prediffusion is a reliable method for testing colistin susceptibility in porcine *E. coli* strains. *Vet Microbiol* 144:359–362. <https://doi.org/10.1016/j.vetmic.2010.01.010>.
  64. Kieffer N, Poirel L, Nordmann P, Madec JY, Haenni M. 2015. Emergence of colistin resistance in *Klebsiella pneumoniae* from veterinary medicine. *J Antimicrob Chemother* 70:1265–1267. <https://doi.org/10.1093/jac/dku485>.
  65. Olaitan AO, Thongmalayvong B, Akkhavong K, Somphavong S, Paboriboune P, Khounsy S, Morand S, Rolain JM. 2015. Clonal transmission of a colistin-resistant *Escherichia coli* from a domesticated pig to a human in Laos. *J Antimicrob Chemother* 70:3402–3404. <https://doi.org/10.1093/jac/dkv252>.
  66. Walsh TR, Wu Y. 2016. China bans colistin as a feed additive for animals. *Lancet Infect Dis* 16:1102–1103. [https://doi.org/10.1016/S1473-3099\(16\)30329-2](https://doi.org/10.1016/S1473-3099(16)30329-2).
  67. European Medicines Agency. 26 May 2016. Updated advice on the use of colistin products in animals within the European Union: development of resistance and possible impact on human and animal health. EMA/231573/2016. [http://www.ema.europa.eu/docs/en\\_GB/document\\_library/Scientific\\_guideline/2016/05/WC500207233.pdf](http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2016/05/WC500207233.pdf). Accessed 5 September 2016.
  68. Capone A, Giannella M, Fortini D, Giordano A, Meledandri M, Ballardini M, Venditti M, Bordi E, Capozzi D, Balice MP, Tarasi A, Parisi G, Lappa A, Carattoli A, Petrosillo N. 2013. High rate of colistin resistance among patients with carbapenem-resistant *Klebsiella pneumoniae* infection accounts for an excess of mortality. *Clin Microbiol Infect* 19:E23–E30. <https://doi.org/10.1111/1469-0691.12070>.
  69. Landman D, Salamera J, Quale J. 2013. Irreproducible and uninterpretable polymyxin B MICs for *Enterobacter cloacae* and *Enterobacter aerogenes*. *J Clin Microbiol* 51:4106–4111. <https://doi.org/10.1128/JCM.02129-13>.
  70. Hindler JA, Humphries RM. 2013. Colistin MIC variability by method for contemporary clinical isolates of multidrug-resistant Gram-negative bacilli. *J Clin Microbiol* 51:1678–1684. <https://doi.org/10.1128/JCM.03385-12>.
  71. European Committee on Antimicrobial Susceptibility Testing (EUCAST). 2014. Breakpoint tables for interpretation of MICs and zone diameters, version 2.0. [http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST\\_files/Breakpoint\\_tables/Breakpoint\\_table\\_v\\_2.0\\_120221.pdf](http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Breakpoint_tables/Breakpoint_table_v_2.0_120221.pdf).
  72. Clinical and Laboratory Standards Institute (CLSI). 2014. Performance standards for antimicrobial susceptibility testing; 24th informational supplement. CLSI document M100-S24. Clinical and Laboratory Standards Institute, Wayne, PA.
  73. Clinical and Laboratory Standards Institute (CLSI). 2012. Methods for dilution of antimicrobial susceptibility tests for bacteria that grow aerobically; approved standard, 9th ed. Document M07-A9. Clinical and Laboratory Standards Institute, Wayne, PA.
  74. Schurek KN, Sampaio JL, Kiffer CR, Sinto S, Mendes CM, Hancock RE. 2009. Involvement of *pmrAB* and *phoPQ* in polymyxin B adaptation and inducible resistance in non-cystic fibrosis clinical isolates of *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother* 53:4345–4351. <https://doi.org/10.1128/AAC.01267-08>.
  75. Behera B, Mathur P, Das A, Kapil A, Gupta B, Bhoi S, Farooque K, Sharma V, Misra MC. 2010. Evaluation of susceptibility testing methods for polymyxin. *Int J Infect Dis* 14:e596–e601. <https://doi.org/10.1016/j.ijid.2009.09.001>.
  76. Lo-Ten-Foe JR, de Smet AM, Diederer BM, Kluytmans JA, van Keulen PH. 2007. Comparative evaluation of the VITEK 2, disk diffusion, Etest, broth microdilution, and agar dilution susceptibility testing methods for colistin in clinical isolates, including heteroresistant *Enterobacter cloacae* and *Acinetobacter baumannii* strains. *Antimicrob Agents Chemother* 51:3726–3730. <https://doi.org/10.1128/AAC.01406-06>.
  77. Hogardt M, Schmoltdt S, Gotzfried M, Adler K, Heesemann J. 2004. Pitfalls of polymyxin antimicrobial susceptibility testing of *Pseudomonas aeruginosa* isolated from cystic fibrosis patients. *J Antimicrob Chemother* 54:1057–1061. <https://doi.org/10.1093/jac/dkh470>.
  78. Moskowitz SM, Garber E, Chen Y, Clock SA, Tabibi S, Miller AK, Doctor M, Saiman L. 2010. Colistin susceptibility testing: evaluation of reliability for cystic fibrosis isolates of *Pseudomonas aeruginosa* and *Stenotrophomonas maltophilia*. *J Antimicrob Chemother* 65:1416–1423. <https://doi.org/10.1093/jac/dkq131>.
  79. Tan TY, Ng LS. 2006. Comparison of three standardized disc susceptibility testing methods for colistin. *J Antimicrob Chemother* 58:864–867. <https://doi.org/10.1093/jac/dkl330>.
  80. Maalej SM, Meziou MR, Rhimi FM, Hammami A. 2011. Comparison of disc diffusion, Etest and agar dilution for susceptibility testing of colistin against *Enterobacteriaceae*. *Lett Appl Microbiol* 53:546–551. <https://doi.org/10.1111/j.1472-765X.2011.03145.x>.
  81. Jayol A, Nordmann P, Brink A, Poirel L. 2015. Heteroresistance to colistin in *Klebsiella pneumoniae* associated with alterations in the PhoPQ regulatory system. *Antimicrob Agents Chemother* 59:2780–2784. <https://doi.org/10.1128/AAC.05055-14>.
  82. Arroyo LA, Garcia-Curiel A, Pachon-Ibanez ME, Llanos AC, Ruiz M, Pachon J, Aznar J. 2005. Reliability of the E-test method for detection of colistin resistance in clinical isolates of *Acinetobacter baumannii*. *J Clin Microbiol* 43:903–905. <https://doi.org/10.1128/JCM.43.2.903-905.2005>.
  83. Tan TY, Ng SY. 2007. Comparison of Etest, Vitek and agar dilution for susceptibility testing of colistin. *Clin Microbiol Infect* 13:541–544. <https://doi.org/10.1111/j.1469-0691.2007.01708.x>.
  84. Jorgensen JH, Ferraro MJ. 2009. Antimicrobial susceptibility testing: a review of general principles and contemporary practices. *Clin Infect Dis* 49:1749–1755. <https://doi.org/10.1086/647952>.
  85. Siniirtas M, Akalin H, Gedikoglu S. 2009. Investigation of colistin sensitivity via three different methods in *Acinetobacter baumannii* isolates with multiple antibiotic resistance. *Int J Infect Dis* 13:e217–e220. <https://doi.org/10.1016/j.ijid.2008.12.012>.
  86. Lee SY, Shin JH, Lee K, Joo MY, Park KH, Shin MG, Suh SP, Ryang DW, Kim SH. 2013. Comparison of the Vitek 2, MicroScan, and Etest methods with the agar dilution method in assessing colistin susceptibility of bloodstream isolates of *Acinetobacter* species from a Korean university hospital. *J Clin Microbiol* 51:1924–1926. <https://doi.org/10.1128/JCM.00427-13>.
  87. Perez LR. 2015. Evaluation of polymyxin susceptibility profile among KPC-producing *Klebsiella pneumoniae* using Etest and MicroScan Walk-Away automated system. *APMIS* 123:951–954. <https://doi.org/10.1111/apm.12438>.
  88. Olaitan AO, Morand S, Rolain JM. 2014. Mechanisms of polymyxin resistance: acquired and intrinsic resistance in bacteria. *Front Microbiol* 5:643. <https://doi.org/10.3389/fmicb.2014.00643>.
  89. Girardello R, Bispo PJ, Yamanaka TM, Gales AC. 2012. Cation concentration variability of four distinct Mueller-Hinton agar brands influences polymyxin B susceptibility results. *J Clin Microbiol* 50:2414–2418. <https://doi.org/10.1128/JCM.06686-11>.
  90. Chen CC, Feingold DS. 1972. Locus of divalent cation inhibition of the bactericidal action of polymyxin B. *Antimicrob Agents Chemother* 2:331–335. <https://doi.org/10.1128/AAC.2.5.331>.
  91. Newton BA. 1953. Reversal of the antibacterial activity of polymyxin by divalent cations. *Nature* 172:160–161. <https://doi.org/10.1038/172160a0>.
  92. Matzneller P, Strommer S, Osterreicher Z, Mitteregger D, Zeitlinger M. 2015. Target site antimicrobial activity of colistin might be misestimated if tested in conventional growth media. *Eur J Clin Microbiol Infect Dis* 34:1989–1994. <https://doi.org/10.1007/s10096-015-2441-7>.
  93. He J, Ledesma KR, Lam WY, Figueroa DA, Lim TP, Chow DS, Tam VH.



2010. Variability of polymyxin B major components in commercial formulations. *Int J Antimicrob Agents* 35:308–310. <https://doi.org/10.1016/j.ijantimicag.2009.11.005>.
94. Humphries RM. 2015. Susceptibility testing of the polymyxins: where are we now? *Pharmacotherapy* 35:22–27. <https://doi.org/10.1002/phar.1505>.
  95. Tam VH, Cao H, Ledesma KR, Hu M. 2011. In vitro potency of various polymyxin B components. *Antimicrob Agents Chemother* 55:4490–4491. <https://doi.org/10.1128/AAC.00119-11>.
  96. Landman D, Georgescu C, Martin DA, Quale J. 2008. Polymyxins revisited. *Clin Microbiol Rev* 21:449–465. <https://doi.org/10.1128/CMR.00006-08>.
  97. Karvanen MC, Mohamad A, Lagerback P. 2011. Colistin is extensively lost during normal experimental conditions, abstr D-690, p 160. *Abstr 51st Intersci Conf Antimicrob Agents Chemother*. American Society for Microbiology, Washington, DC.
  98. Albur M, Noel A, Bowker K, Macgowan A. 2014. Colistin susceptibility testing: time for a review. *J Antimicrob Chemother* 69:1432–1434. <https://doi.org/10.1093/jac/dkt503>.
  99. Sader HS, Rhomberg PR, Flamm RK, Jones RN. 2012. Use of a surfactant (polysorbate 80) to improve MIC susceptibility testing results for polymyxin B and colistin. *Diagn Microbiol Infect Dis* 74:412–414. <https://doi.org/10.1016/j.diagmicrobio.2012.08.025>.
  100. Brown MR, Winsley BE. 1968. Synergistic action of polysorbate 80 and polymyxin B sulphate on *Pseudomonas aeruginosa*. *J Gen Microbiol* 50(Suppl):ix.
  101. Brown MR, Geaton EM, Gilbert P. 1979. Additivity of action between polysorbate 80 and polymyxin B towards spheroplasts of *Pseudomonas aeruginosa* NCTC 6750. *J Pharm Pharmacol* 31:168–170. <https://doi.org/10.1111/j.2042-7158.1979.tb13463.x>.
  102. Sader HS, Rhomberg PR, Farrell DJ, Jones RN. 2015. Differences in potency and categorical agreement between colistin and polymyxin B when testing 15,377 clinical strains collected worldwide. *Diagn Microbiol Infect Dis* 83:379–381. <https://doi.org/10.1016/j.diagmicrobio.2015.08.013>.
  103. Sautrey G, Duval RE, Chevalley A, Fontanay S, Clarot I. 2015. Capillary electrophoresis for fast detection of heterogeneous population in colistin-resistant Gram-negative bacteria. *Electrophoresis* 36:2630–2633. <https://doi.org/10.1002/elps.201500064>.
  104. Tamayo M, Santiso R, Otero F, Bou G, Lepe JA, McConnell MJ, Cisneros JM, Gosalvez J, Fernandez JL. 2013. Rapid determination of colistin resistance in clinical strains of *Acinetobacter baumannii* by use of the Micromax assay. *J Clin Microbiol* 51:3675–3682. <https://doi.org/10.1128/JCM.01787-13>.
  105. Nordmann P, Jayol A, Poirel L. 2016. Rapid detection of polymyxin resistance in *Enterobacteriaceae*. *Emerg Infect Dis* 22:1038–1043. <https://doi.org/10.3201/eid2206.151840>.
  106. Jayol A, Dubois V, Poirel L, Nordmann P. 2016. Rapid detection of polymyxin-resistant *Enterobacteriaceae* from blood cultures. *J Clin Microbiol* 54:2273–2277. <https://doi.org/10.1128/JCM.00918-16>.
  107. Nordmann P, Jayol A, Poirel L. 2016. A universal culture medium for screening polymyxin-resistant Gram-negative isolates. *J Clin Microbiol* 54:1395–1399. <https://doi.org/10.1128/JCM.00446-16>.
  108. Levine M. 1943. The effect of concentration of dyes on differentiation of enteric bacteria on eosin-methylene-blue agar. *J Bacteriol* 45:471–475.
  109. Moffatt JH, Harper M, Harrison P, Hale JD, Vinogradov E, Seemann T, Henry R, Crane B, St Michael F, Cox AD, Adler B, Nation RL, Li J, Boyce JD. 2010. Colistin resistance in *Acinetobacter baumannii* is mediated by complete loss of lipopolysaccharide production. *Antimicrob Agents Chemother* 54:4971–4977. <https://doi.org/10.1128/AAC.00834-10>.
  110. Bontron S, Poirel L, Nordmann P. 2016. Real-time PCR for detection of plasmid-mediated polymyxin resistance (*mcr-1*) from cultured bacteria and stools. *J Antimicrob Chemother* 71:2318–2320. <https://doi.org/10.1093/jac/dkw139>.
  111. Aquilini E, Merino S, Knirel YA, Regue M, Tomas JM. 2014. Functional identification of *Proteus mirabilis* *eptC* gene encoding a core lipopolysaccharide phosphoethanolamine transferase. *Int J Mol Sci* 15:6689–6702. <https://doi.org/10.3390/ijms15046689>.
  112. Jiang SS, Liu MC, Teng LJ, Wang WB, Hsueh PR, Liaw SJ. 2010. *Proteus mirabilis* *pmrl*, an RppA-regulated gene necessary for polymyxin B resistance, biofilm formation, and urothelial cell invasion. *Antimicrob Agents Chemother* 54:1564–1571. <https://doi.org/10.1128/AAC.01219-09>.
  113. Lin QY, Tsai YL, Liu MC, Lin WC, Hsueh PR, Liaw SJ. 2014. *Serratia marcescens* *arn*, a PhoP-regulated locus necessary for polymyxin B resistance. *Antimicrob Agents Chemother* 58:5181–5190. <https://doi.org/10.1128/AAC.00013-14>.
  114. Gunn JS. 2008. The *Salmonella* PmrAB regulon: lipopolysaccharide modifications, antimicrobial peptide resistance and more. *Trends Microbiol* 16:284–290. <https://doi.org/10.1016/j.tim.2008.03.007>.
  115. Yan A, Guan Z, Raetz CR. 2007. An undecaprenyl phosphate-aminorabinose flippase required for polymyxin resistance in *Escherichia coli*. *J Biol Chem* 282:36077–36089. <https://doi.org/10.1074/jbc.M706172200>.
  116. Cannatelli A, Di Pilato V, Giani T, Arena F, Ambretti S, Gaibani P, D'Andrea MM, Rossolini GM. 2014. In vivo evolution to colistin resistance by PmrB sensor kinase mutation in KPC-producing *Klebsiella pneumoniae* is associated with low-dosage colistin treatment. *Antimicrob Agents Chemother* 58:4399–4403. <https://doi.org/10.1128/AAC.02555-14>.
  117. Cheng YH, Lin TL, Pan YJ, Wang YP, Lin YT, Wang JT. 2015. Colistin resistance mechanisms in *Klebsiella pneumoniae* strains from Taiwan. *Antimicrob Agents Chemother* 59:2909–2913. <https://doi.org/10.1128/AAC.04763-14>.
  118. Choi MJ, Ko KS. 2014. Mutant prevention concentrations of colistin for *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* clinical isolates. *J Antimicrob Chemother* 69:275–277. <https://doi.org/10.1093/jac/dkt315>.
  119. Jayol A, Poirel L, Brink A, Villegas MV, Yilmaz M, Nordmann P. 2014. Resistance to colistin associated with a single amino acid change in protein PmrB among *Klebsiella pneumoniae* isolates of worldwide origin. *Antimicrob Agents Chemother* 58:4762–4766. <https://doi.org/10.1128/AAC.00084-14>.
  120. Olaitan AO, Diene SM, Kempf M, Berrazeg M, Bakour S, Gupta SK, Thongmalayong B, Akkhavong K, Somphavong S, Paboriboune P, Chaisiri K, Komalamisra C, Adelowo OO, Fagade OE, Banjo OA, Oke AJ, Adler A, Assous MV, Morand S, Raoult D, Rolain JM. 2014. Worldwide emergence of colistin resistance in *Klebsiella pneumoniae* from healthy humans and patients in Lao PDR, Thailand, Israel, Nigeria and France owing to inactivation of the PhoP/PhoQ regulator *mgrB*: an epidemiological and molecular study. *Int J Antimicrob Agents* 44:500–507. <https://doi.org/10.1016/j.ijantimicag.2014.07.020>.
  121. Diene SM, Merhej V, Henry M, El Filali A, Roux V, Robert C, Azza S, Gavory F, Barbe V, La Scola B, Raoult D, Rolain JM. 2013. The rhizome of the multidrug-resistant *Enterobacter aerogenes* genome reveals how new “killer bugs” are created because of a sympatric lifestyle. *Mol Biol Evol* 30:369–383. <https://doi.org/10.1093/molbev/mss236>.
  122. Olaitan AO, Dia NM, Gautret P, Benkouiten S, Belhouchat K, Drali T, Parola P, Brouqui P, Memish Z, Raoult D, Rolain JM. 2015. Acquisition of extended-spectrum cephalosporin- and colistin-resistant *Salmonella enterica* subsp. *enterica* serotype Newport by pilgrims during Hajj. *Int J Antimicrob Agents* 45:600–604. <https://doi.org/10.1016/j.ijantimicag.2015.01.010>.
  123. Sun S, Negrea A, Rhen M, Andersson DI. 2009. Genetic analysis of colistin resistance in *Salmonella enterica* serovar Typhimurium. *Antimicrob Agents Chemother* 53:2298–2305. <https://doi.org/10.1128/AAC.01016-08>.
  124. Olaitan AO, Morand S, Rolain JM. 2016. Emergence of colistin-resistant bacteria in humans without colistin usage: a new worry and cause for vigilance. *Int J Antimicrob Agents* 47:1–3. <https://doi.org/10.1016/j.ijantimicag.2015.11.009>.
  125. Quesada A, Porrero MC, Tellez S, Palomo G, Garcia M, Dominguez L. 2015. Polymorphism of genes encoding PmrAB in colistin-resistant strains of *Escherichia coli* and *Salmonella enterica* isolated from poultry and swine. *J Antimicrob Chemother* 70:71–74. <https://doi.org/10.1093/jac/dku320>.
  126. Groisman EA. 2001. The pleiotropic two-component regulatory system PhoP-PhoQ. *J Bacteriol* 183:1835–1842. <https://doi.org/10.1128/JB.183.6.1835-1842.2001>.
  127. Park SY, Groisman EA. 2014. Signal-specific temporal response by the *Salmonella* PhoP/PhoQ regulatory system. *Mol Microbiol* 91:135–144. <https://doi.org/10.1111/mmi.12449>.
  128. Wright MS, Suzuki Y, Jones MB, Marshall SH, Rudin SD, van Duin D, Kaye K, Jacobs MR, Bonomo RA, Adams MD. 2015. Genomic and transcriptomic analyses of colistin-resistant clinical isolates of *Klebsiella pneumoniae* reveal multiple pathways of resistance. *Antimicrob Agents Chemother* 59:536–543. <https://doi.org/10.1128/AAC.04037-14>.
  129. Lippa AM, Goulian M. 2009. Feedback inhibition in the PhoQ/PhoP

- signaling system by a membrane peptide. *PLoS Genet* 5:e1000788. <https://doi.org/10.1371/journal.pgen.1000788>.
130. Cannatelli A, Giani T, D'Andrea MM, Di Pilato V, Arena F, Conte V, Tryfinopoulou K, Vatopoulos A, Rossolini G. 2014. MgrB inactivation is a common mechanism of colistin resistance in KPC-producing *Klebsiella pneumoniae* of clinical origin. *Antimicrob Agents Chemother* 58:5696–5703. <https://doi.org/10.1128/AAC.03110-14>.
  131. Poirel L, Jayol A, Bontron S, Villegas MV, Ozdamar M, Turkoglu S, Nordmann P. 2015. The *mgrB* gene as a key target for acquired resistance to colistin in *Klebsiella pneumoniae*. *J Antimicrob Chemother* 70:75–80. <https://doi.org/10.1093/jac/dku323>.
  132. Lopez-Camacho E, Gomez-Gil R, Tobes R, Manrique M, Lorenzo M, Galvan B, Salvarelli E, Moatassim Y, Salanueva JJ, Pareja E, Codoner FM, Alvarez-Tejado M, Garcillan-Barcia MP, De la Cruz F, Mingorance J. 2014. Genomic analysis of the emergence and evolution of multidrug resistance during a *Klebsiella pneumoniae* outbreak including carbapenem and colistin resistance. *J Antimicrob Chemother* 69:632–636. <https://doi.org/10.1093/jac/dkt419>.
  133. Jayol A, Poirel L, Villegas MV, Nordmann P. 2015. Modulation of *mgrB* gene expression as a source of colistin resistance in *Klebsiella oxytoca*. *Int J Antimicrob Agents* 46:108–110. <https://doi.org/10.1016/j.ijantimicag.2015.02.015>.
  134. Olaitan AO, Rolain JM. 2015. Interruption of *mgrB* in the mediation of colistin resistance in *Klebsiella oxytoca*. *Int J Antimicrob Agents* 46:354–355. <https://doi.org/10.1016/j.ijantimicag.2015.06.003>.
  135. Jayol A, Nordmann P, Desroches M, Descousser JW, Poirel L. 2016. Acquisition of broad-spectrum cephalosporin resistance leading to colistin resistance in *Klebsiella pneumoniae*. *Antimicrob Agents Chemother* 60:3199–3201. <https://doi.org/10.1128/AAC.00237-16>.
  136. Zowawi HM, Forde BM, Alfaresi M, Alzarouni A, Farahat Y, Chong TM, Yin WF, Chan KG, Li J, Schembri MA, Beatson SA, Paterson DL. 2015. Stepwise evolution of pandrug-resistance in *Klebsiella pneumoniae*. *Sci Rep* 5:15082. <https://doi.org/10.1038/srep15082>.
  137. Cheng YH, Lin TL, Lin YT, Wang JT. 2016. Amino acid substitutions of CrrB responsible for resistance to colistin through CrrC in *Klebsiella pneumoniae*. *Antimicrob Agents Chemother* 60:3709–3716. <https://doi.org/10.1128/AAC.00009-16>.
  138. De Majumdar S, Yu J, Fookes M, McAteer SP, Llobet E, Finn S, Spence S, Monahan A, Kissenpennig A, Ingram RJ, Bengoechea J, Gally DL, Fanning S, Elborn JS, Schneiders T. 2015. Elucidation of the RamA regulon in *Klebsiella pneumoniae* reveals a role in LPS regulation. *PLoS Pathog* 11:e1004627. <https://doi.org/10.1371/journal.ppat.1004627>.
  139. Liu YY, Wang Y, Walsh TR, Yi LX, Zhang R, Spencer J, Doi Y, Tian G, Dong B, Huang X, Yu LF, Gu D, Ren H, Chen X, Lv L, He D, Zhou H, Liang Z, Liu JH, Shen J. 2016. Emergence of plasmid-mediated colistin resistance mechanism MCR-1 in animals and human beings in China: a microbiological and molecular biological study. *Lancet Infect Dis* 16:161–168. [https://doi.org/10.1016/S1473-3099\(15\)00424-7](https://doi.org/10.1016/S1473-3099(15)00424-7).
  140. Sherman EX, Hufnagel DA, Weiss DS. 2016. MCR-1 confers cross-resistance to lysozyme. *Lancet Infect Dis* 16:1226–1227. [https://doi.org/10.1016/S1473-3099\(16\)30395-4](https://doi.org/10.1016/S1473-3099(16)30395-4).
  141. Stojanoski V, Sankaran B, Prasad BV, Poirel L, Nordmann P, Palzkill T. 2016. Structure of the catalytic domain of the colistin resistance enzyme MCR-1. *BMC Biol* 14:81. <https://doi.org/10.1186/s12915-016-0303-0>.
  142. Shen Z, Wang Y, Shen Y, Shen J, Wu C. 2016. Early emergence of *mcr-1* in *Escherichia coli* from food-producing animals. *Lancet Infect Dis* 16:293. [https://doi.org/10.1016/S1473-3099\(16\)00061-X](https://doi.org/10.1016/S1473-3099(16)00061-X).
  143. Zurfluh K, Poirel L, Nordmann P, Nuesch-Inderbinen M, Hachler H, Stephan R. 2016. Occurrence of the plasmid-borne *mcr-1* colistin resistance gene in ESBL-producing *Enterobacteriaceae* in river water and imported vegetable samples in Switzerland. *Antimicrob Agents Chemother* 60:2594–2595. <https://doi.org/10.1128/AAC.00066-16>.
  144. Poirel L, Nordmann P. 2016. Plasmid-encoded colistin resistance: the animal world as the culprit? *J Antimicrob Chemother* 71:2326–2327. <https://doi.org/10.1093/jac/dkw074>.
  145. Thanh DP, Tuyen HT, Nguy T, Nguyen ET, The HC, Wick RR, Thwaites G, Baker S, Holt KE. 2016. Inducible colistin resistance via a disrupted plasmid-borne *mcr-1* gene in a 2008 Vietnamese *Shigella sonnei* isolate. *J Antimicrob Chemother* 71:2314–2317. <https://doi.org/10.1093/jac/dkw173>.
  146. Yao X, Doi Y, Zeng L, Lv L, Liu JH. 2016. Carbapenem-resistant and colistin-resistant *Escherichia coli* co-producing NDM-9 and MCR-1. *Lancet Infect Dis* 16:288–289. [https://doi.org/10.1016/S1473-3099\(16\)00057-8](https://doi.org/10.1016/S1473-3099(16)00057-8).
  147. Du H, Chen L, Tang YW, Kreiswirth BN. 2016. Emergence of the *mcr-1* colistin resistance gene in carbapenem-resistant *Enterobacteriaceae*. *Lancet Infect Dis* 16:287–288. [https://doi.org/10.1016/S1473-3099\(16\)00056-6](https://doi.org/10.1016/S1473-3099(16)00056-6).
  148. Zhong LL, Zhang YF, Doi Y, Huang X, Zhang XF, Zeng KJ, Shen C, Patil S, Xing Y, Zou Y, Tian GB. 2017. Coproduction of MCR-1 and NDM-1 by colistin-resistant *Escherichia coli* isolated from a healthy individual. *Antimicrob Agents Chemother* 61:e01962-16. <https://doi.org/10.1128/AAC.01962-16>.
  149. Poirel L, Kieffer N, Liassine N, Thanh D, Nordmann P. 2016. Plasmid-mediated carbapenem and colistin resistance in a clinical isolate of *Escherichia coli*. *Lancet Infect Dis* 16:281. [https://doi.org/10.1016/S1473-3099\(16\)00006-2](https://doi.org/10.1016/S1473-3099(16)00006-2).
  150. Falgenhauer L, Waezsada SE, Yao Y, Imirzalioglu C, Kasbohrer A, Roesler U, Michael GB, Schwarz S, Werner G, Kreienbrock L, Chakraborty T. 2016. Colistin resistance gene *mcr-1* in extended-spectrum beta-lactamase-producing and carbapenemase-producing Gram-negative bacteria in Germany. *Lancet Infect Dis* 16:282–283. [https://doi.org/10.1016/S1473-3099\(16\)00009-8](https://doi.org/10.1016/S1473-3099(16)00009-8).
  151. Mulvey MR, Mataseje LF, Robertson J, Nash JH, Boerlin P, Toye B, Irwin R, Melano RG. 2016. Dissemination of the *mcr-1* colistin resistance gene. *Lancet Infect Dis* 16:289–290. [https://doi.org/10.1016/S1473-3099\(16\)00067-0](https://doi.org/10.1016/S1473-3099(16)00067-0).
  152. Liu BT, Song FJ, Zou M, Hao ZH, Shan H. 2017. Emergence of colistin resistance gene *mcr-1* in *Cronobacter sakazakii* producing NDM-9 and *Escherichia coli* from the same animal. *Antimicrob Agents Chemother* 61:e01444-16. <https://doi.org/10.1128/AAC.01444-16>.
  153. Zurfluh K, Tasara T, Poirel L, Nordmann P, Stephan R. 2016. Draft genome sequence of *Escherichia coli* S51, a chicken isolate harboring a chromosomally encoded *mcr-1* gene. *Genome Announc* 4:e00796-16. <https://doi.org/10.1128/genomeA.00796-16>.
  154. Poirel L, Kieffer N, Brink A, Coetze J, Jayol A, Nordmann P. 2016. Genetic features of MCR-1-producing colistin-resistant *Escherichia coli* isolates, South Africa. *Antimicrob Agents Chemother* 60:4394–4397. <https://doi.org/10.1128/AAC.00444-16>.
  155. Snesrud E, He S, Chandler M, Dekker JP, Hickman AB, McGann P, Dyda F. 2016. A model for transposition of the colistin resistance gene *mcr-1* by IS*Ap1*. *Antimicrob Agents Chemother* 60:6973–6976. <https://doi.org/10.1128/AAC.01457-16>.
  156. Nordmann P, Lienhard R, Kieffer N, Clerc O, Poirel L. 2016. Plasmid-mediated colistin-resistant *Escherichia coli* in bacteremia in Switzerland. *Clin Infect Dis* 62:1322–1323. <https://doi.org/10.1093/cid/ciw124>.
  157. Di Pilato V, Arena F, Tascini C, Cannatelli A, Henrici De Angelis L, Fortunato S, Giani T, Menichetti F, Rossolini GM. 2016. *mcr-1.2*, a new *mcr* variant carried on a transferable plasmid from a colistin-resistant KPC carbapenemase-producing *Klebsiella pneumoniae* strain of sequence type 512. *Antimicrob Agents Chemother* 60:5612–5615. <https://doi.org/10.1128/AAC.01075-16>.
  158. Xavier BB, Lammens C, Ruhel R, Kumar-Singh S, Butaye P, Goossens H, Malhotra-Kumar S. 2016. Identification of a novel plasmid-mediated colistin-resistance gene, *mcr-2*, in *Escherichia coli*, Belgium, June 2016. *Euro Surveill* 21:30280. <https://doi.org/10.2807/1560-7917.ES.21.27.30280>.
  159. Campos MA, Vargas MA, Regueiro V, Llopart CM, Alberti S, Bengoechea JA. 2004. Capsule polysaccharide mediates bacterial resistance to antimicrobial peptides. *Infect Immun* 72:7107–7114. <https://doi.org/10.1128/IAI.72.12.7107-7114.2004>.
  160. Llobet E, Tomas JM, Bengoechea JA. 2008. Capsule polysaccharide is a bacterial decoy for antimicrobial peptides. *Microbiology* 154:3877–3886. <https://doi.org/10.1099/mic.0.2008/022301-0>.
  161. Fresno S, Jimenez N, Izquierdo L, Merino S, Corsaro MM, De Castro C, Parrilli M, Naldi T, Regue M, Tomas JM. 2006. The ionic interaction of *Klebsiella pneumoniae* K2 capsule and core lipopolysaccharide. *Microbiology* 152:1807–1818. <https://doi.org/10.1099/mic.0.28611-0>.
  162. Pilonieta MC, Erickson KD, Ernst RK, Detweiler CS. 2009. A protein important for antimicrobial peptide resistance, Ydel/OmdA, is in the periplasm and interacts with OmpD/NmpC. *J Bacteriol* 191:7243–7252. <https://doi.org/10.1128/JB.00688-09>.
  163. Padilla E, Llobet E, Domenech-Sanchez A, Martinez-Martinez L, Bengoechea JA, Alberti S. 2010. *Klebsiella pneumoniae* AcrAB efflux pump contributes to antimicrobial resistance and virulence. *Antimicrob Agents Chemother* 54:177–183. <https://doi.org/10.1128/AAC.00715-09>.
  164. Srinivasan VB, Rajamohan G. 2013. KpnEF, a new member of the *Klebsiella pneumoniae* cell envelope stress response regulon, is an SMR-type efflux pump involved in broad-spectrum antimicrobial resis-

- tance. *Antimicrob Agents Chemother* 57:4449–4462. <https://doi.org/10.1128/AAC.02284-12>.
165. Ni W, Li Y, Guan J, Zhao J, Cui J, Wang R, Liu Y. 2016. Effects of efflux pump inhibitors on colistin resistance in multidrug resistant Gram-negative bacteria. *Antimicrob Agents Chemother* 60:3215–3218. <https://doi.org/10.1128/AAC.00248-16>.
  166. Lee JY, Ko KS. 2014. Mutations and expression of PmrAB and PhoPQ related with colistin resistance in *Pseudomonas aeruginosa* clinical isolates. *Diagn Microbiol Infect Dis* 78:271–276. <https://doi.org/10.1016/j.diagmicrobio.2013.11.027>.
  167. Moskowicz SM, Brannon MK, Dasgupta N, Pier M, Sgambati N, Miller AK, Selgrade SE, Miller SI, Denton M, Conway SP, Johansen HK, Høiby N. 2012. PmrB mutations promote polymyxin resistance of *Pseudomonas aeruginosa* isolated from colistin-treated cystic fibrosis patients. *Antimicrob Agents Chemother* 56:1019–1030. <https://doi.org/10.1128/AAC.05829-11>.
  168. Barrow K, Kwon DH. 2009. Alterations in two-component regulatory systems of *phoPQ* and *pmrAB* are associated with polymyxin B resistance in clinical isolates of *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother* 53:5150–5154. <https://doi.org/10.1128/AAC.00893-09>.
  169. Abraham N, Kwon DH. 2009. A single amino acid substitution in PmrB is associated with polymyxin B resistance in clinical isolate of *Pseudomonas aeruginosa*. *FEMS Microbiol Lett* 298:249–254. <https://doi.org/10.1111/j.1574-6968.2009.01720.x>.
  170. Miller AK, Brannon MK, Stevens L, Johansen HK, Selgrade SE, Miller SI, Høiby N, Moskowicz SM. 2011. PhoQ mutations promote lipid A modification and polymyxin resistance of *Pseudomonas aeruginosa* found in colistin-treated cystic fibrosis patients. *Antimicrob Agents Chemother* 55:5761–5769. <https://doi.org/10.1128/AAC.05391-11>.
  171. Muller C, Plesiat P, Jeannot K. 2011. A two-component regulatory system interconnects resistance to polymyxins, aminoglycosides, fluoroquinolones, and beta-lactams in *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother* 55:1211–1221. <https://doi.org/10.1128/AAC.01252-10>.
  172. Gutu AD, Sgambati N, Strasbourger P, Brannon MK, Jacobs MA, Haugen E, Kaul RK, Johansen HK, Høiby N, Moskowicz SM. 2013. Polymyxin resistance of *Pseudomonas aeruginosa phoQ* mutants is dependent on additional two-component regulatory systems. *Antimicrob Agents Chemother* 57:2204–2215. <https://doi.org/10.1128/AAC.02353-12>.
  173. Adams MD, Nickel GC, Bajaksouzian S, Lavender H, Murthy AR, Jacobs MR, Bonomo RA. 2009. Resistance to colistin in *Acinetobacter baumannii* associated with mutations in the PmrAB two-component system. *Antimicrob Agents Chemother* 53:3628–3634. <https://doi.org/10.1128/AAC.00284-09>.
  174. Arroyo LA, Herrera CM, Fernandez L, Hankins JV, Trent MS, Hancock RE. 2011. The *pmrCAB* operon mediates polymyxin resistance in *Acinetobacter baumannii* ATCC 17978 and clinical isolates through phosphoethanolamine modification of lipid A. *Antimicrob Agents Chemother* 55:3743–3751. <https://doi.org/10.1128/AAC.00256-11>.
  175. Beceiro A, Llobet E, Aranda J, Bengoechea JA, Doumith M, Hornsey M, Dhanji H, Chart H, Bou G, Livermore DM, Woodford N. 2011. Phosphoethanolamine modification of lipid A in colistin-resistant variants of *Acinetobacter baumannii* mediated by the *pmrAB* two-component regulatory system. *Antimicrob Agents Chemother* 55:3370–3379. <https://doi.org/10.1128/AAC.00079-11>.
  176. Kim Y, Bae IK, Lee H, Jeong SH, Yong D, Lee K. 2014. In vivo emergence of colistin resistance in *Acinetobacter baumannii* clinical isolates of sequence type 357 during colistin treatment. *Diagn Microbiol Infect Dis* 79:362–366. <https://doi.org/10.1016/j.diagmicrobio.2014.03.027>.
  177. Lesho E, Yoon EJ, McGann P, Snesrud E, Kwak Y, Milillo M, Onmus-Leone F, Preston L, St Clair K, Nikolich M, Viscount H, Wortmann G, Zapor M, Grillot-Courvalin C, Courvalin P, Clifford R, Waterman PE. 2013. Emergence of colistin-resistance in extremely drug-resistant *Acinetobacter baumannii* containing a novel *pmrCAB* operon during colistin therapy of wound infections. *J Infect Dis* 208:1142–1151. <https://doi.org/10.1093/infdis/jit293>.
  178. Park YK, Choi JY, Shin D, Ko KS. 2011. Correlation between overexpression and amino acid substitution of the PmrAB locus and colistin resistance in *Acinetobacter baumannii*. *Int J Antimicrob Agents* 37:525–530. <https://doi.org/10.1016/j.ijantimicag.2011.02.008>.
  179. Pournaras S, Poulou A, Dafopoulou K, Chabane YN, Kristo I, Makris D, Hardouin J, Cosette P, Tsakris A, Dé E. 2014. Growth retardation, reduced invasiveness, and impaired colistin-mediated cell death associated with colistin resistance development in *Acinetobacter baumannii*. *Antimicrob Agents Chemother* 58:828–832. <https://doi.org/10.1128/AAC.01439-13>.
  180. Rolain JM, Diene SM, Kempf M, Gimenez G, Robert C, Raoult D. 2013. Real-time sequencing to decipher the molecular mechanism of resistance of a clinical pan-drug-resistant *Acinetobacter baumannii* isolate from Marseille, France. *Antimicrob Agents Chemother* 57:592–596. <https://doi.org/10.1128/AAC.01314-12>.
  181. Moffatt JH, Harper M, Adler B, Nation RL, Li J, Boyce JD. 2011. Insertion sequence IS*Aba11* is involved in colistin resistance and loss of lipopolysaccharide in *Acinetobacter baumannii*. *Antimicrob Agents Chemother* 55:3022–3024. <https://doi.org/10.1128/AAC.01732-10>.
  182. Ah YM, Kim AJ, Lee JY. 2014. Colistin resistance in *Klebsiella pneumoniae*. *Int J Antimicrob Agents* 44:8–15. <https://doi.org/10.1016/j.ijantimicag.2014.02.016>.
  183. Gales AC, Jones RN, Sader HS. 2011. Contemporary activity of colistin and polymyxin B against a worldwide collection of Gram-negative pathogens: results from the SENTRY Antimicrobial Surveillance Program (2006–09). *J Antimicrob Chemother* 66:2070–2074. <https://doi.org/10.1093/jac/dkr239>.
  184. Walky A, DeCorby M, Nichol K, Karlowsky JA, Hoban DJ, Zhanel GG. 2009. In vitro activity of colistin (polymyxin E) against 3,480 isolates of Gram-negative bacilli obtained from patients in Canadian hospitals in the CANWARD study, 2007–2008. *Antimicrob Agents Chemother* 53:4924–4926. <https://doi.org/10.1128/AAC.00786-09>.
  185. Sutherland CA, Nicolau DP. 2015. Susceptibility profile of ceftolozane/tazobactam and other parenteral antimicrobials against *Escherichia coli*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa* from U.S. hospitals. *Clin Ther* 37:1564–1571. <https://doi.org/10.1016/j.clinthera.2015.05.501>.
  186. Tijet N, Sheth PM, Lastovetska O, Chung C, Patel SN, Melano RG. 2014. Molecular characterization of *Klebsiella pneumoniae* carbapenemase (KPC)-producing *Enterobacteriaceae* in Ontario, Canada, 2008–2011. *PLoS One* 9:e116421. <https://doi.org/10.1371/journal.pone.0116421>.
  187. Bratu S, Tolaneu P, Karumudi U, Quale J, Mooty M, Nichani S, Landman D. 2005. Carbapenemase-producing *Klebsiella pneumoniae* in Brooklyn, NY: molecular epidemiology and in vitro activity of polymyxin B and other agents. *J Antimicrob Chemother* 56:128–132. <https://doi.org/10.1093/jac/dki175>.
  188. Bogdanovich T, Adams-Haduch JM, Tian GB, Nguyen MH, Kwak EJ, Muto CA, Doi Y. 2011. Colistin-resistant, *Klebsiella pneumoniae* carbapenemase (KPC)-producing *Klebsiella pneumoniae* belonging to the international epidemic clone ST258. *Clin Infect Dis* 53:373–376. <https://doi.org/10.1093/cid/cir401>.
  189. Marchaim D, Chopra T, Pogue JM, Perez F, Hujer AM, Rudin S, Endimiani A, Navon-Venezia S, Hothi J, Slim J, Blunden C, Shango M, Lephart PR, Salimnia H, Reid D, Moshos J, Hafeez W, Bheemreddy S, Chen TY, Dhar S, Bonomo RA, Kaye KS. 2011. Outbreak of colistin-resistant, carbapenem-resistant *Klebsiella pneumoniae* in metropolitan Detroit, Michigan. *Antimicrob Agents Chemother* 55:593–599. <https://doi.org/10.1128/AAC.01020-10>.
  190. Garza-Ramos U, Barrios H, Reyna-Flores F, Sanchez-Perez A, Tamayo-Legorreta E, Ibarra-Pacheco A, Salazar-Salinas J, Nunez-Ceballos R, Silva-Sanchez J. 2014. Characteristics of KPC-2-producing *Klebsiella pneumoniae* (ST258) clinical isolates from outbreaks in 2 Mexican medical centers. *Diagn Microbiol Infect Dis* 79:483–485. <https://doi.org/10.1016/j.diagmicrobio.2014.05.010>.
  191. Arduino SM, Quiroga MP, Ramirez MS, Merckier AK, Errecalde L, Di Martino A, Smayevsky J, Kaufman S, Centron D. 2012. Transposons and integrons in colistin-resistant clones of *Klebsiella pneumoniae* and *Acinetobacter baumannii* with epidemic or sporadic behaviour. *J Med Microbiol* 61:1417–1420. <https://doi.org/10.1099/jmm.0.038968-0>.
  192. Weterings V, Zhou K, Rossen JW, van Stenis D, Thewessen E, Kluytmans J, Veenemans J. 2015. An outbreak of colistin-resistant *Klebsiella pneumoniae* carbapenemase-producing *Klebsiella pneumoniae* in the Netherlands (July to December 2013), with inter-institutional spread. *Eur J Clin Microbiol Infect Dis* 34:1647–1655. <https://doi.org/10.1007/s10096-015-2401-2>.
  193. Toth A, Damjanova I, Puskas E, Janvari L, Farkas M, Dobak A, Borocz K, Paszti J. 2010. Emergence of a colistin-resistant KPC-2-producing *Klebsiella pneumoniae* ST258 clone in Hungary. *Eur J Clin Microbiol Infect Dis* 29:765–769. <https://doi.org/10.1007/s10096-010-0921-3>.
  194. Kontopoulou K, Protonotariou E, Vasilakos K, Kriti M, Koteli A, Antoniadou E, Sofianou D. 2010. Hospital outbreak caused by *Klebsiella pneu-*



- moniae* producing KPC-2 beta-lactamase resistant to colistin. J Hosp Infect 76:70–73. <https://doi.org/10.1016/j.jhin.2010.03.021>.
195. Antoniadou A, Kontopidou F, Poulakou G, Koratzanis E, Galani I, Papadomichelakis E, Kopterides P, Souli M, Armaganidis A, Giamarellou H. 2007. Colistin-resistant isolates of *Klebsiella pneumoniae* emerging in intensive care unit patients: first report of a multiclonal cluster. J Antimicrob Chemother 59:786–790. <https://doi.org/10.1093/jac/dkl562>.
  196. Mezzatesta ML, Gona F, Caio C, Petrolito V, Sciortino D, Sciacca A, Santangelo C, Stefani S. 2011. Outbreak of KPC-3-producing, and colistin-resistant, *Klebsiella pneumoniae* infections in two Sicilian hospitals. Clin Microbiol Infect 17:1444–1447. <https://doi.org/10.1111/j.1469-0691.2011.03572.x>.
  197. Mammina C, Bonura C, Di Bernardo F, Aleo A, Fasciana T, Sodano C, Saporito MA, Verde MS, Tetamo R, Palma DM. 2012. Ongoing spread of colistin-resistant *Klebsiella pneumoniae* in different wards of an acute general hospital, Italy, June to December 2011. Euro Surveill 17:20248.
  198. Giani T, Arena F, Vaggelli G, Conte V, Chiarelli A, Henrici De Angelis L, Fornaini R, Grazzini M, Niccolini F, Pecile P, Rossolini GM. 2015. Large nosocomial outbreak of colistin-resistant, carbapenemase-producing *Klebsiella pneumoniae* traced to clonal expansion of an *mgrB* deletion mutant. J Clin Microbiol 53:3341–3344. <https://doi.org/10.1128/JCM.01017-15>.
  199. Parisi SG, Bartolini A, Santacatterina E, Castellani E, Ghirardo R, Berto A, Franchin E, Menegotto N, De Canale E, Tommasini T, Rinaldi R, Basso M, Stefani S, Palu G. 2015. Prevalence of *Klebsiella pneumoniae* strains producing carbapenemases and increase of resistance to colistin in an Italian teaching hospital from January 2012 to December 2014. BMC Infect Dis 15:244. <https://doi.org/10.1186/s12879-015-0996-7>.
  200. Mezzatesta ML, Caio C, Gona F, Cormaci R, Salerno I, Zingali T, Denaro C, Gennaro M, Quattrone C, Stefani S. 2014. Carbapenem and multidrug resistance in Gram-negative bacteria in a single centre in Italy: considerations on in vitro assay of active drugs. Int J Antimicrob Agents 44:112–116. <https://doi.org/10.1016/j.ijantimicag.2014.04.014>.
  201. Monaco M, Giani T, Raffone M, Arena F, Garcia-Fernandez A, Pollini S, Grundmann H, Pantosti A, Rossolini GM. 2014. Colistin resistance superimposed to endemic carbapenem-resistant *Klebsiella pneumoniae*: a rapidly evolving problem in Italy, November 2013 to April 2014. Euro Surveill 19:20939. <https://doi.org/10.2807/1560-7917.ES2014.19.42.20939>.
  202. Meletis G, Oustas E, Botziori C, Kakasi E, Koteli A. 2015. Containment of carbapenem resistance rates of *Klebsiella pneumoniae* and *Acinetobacter baumannii* in a Greek hospital with a concomitant increase in colistin, gentamicin and tigecycline resistance. New Microbiol 38: 417–421.
  203. Zagorianou A, Sianou E, Iosifidis E, Dimou V, Protonotariou E, Miyakis S, Roilides E, Sofianou D. 2012. Microbiological and molecular characteristics of carbapenemase-producing *Klebsiella pneumoniae* endemic in a tertiary Greek hospital during 2004–2010. Euro Surveill 17:20088.
  204. Pena I, Picazo JJ, Rodriguez-Avial C, Rodriguez-Avial I. 2014. Carbapenemase-producing *Enterobacteriaceae* in a tertiary hospital in Madrid, Spain: high percentage of colistin resistance among VIM-1-producing *Klebsiella pneumoniae* ST11 isolates. Int J Antimicrob Agents 43:460–464. <https://doi.org/10.1016/j.ijantimicag.2014.01.021>.
  205. Jayol A, Poirel L, Dortet L, Nordmann P. 2016. National survey of colistin resistance among carbapenemase-producing *Enterobacteriaceae* and outbreak caused by colistin-resistant OXA-48-producing *Klebsiella pneumoniae*, France, 2014. Euro Surveill 21:30339. <https://doi.org/10.2807/1560-7917.ES.2016.21.37.30339>.
  206. Suh JY, Son JS, Chung DR, Peck KR, Ko KS, Song JH. 2010. Nonclonal emergence of colistin-resistant *Klebsiella pneumoniae* isolates from blood samples in South Korea. Antimicrob Agents Chemother 54: 560–562. <https://doi.org/10.1128/AAC.00762-09>.
  207. Tan TY, Ng SY. 2006. The in-vitro activity of colistin in Gram-negative bacteria. Singapore Med J 47:621–624.
  208. Chen S, Hu F, Zhang X, Xu X, Liu Y, Zhu D, Wang H. 2011. Independent emergence of colistin-resistant *Enterobacteriaceae* clinical isolates without colistin treatment. J Clin Microbiol 49:4022–4023. <https://doi.org/10.1128/JCM.01233-11>.
  209. Chiu SK, Wu TL, Chuang YC, Lin JC, Fung CP, Lu PL, Wang JT, Wang LS, Siu LK, Yeh KM. 2013. National surveillance study on carbapenem non-susceptible *Klebsiella pneumoniae* in Taiwan: the emergence and rapid dissemination of KPC-2 carbapenemase. PLoS One 8:e69428. <https://doi.org/10.1371/journal.pone.0069428>.
  210. Matthaïou DK, Michalopoulos A, Rafailidis Pl, Karageorgopoulos DE, Papaïoannou V, Ntani G, Samonis G, Falagas ME. 2008. Risk factors associated with the isolation of colistin-resistant Gram-negative bacteria: a matched case-control study. Crit Care Med 36:807–811. <https://doi.org/10.1097/CCM.0B013E3181652FAE>.
  211. Kontopidou F, Plachouras D, Papadomichelakis E, Koukos G, Galani I, Poulakou G, Dimopoulos G, Antoniadou A, Armaganidis A, Giamarellou H. 2013. Colonization and infection by colistin-resistant Gram-negative bacteria in a cohort of critically ill patients. Clin Microbiol Infect 17: E9–E11. <https://doi.org/10.1111/j.1469-0691.2011.03649.x>.
  212. Halaby T, Al Naiemi N, Kluytmans J, van der Palen J, Vandembroucke-Grauls CM. 2013. Emergence of colistin resistance in *Enterobacteriaceae* after the introduction of selective digestive tract decontamination in an intensive care unit. Antimicrob Agents Chemother 57:3224–3229. <https://doi.org/10.1128/AAC.02634-12>.
  213. Strenger V, Gschliesser T, Grisold A, Zarfel G, Feierl G, Masoud L, Hoenigl M, Resch B, Muller W, Urlesberger B. 2011. Orally administered colistin leads to colistin-resistant intestinal flora and fails to prevent faecal colonisation with extended-spectrum beta-lactamase-producing enterobacteria in hospitalised newborns. Int J Antimicrob Agents 37: 67–69. <https://doi.org/10.1016/j.ijantimicag.2010.09.010>.
  214. Durante-Mangoni E, Del Franco M, Andini R, Bernardo M, Giannouli M, Zarrilli R. 2015. Emergence of colistin resistance without loss of fitness and virulence after prolonged colistin administration in a patient with extensively drug-resistant *Acinetobacter baumannii*. Diagn Microbiol Infect Dis 82:222–226. <https://doi.org/10.1016/j.diagmicrobio.2015.03.013>.
  215. Silvestri L, Taylor N, van Saene HK, Bakker J. 2014. Colistin, SDD and resistance: nihil novi sub sole. Intensive Care Med 40:1065. <https://doi.org/10.1007/s00134-014-3321-9>.
  216. Zhao F, Zong Z. 2016. *Kluyvera ascorbata* strain from hospital sewage carrying the *mcr-1* colistin resistance gene. Antimicrob Agents Chemother 60:7498–7501.
  217. Skov RL, Monnet DL. 2016. Plasmid-mediated colistin resistance (*mcr-1* gene): three months later, the story unfolds. Euro Surveill 21:30155. <https://doi.org/10.2807/1560-7917.ES.2016.21.9.30155>.
  218. Hu Y, Liu F, Lin IY, Gao GF, Zhu B. 2016. Dissemination of the *mcr-1* colistin resistance gene. Lancet Infect Dis 16:146–147. [https://doi.org/10.1016/S1473-3099\(15\)00533-2](https://doi.org/10.1016/S1473-3099(15)00533-2).
  219. Ruppe E, Chatelier EL, Pons N, Andreumont A, Ehrlich SD. 2016. Dissemination of the *mcr-1* colistin resistance gene. Lancet Infect Dis 16: 290–291. [https://doi.org/10.1016/S1473-3099\(16\)00666-9](https://doi.org/10.1016/S1473-3099(16)00666-9).
  220. Zhi C, Lv L, Yu LF, Doi Y, Liu JH. 2016. Dissemination of the *mcr-1* colistin resistance gene. Lancet Infect Dis 16:292–293. [https://doi.org/10.1016/S1473-3099\(16\)00063-3](https://doi.org/10.1016/S1473-3099(16)00063-3).
  221. Zhang R, Huang Y, Chan EW, Zhou H, Chen S. 2016. Dissemination of the *mcr-1* colistin resistance gene. Lancet Infect Dis 16:291–292. [https://doi.org/10.1016/S1473-3099\(16\)00062-1](https://doi.org/10.1016/S1473-3099(16)00062-1).
  222. Olaitan AO, Chabou S, Okdah L, Morand S, Rolain JM. 2016. Dissemination of the *mcr-1* colistin resistance gene. Lancet Infect Dis 16:147. [https://doi.org/10.1016/S1473-3099\(15\)00540-X](https://doi.org/10.1016/S1473-3099(15)00540-X).
  223. Malhotra-Kumar S, Xavier BB, Das AJ, Lammens C, Hoang HT, Pham NT, Goossens H. 2016. Colistin-resistant *Escherichia coli* harbouring *mcr-1* isolated from food animals in Hanoi, Vietnam. Lancet Infect Dis 16: 286–287. [https://doi.org/10.1016/S1473-3099\(16\)00014-1](https://doi.org/10.1016/S1473-3099(16)00014-1).
  224. Stoesser N, Mathers AJ, Moore CE, Day NP, Crook DW. 2016. Colistin resistance gene *mcr-1* and pHNSHP45 plasmid in human isolates of *Escherichia coli* and *Klebsiella pneumoniae*. Lancet Infect Dis 16: 285–286. [https://doi.org/10.1016/S1473-3099\(16\)00010-4](https://doi.org/10.1016/S1473-3099(16)00010-4).
  225. Petrillo M, Angers-Loustau A, Kreysa J. 2016. Possible genetic events producing colistin resistance gene *mcr-1*. Lancet Infect Dis 16:280. [https://doi.org/10.1016/S1473-3099\(16\)00005-0](https://doi.org/10.1016/S1473-3099(16)00005-0).
  226. Suzuki S, Ohnishi M, Kawanishi M, Akiba M, Kuroda M. 2016. Investigation of a plasmid genome database for colistin-resistance gene *mcr-1*. Lancet Infect Dis 16:284–285. [https://doi.org/10.1016/S1473-3099\(16\)00008-6](https://doi.org/10.1016/S1473-3099(16)00008-6).
  227. Haenni M, Poirel L, Kieffer N, Chatre P, Saras E, Metayer V, Dumoulin R, Nordmann P, Madec JY. 2016. Co-occurrence of extended spectrum  $\beta$ -lactamase and MCR-1 encoding genes on plasmids. Lancet Infect Dis 16:281–282. [https://doi.org/10.1016/S1473-3099\(16\)00007-4](https://doi.org/10.1016/S1473-3099(16)00007-4).
  228. Perrin-Guyomard A, Bruneau M, Houe P, Deleu K, Legrand P, Poirier C, Soumet C, Sanders P. 2016. Prevalence of *mcr-1* in commensal *Escherichia coli* from French livestock, 2007 to 2014. Euro Surveill 21:30135. <https://doi.org/10.2807/1560-7917.ES.2016.21.6.30135>.
  229. Cannatelli A, Giani T, Antonelli A, Principe L, Luzzaro F, Rossolini GM.

2016. First detection of the *mcr-1* colistin resistance gene in *Escherichia coli*, Italy. *Antimicrob Agents Chemother* 60:3257–3258. <https://doi.org/10.1128/AAC.00246-16>.
230. Giufrè M, Monaco M, Accogli M, Pantosti A, Cerquetti M, PAMURSA Study Group. 2016. Emergence of the colistin resistance *mcr-1* determinant in commensal *Escherichia coli* from residents of long-term-care facilities in Italy. *J Antimicrob Chemother* 71:2329–2331. <https://doi.org/10.1093/jac/dkw195>.
231. Doumith M, Godbole G, Ashton P, Larkin L, Dallman T, Day M, Day M, Muller-Pebody B, Ellington MJ, de Pinna E, Johnson AP, Hopkins KL, Woodford N. 2016. Detection of the plasmid-mediated *mcr-1* gene conferring colistin resistance in human and food isolates of *Salmonella enterica* and *Escherichia coli* in England and Wales. *J Antimicrob Chemother* 71:2300–2305. <https://doi.org/10.1093/jac/dkw093>.
232. Kluytmans-van den Bergh MF, Huizinga P, Bonten MJ, Bos M, De Bruyne K, Friedrich AW, Rossen JW, Savelkoul PH, Kluytmans JA. 2016. Presence of *mcr-1*-positive *Enterobacteriaceae* in retail chicken meat but not in humans in the Netherlands since 2009. *Euro Surveill* 21:30149. <https://doi.org/10.2807/1560-7917.ES.2016.21.9.30149>.
233. Bonten MJ. 2016. Antimicrobial resistance: is it really all going wrong now? *Ned Tijdschr Geneesk* 160:D81.
234. Arcilla MS, van Hattem JM, Matamoros S, Melles DC, Penders J, de Jong MD, Schultsz C. 2016. Dissemination of the *mcr-1* colistin resistance gene. *Lancet Infect Dis* 16:147–149. [https://doi.org/10.1016/S1473-3099\(15\)00541-1](https://doi.org/10.1016/S1473-3099(15)00541-1).
235. Veldman K, van Essen-Zandbergen A, Rapallini M, Wit B, Heymans R, van Pelt W, Mevius D. 2016. Location of colistin resistance gene *mcr-1* in *Enterobacteriaceae* from livestock and meat. *J Antimicrob Chemother* 71:2340–2342. <https://doi.org/10.1093/jac/dkw181>.
236. Hasman H, Hammerum AM, Hansen F, Hendriksen RS, Olesen B, Agerso Y, Zankari E, Leekitcharoenphon P, Stegger M, Kaas RS, Cavaco LM, Hansen DS, Aarestrup FM, Skov RL. 2015. Detection of *mcr-1* encoding plasmid-mediated colistin-resistant *Escherichia coli* isolates from human bloodstream infection and imported chicken meat, Denmark 2015. *Euro Surveill* 20:30085. <https://doi.org/10.2807/1560-7917.ES.2015.20.49.30085>.
237. McGann P, Snesrud E, Maybank R, Corey B, Ong AC, Clifford R, Hinkle M, Whitman T, Lesho E, Schaefer KE. 2016. *Escherichia coli* harboring *mcr-1* and *bla<sub>CTX-M</sub>* on a novel IncF plasmid: first report of *mcr-1* in the United States. *Antimicrob Agents Chemother* 60:4420–4421. <https://doi.org/10.1128/AAC.01103-16>.
238. Grami R, Mansour W, Mehri W, Bouallegue O, Boujaafar N, Madec JY, Haenni M. 2016. Impact of food animal trade on the spread of *mcr-1*-mediated colistin resistance, Tunisia, July 2015. *Euro Surveill* 21:30144. <https://doi.org/10.2807/1560-7917.ES.2016.21.8.30144>.
239. Elnahriry SS, Khalifa HO, Soliman AM, Ahmed AM, Moustafa AH, Shimamoto T. 2016. Emergence of plasmid-mediated colistin resistance gene, *mcr-1*, in a clinical *Escherichia coli* isolate from Egypt. *Antimicrob Agents Chemother* 60:3249–3250. <https://doi.org/10.1128/AAC.00269-16>.
240. Perreten V, Strauss C, Collaud A, Gerber D. 2016. Colistin resistance gene *mcr-1* in avian pathogenic *Escherichia coli* in South Africa. *Antimicrob Agents Chemother* 60:4414–4415. <https://doi.org/10.1128/AAC.00548-16>.
241. Fernandes MR, Moura Q, Sartori L, Silva KC, Cunha MP, Esposito F, Lopes R, Otutumi LK, Gonçalves DD, Dropa M, Matté MH, Monte DF, Landgraf M, Francisco GR, Bueno MF, de Oliveira Garcia D, Knöbl T, Moreno AM, Lincopan N. 2016. Silent dissemination of colistin-resistant *Escherichia coli* in South America could contribute to the global spread of the *mcr-1* gene. *Euro Surveill* 21:30214. <https://doi.org/10.2807/1560-7917.ES.2016.21.17.30214>.
242. Zeng KJ, Doi Y, Patil S, Huang X, Tian GB. 2016. Emergence of plasmid-mediated *mcr-1* gene in colistin-resistant *Enterobacter aerogenes* and *Enterobacter cloacae*. *Antimicrob Agents Chemother* 60:3862–3863. <https://doi.org/10.1128/AAC.00345-16>.
243. Tse H, Yuen KY. 2016. Dissemination of the *mcr-1* colistin resistance gene. *Lancet Infect Dis* 16:145–146. [https://doi.org/10.1016/S1473-3099\(15\)00532-0](https://doi.org/10.1016/S1473-3099(15)00532-0).
244. Webb HE, Granier SA, Marault M, Millemann Y, den Bakker HC, Nightingale KK, Bugarel M, Ison SA, Scott HM, Loneragan GH. 2016. Dissemination of the *mcr-1* colistin resistance gene. *Lancet Infect Dis* 16:144–145. [https://doi.org/10.1016/S1473-3099\(15\)00538-1](https://doi.org/10.1016/S1473-3099(15)00538-1).
245. Iraz M, Ozad Duzgun A, Sandalli C, Doymaz MZ, Akkoyunlu Y, Saral A, Peleg AY, Ozgumus OB, Beris FS, Karaoglu H, Copur Cicek A. 2015. Distribution of beta-lactamase genes among carbapenem-resistant *Klebsiella pneumoniae* strains isolated from patients in Turkey. *Ann Lab Med* 35:595–601. <https://doi.org/10.3343/alm.2015.35.6.595>.
246. Hussein K, Sprecher H, Mashiach T, Oren I, Kassis I, Finkelstein R. 2009. Carbapenem resistance among *Klebsiella pneumoniae* isolates: risk factors, molecular characteristics, and susceptibility patterns. *Infect Control Hosp Epidemiol* 30:666–671. <https://doi.org/10.1086/598244>.
247. Lee JY, Chung ES, Na IY, Kim H, Shin D, Ko KS. 2014. Development of colistin resistance in *pmrA*-, *phoP*-, *parR*- and *cprR*-inactivated mutants of *Pseudomonas aeruginosa*. *J Antimicrob Chemother* 69:2966–2971. <https://doi.org/10.1093/jac/dku238>.

**Laurent Poirel**, Ph.D., is an Associate Professor in the Medical and Molecular Microbiology Unit, Department of Medicine, University of Fribourg, Switzerland. His research interests focus on antibiotic resistance in Gram-negative bacteria, with a special focus on the identification of molecular mechanisms responsible for acquisition of carbapenem and polymyxin resistance and the genetics of acquisition of resistance genes.



**Aurélien Jayol**, Pharm.D., is a Microbiologist currently preparing a Ph.D. thesis at the University of Fribourg, Switzerland. Her research interests focus on antibiotic resistance, especially the molecular mechanisms responsible for acquisition of colistin resistance in *Enterobacteriaceae* and the use of diagnostic tests for detection of colistin resistance.



**Patrice Nordmann**, M.D., Ph.D., is Chair Professor and Head of the Medical and Molecular Microbiology Unit of the French INSERM European Unit (University of Fribourg) of the National Reference Center for Emerging Antibiotic Resistance (Switzerland), Department of Medicine, University of Fribourg, Switzerland. His research interests focus on antibiotic resistance in Gram-negative bacteria, encompassing identification of molecular mechanisms, performance of epidemiological surveys, development of rapid diagnostic tests for antibiotic resistance, and development of novel antibiotic molecules.

