



# Dépistage et caractérisation de bactéries multirésistantes aux antibiotiques au sein d'un réservoir aviaire méditerranéen

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Thèse pour obtenir le grade de docteur

# Dépistage et caractérisation de bactéries multirésistantes aux antibiotiques au sein d'un réservoir aviaire méditerranéen

Présentée par **Salim Aberkane**

Soutenue le 12 janvier 2017 devant le jury composé de

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## I. Résumé

La résistance bactérienne aux antibiotiques est devenue un problème majeur de santé publique impliquant des actions de surveillance et de lutte contre sa diffusion. L'épidémiologie de la résistance aux antibiotiques au sein des pathogènes cliniques est indispensable notamment à la prise en charge thérapeutique. Cependant, elle est également pertinente au sein des bactéries animales et environnementales afin d'en apprécier l'ampleur et d'en appréhender la diffusion. Alors que de nombreux travaux ont été conduits sur le microbiote des animaux de compagnie, les études portant sur la faune sauvage restent rares. Or, il apparaît opportun de l'intégrer dans l'étude de la dynamique des bactéries antibiorésistantes afin d'apprécier son rôle épidémiologique dans leur dissémination et d'évaluer les risques zoonotiques qui en découlent.

Sur la base de notre revue de la littérature, nous avons mis en évidence un lien étroit existant entre les activités humaines et la présence de bactéries antibiorésistantes dans la faune sauvage. Ceci nous a conduits à discuter de l'existence probable de voies d'échanges entre le compartiment humain et animal.

Sur la base d'une étude ayant démontré la présence dans le sud de la France d'un réservoir aviaire d'*Escherichia coli* producteurs de bêta-lactamases à spectre élargi, nous avons exploré le microbiote cloacal de deux espèces de goélands, différentes par leurs niches écologiques et leur mode d'alimentation, en tant que réservoir potentiel de bactéries multirésistantes aux antibiotiques.

Dans un premier temps nous nous sommes intéressés à la présence de *Proteus mirabilis* producteurs d'AmpC acquises dans le microbiote des goélands au cours des deux années d'étude. Ces isolats étaient producteurs de céphalosporinases de type CMY-2 dont le support génétique était un élément intégratif et conjugatif (ICE) de la famille SXT/R391-like. Deux souches cliniques humaines avaient les mêmes enzymes, supports et fond génétiques que des souches aviaires. Ceci permet de supposer que ces goélands constituent un réservoir de *P. mirabilis* porteurs du gène *bla*<sub>CMY-2</sub>, et que les structures de type ICE SXT/R391-like joueraient un rôle important dans la dissémination et la persistance de ce gène de résistance.

Nous avons également isolé des souches d'*Escherichia coli* productrices de carbapénèmases acquises, qui constituent actuellement l'une des menaces les plus préoccupantes pour la santé publique en termes d'antibiorésistance. Ces souches proviennent uniquement de goélands leucophées et sont porteuses du gène *bla*<sub>VIM-1</sub>. L'analyse de leur patrimoine génétique montre qu'elles sont liées à des souches humaines sensibles. Nous n'avons en effet pas isolé de souches humaines productrices de carbapénèmases de type VIM dans le même temps. Cette découverte pose la question d'un réservoir aviaire potentiel et d'une menace de diffusion.

Lors du screening nous avons identifié une souche de *Vibrio cholerae* non-O1/non-O139 résistante aux carbapénèmes et provenant d'un goéland leucophée. Elle possédait des gènes *bla*<sub>VIM-1</sub> et *bla*<sub>VIM-4</sub> qui faisaient partie d'un intégroon de classe 1, situé sur un plasmide IncA/C. Il s'agit de la première description d'une souche de *V. cholerae* productrice de ce type de carbapénèmases.

Ce travail démontre la complexité de la circulation de l'antibiorésistance au sein du microbiote étudié. Il ouvre de nombreuses perspectives d'un point de vue épidémiologique mais également fondamental sur les mécanismes et les supports génétique de cette antibiorésistance. En effet, il illustre bien les apports importants des outils d'épidémiologie moléculaire dans la surveillance de l'émergence et la compréhension de la dynamique de transmission et de diffusion des bactéries multirésistantes dans la faune sauvage.

## Abstract

Bacterial resistance has become a major public health problem leading to a strengthening of spread surveillance and control. The epidemiology of antimicrobial resistance (AMR) in clinical pathogens is essential for therapeutic management. It is also relevant in animal and environmental bacteria to determine and understand AMR existence and diffusion. While much work has been done on the microbiota of companion animals, studies involving wildlife are scarce. It is essential to consider wildlife when studying AMR dynamics to assess its epidemiological role in AMR spread and understand the zoonotic risk which ensues from it.

With our literature review, we highlight the close link between human activities and the presence of AMR in wildlife. It led us to discuss the pathways between the human and animal compartments.

A previous study reported the presence of an avian reservoir of extended spectrum beta-lactamases-producing *Escherichia coli* in the South of France. Based on this finding, we explored the microbiota of two gull species, differentiated by their ecological niches and diet, as a potential reservoir of AMR.

First, we investigated the presence of acquired AmpC-producing *Proteus mirabilis* in the gulls' microbiota over two years. The isolates produced CMY-2 cephalosporinases with the genetic support of an integrative and conjugative element (ICE) which belongs to the SXT/R391-like family. Two human strains had the same enzymes, genetic support and genetic background as the avian isolates. This suggests that these gulls may act as a reservoir of *bla*<sub>CMY-2</sub>-carrying *P. mirabilis*, and the SXT/R391-like ICEs may play an important role in this gene's dissemination and persistence.

We also isolated acquired carbapenemases-producing *E. coli*, which is currently one of the most serious AMR threats to public health. These strains, which carried the *bla*<sub>VIM-1</sub> gene, were recovered from yellow-legged gulls. The phylogenetic analyses showed that the gulls are significantly linked with human susceptible isolates. However, VIM carbapenemase producing-human isolate was not isolated in the same time period. This discovery raises the question of a potential avian reservoir and the threat of diffusion.

During the screening, we identified a carbapenem resistant non-O1/non-O139 *Vibrio cholerae* strain, recovered from a yellow-legged gull. It carried both *bla*<sub>VIM-1</sub> and *bla*<sub>VIM-4</sub> genes which were part of a class 1 integron structure located in an IncA/C plasmid. This is the first description of a *V. cholera* strain producing this type of carbapenemase.

This work demonstrates the complexity of the AMR circulation in the microbiota studied. It opens many perspectives from an epidemiological and fundamental point of view on the mechanisms and genetic supports of AMR. It further illustrates the contribution of molecular epidemiology tools in the understanding of the dynamics of transmission and diffusion and the surveillance of the emergence of AMR in wildlife.

## II. Introduction

Après une phase d'optimisme victorieux ayant suivi le développement de nouveaux vaccins et antibiotiques, les dernières décades ont vu l'émergence ou la réémergence d'agents pathogènes affectant les populations humaines et animales domestiques (1, 2). Parmi ces émergences, celle des bactéries résistantes aux antibiotiques occupent une place à part. En effet, ce phénomène d'émergence et de diffusion de l'antibiorésistance est la conséquence directe de l'utilisation abusive et inappropriée des molécules antibiotiques qui semblaient avoir remis durablement les maladies infectieuses bactériennes au rang de causes mineures de mortalité et de morbidité au sein des populations humaine. Or aujourd'hui cette situation s'est aggravée. L'organisme mondial de la santé (OMS) a identifié ce phénomène comme une menace majeure pour la santé humaine (3) mais aussi ayant un impact économique délétère croissant dans les pays industrialisés comme dans les pays en développement (4).

Cependant, tandis qu'une grande partie des agents pathogènes à l'origine des crises sanitaires récentes, tels que le VIH ou le virus Ebola, ont une origine zoonotique (i.e. ils circulaient à l'origine au sein de la faune sauvage) (2, 5), l'origine des bactéries antibiorésistantes est aussi diverse que difficile à déterminer. En effet, les éléments génétiques conférant les résistances peuvent être échangés entre différentes espèces et souches bactériennes. Ces éléments peuvent préexister à l'utilisation humaine des antibiotiques (6). La plupart des champignons et bactéries qui produisent des antibiotiques possèdent des mécanismes de résistances contre ces derniers (7). Les résistances peuvent également évoluer directement en réponse à une exposition aux antibiotiques, cependant dans ce cas celle-ci peut avoir eu lieu au sein d'un hôte humain comme chez un animal domestique, voire dans l'environnement (8). Ainsi, alors que les crises sanitaires et économiques associées aux agents pathogènes zoonotiques ont fait prendre conscience de la nécessité d'étudier conjointement la dynamique des maladies associées au sein des populations humaines, d'animaux domestiques et de la faune sauvage, ce type d'approche « One Health » a en revanche été très peu développé dans le cadre de l'étude des bactéries antibiorésistantes. L'OMS, l'organisation des nations unies pour l'alimentation et l'agriculture (FAO) et l'organisation mondiale de la santé animale (OIE) ont souligné la nécessité de collaborer étroitement



pour lutter contre l'émergence de l'antibiorésistance. Cependant cette approche n'inclut pour le moment et dans la majorité des cas que les animaux domestiques et non la faune sauvage (6, 9). Pourtant, cette dernière apparaît à la lumière des données existantes comme un compartiment potentiellement clé dans les dynamiques des bactéries antibiorésistantes (6).

Il existe actuellement très peu de données sur les bactéries antibiorésistantes présentes au sein de la faune sauvage, par rapport aux connaissances existantes au sein des populations humaines et animales domestiques. La grande majorité des recherches menées à ce jour ont mis en évidence la présence de bactéries résistantes à un ou plusieurs antibiotiques dans presque tous les écosystèmes, jusqu'aux plus reculés (10, 11). La quantité et la diversité de ces bactéries augmentent généralement avec la proximité des activités humaines (12, 13). Ces études ont permis de détecter des bactéries antibiorésistantes chez des espèces très variées allant du rongeur (14), au loup (15) en passant par la tortue marine (16). Les animaux sauvages apparaissent donc régulièrement porteurs et excréteurs de bactéries antibiorésistantes.

Les proportions de bactéries antibiorésistantes et les types de résistances rencontrés dans la faune sauvage varient considérablement en fonction des espèces et des sites. Cependant, il existe actuellement peu d'études de comparaison des profils de résistances en un même site chez plusieurs espèces ou en plusieurs sites chez une même espèce. Une étude comparant les antibiorésistances présentes chez les *Escherichia coli* isolées chez différentes espèces d'oiseaux a montré que les plus touchés étaient les oiseaux vivant en milieu urbain, les oiseaux aquatiques et les rapaces (17). Chacune de ces catégories pourrait présenter un risque accru de contact avec des bactéries antibiorésistantes du fait de son écologie. Les rapaces concentrent généralement les agents pathogènes présents chez leurs proies, les oiseaux urbains peuvent être directement en lien avec les bactéries infectant l'homme via les déchets anthropiques ou les eaux usées, tandis que les oiseaux aquatiques peuvent être en contact avec les bactéries résistantes qui persistent dans l'eau. En ce qui concerne les différents habitats, ceux qui sont les plus proches des activités humaines sont généralement ceux où la plus grande diversité et prévalence de résistance est observée (12, 13). Cependant, rares sont les études qui s'intéressent à un continuum d'habitats et permettant d'avoir une vision précise de la dynamique de l'antibiorésistance

dans l'espace à partir de sources potentielles. Deux études pionnières dans ce domaine ont comparé les bactéries antibiorésistantes présentes chez les rongeurs dans différents habitats. Une première étude effectuée en milieu rural a souligné une corrélation positive entre les densités d'élevage sur le site d'étude et la proportion de bactéries antibiorésistantes (18). Une seconde étude également menée sur des rongeurs a montré un gradient de prévalence de bactéries antibiorésistances allant des espaces protégés, tels que les réserves, les moins touchés, aux abords des porcheries, les plus touchés (19).

Les types d'habitats et l'écologie des espèces apparaissent donc jouer un rôle important dans la diversité et la quantité des bactéries antibiorésistantes présentes. Cependant les données sur l'impact de ces facteurs sont très limitées. Par ailleurs les habitats fréquentés par les espèces et leur écologie sont également susceptibles de déterminer leur rôle dans la dynamique des bactéries antibiorésistantes dont elles sont porteuses. Il a ainsi été démontré que différentes espèces capables de se déplacer sur de très longues distances telles que le loup ibérique (*Canis lupus signatus*) (15) et différents oiseaux migrateurs (20, 21) peuvent porter des bactéries antibiorésistantes. Ces derniers pourraient donc avoir un rôle de dispersion de ces agents pathogènes plus ou moins important en fonction de la durée d'excrétion des bactéries et des déplacements effectifs des individus. De plus certaines espèces étant directement en contact avec l'homme comme des rongeurs et des oiseaux, ou encore le renard, pourraient représenter des ponts épidémiologiques entre le compartiment humain et le compartiment sauvage. Il a notamment été démontré que des souches antibiorésistantes très proches de celles détectées au sein des populations humaines étaient présentes chez des oiseaux sauvages (21-23). Cependant la compréhension des échanges entre ces compartiments nécessite la comparaison phylogénétique des souches résistantes trouvées chez les humains et au sein de la faune sauvage, ce qui a été rarement mis en œuvre.

Au vu des données disponibles il apparaît donc que :

- La faune sauvage est porteuse de bactéries antibiorésistantes.
- Les types de résistances et leur prévalence diffèrent en fonction de l'écologie des espèces et des habitats occupés mais les connaissances manquent pour comprendre ces différences.

- Certaines souches antibiorésistantes observées dans la faune sauvage sont très proches des souches présentes au sein des populations humaines. Il existe donc des échanges de bactéries antibiorésistantes entre faune sauvage et populations humaines mais ceux-ci restent mal compris.
- Certaines espèces pouvant se déplacer sur de longues distances pourraient potentiellement avoir un rôle dans la dispersion des bactéries antibiorésistantes.

Dans ce contexte, il apparaît pertinent d'intégrer la faune sauvage dans l'étude de la dynamique des bactéries antibiorésistantes notamment pour comprendre son rôle potentiel dans leur dissémination. Il semble également important de la prendre en compte dans l'évaluation des risques liés à la présence de ces agents biologiques pathogènes pour l'Homme.

L'objectif global de ce travail a été d'étudier la circulation des bactéries multirésistantes aux antibiotiques à l'interface entre populations humaines et faune sauvage, afin de comprendre son rôle dans la dissémination de l'antibiorésistance.

Sur la base de notre revue de la littérature portant sur le rôle de la faune sauvage dans la dynamique des bactéries résistantes aux antibiotiques, l'étude de Bonnedhal et al. a particulièrement attiré notre attention. En effet, cette étude menée en 2009 dans le Sud de la France (21), a mis en évidence un réservoir aviaire (goélands leucophées, *Larus michahellis*) de souches d'*E. coli* productrices d'enzymes de haut niveau de résistance aux céphalosporines de troisième génération (C3G), des bêta-lactamases à spectre élargi (BLSE) de type CTX-M. En revanche, les auteurs n'ont pas précisé l'existence d'autres bactéries multirésistantes aux antibiotiques. Ainsi à l'instar de ce réservoir aviaire d'*E. coli* producteur de BLSE, l'objectif de notre étude a été d'explorer ce même microbiote cloacal des goélands en tant que réservoir potentiel d'autres bactéries multirésistantes. Nous avons inclus une autre espèce d'oiseaux, des goélands railleurs (*Chroicocephalus genei*). Ces derniers sont différents des goélands leucophées par leur niche écologique et leur mode d'alimentation. En effet, ils sont beaucoup moins au contact de l'homme et se nourrissent exclusivement de poissons marins, contrairement aux goélands leucophées qui sont plus anthropiques et se nourrissent dans les décharges.

Nos objectifs spécifiques sont les suivants :

A partir du microbiote cloacal des deux espèces de goélands (railleur et leucopnée)

1. Dépister et caractériser des isolats de *Proteus mirabilis* producteurs de céphalosporinases hyperproduites.
2. Etudier la persistance de ces *P. mirabilis* dans le temps.
3. Dépister et caractériser des isolats d'*E. coli* résistants aux carbapénèmes.
4. Caractériser un isolat de *Vibrio cholerae* résistants aux carbapénèmes.

# **Chapitre I**

## **Revue de littérature**

## **Préambule**

La diffusion des résistances aux antibiotiques est devenue un problème majeur de santé humaine avec comme conséquences une augmentation des coûts économiques et un risque d'impasses thérapeutiques.

La compréhension et la lutte contre cette diffusion inclue non seulement des études en clinique humaine et vétérinaire, mais aussi environnementales et de la faune sauvage. En effet, les hypothèses selon lesquelles la faune pourrait jouer un rôle important dans la dynamique des bactéries résistantes aux antibiotiques sont de plus en plus nombreuses, alors que les données empiriques restent rares.

Cette revue analyse les données de la littérature disponible pour identifier les principales informations que nous possédons dans ce domaine et suggérer des voies de recherche afin de combler les lacunes de nos connaissances actuelles.

Pour atteindre notre objectif, nous nous sommes posés quatre questions : i) Quelles sont les bactéries résistantes les plus fréquemment retrouvées dans la faune sauvage? ii) Comment peut s'effectuer la circulation de la résistance et/ou ses supports génétiques entre la faune sauvage et les autres compartiments (humain, animaux domestiques et environnement) ? iii) Dans quels habitats trouve-t-on ces bactéries résistantes ? iv) Est-ce que ces résistances sont associées à certaines caractéristiques écologiques de l'hôte ?

Nous avons mis en évidence le lien étroit existant entre l'impact des activités humaines sur les habitats naturels et le portage de bactéries résistantes aux antibiotiques par la faune sauvage. En outre, nous avons souligné que les espèces omnivores, carnivores et anthropophiles ont un risque élevé d'être porteuses et donc potentiellement de répandre ces bactéries résistantes. Enfin, nous discutons l'existence probable de voies d'échanges de bactéries résistantes entre populations humaines et faune sauvage, et soulignons l'importance de prendre en compte le transfert horizontal de gènes de résistance dans l'étude de ces voies.

Article I

**Review: Antimicrobial resistance in wildlife**

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## REVIEW

## Antimicrobial resistance in wildlife

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## Summary

1. The spread of antimicrobial resistance is of major concern for human health and leads to growing economic costs. While it is increasingly hypothesized that wildlife could play an important role in antimicrobial-resistant bacteria dynamics, empirical data remain scarce.

2. The present work builds on a systematic review of the available data in order to highlight the main information we have and to suggest research pathways that should be followed if we aim to fill the gaps in our current knowledge.

3. To achieve this goal, we address four questions: (i) Which resistant bacteria are the most frequently observed in wildlife? (ii) How are resistant bacteria exchanged between wildlife and the other hosts involved? (iii) In which habitats are those resistant bacteria found? (iv) Are resistances associated with certain ecological traits of the host?

4. *Synthesis and applications.* We highlight the strong link existing between the impact of human activities on natural habitats and the carriage of antimicrobial-resistant bacteria by wildlife. Furthermore, we underline that omnivorous, anthropophilic and carnivorous species are at high risk of being carriers and potentially spreaders of antimicrobial-resistant bacteria. Identifying among those groups key sentinel species may be of particular interest to implement ecosystem contamination surveillance. Finally, we discuss possible exchange routes for antimicrobial-resistant bacteria between humans and wildlife. Considering that water is of major importance in those exchanges, a critical way to control antimicrobial resistance spread may be to limit aquatic environment contamination by antimicrobial-resistant bacteria and antibiotics.

**Key-words:** antibiotic resistance, antibiotic-resistant bacteria, emerging infectious disease, *Escherichia coli*, health ecology, *Klebsiella pneumoniae*, MRSA, pathogens, *Salmonella spp.*, transmission routes

## Introduction

Antimicrobial resistance (AMR) is a major threat for human health world-wide, impairing our capacity to treat an increasing number of infections (WHO 2014). In addition, it entails a considerable increase in treatment costs,

as resistance makes necessary the use of expensive last-generation molecules and implies extra hospitalization costs. AMR is also a crucial issue in agriculture since it makes more complex the maintenance of domestic animal health, leading to additional economic costs. Furthermore, available data show that numerous wildlife species carry antimicrobial-resistant bacteria (AMRB) in a wide range of habitats, which raises the question of their role

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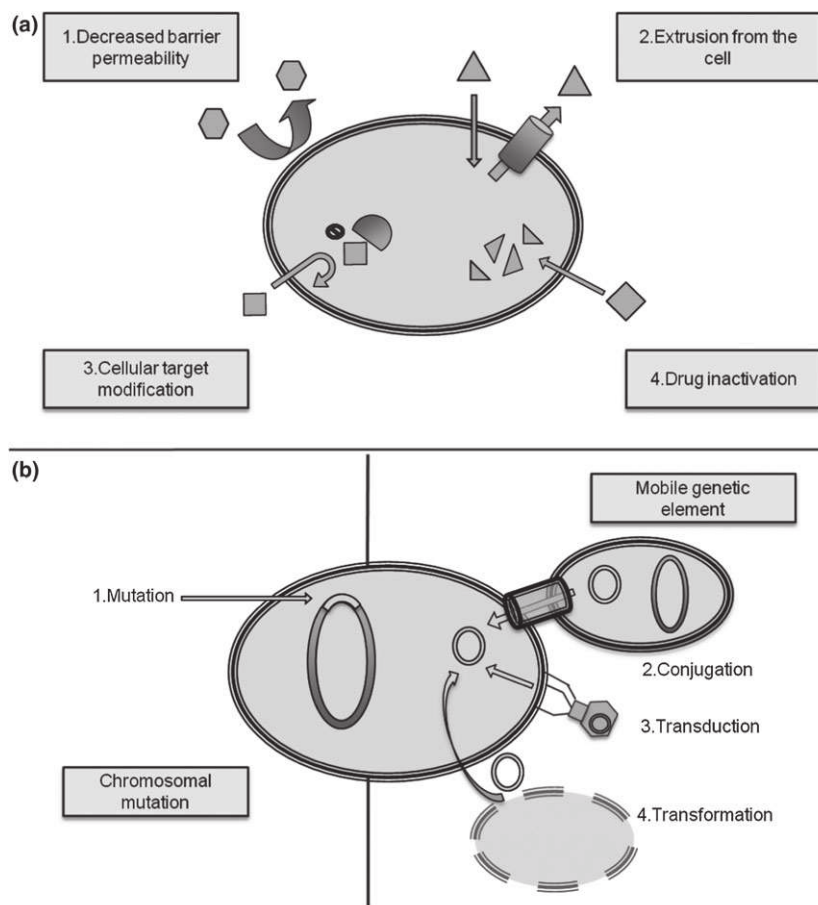
in AMRB dynamics at the interface between human populations, domestic animals and natural ecosystems.

There is thus an urgent need to understand the dynamics of AMR: how it spreads, how it passes from one compartment to another and how and why it is maintained within bacterial populations. These dynamics are greatly complicated both by the large diversity of antimicrobial resistance mechanisms (Fig. 1a) and by the horizontal transfer of resistance genes existing between bacteria (Fig. 1b; Tenover 2006). Indeed, the dissemination of AMR has been largely attributed to inter- and intraspecific DNA exchange, mainly through the horizontal transfer of plasmid-located resistance genes, which is the most important mechanism at the origin of acquisition of resistance in bacterial pathogens of human health concern (Carattoli 2013). Plasmids are extrachromosomal DNA molecules capable of autonomous replication and can confer resistance to the major classes of antimicrobials, including  $\beta$ -lactams, aminoglycosides, tetracyclines, chloramphenicol, sulphonamides, trimethoprim, macrolides and quinolones (Carattoli 2009).

The main measures currently applied in European countries are to cut down on the use of antibiotics in both human and domestic animals, since it has become clear that the two compartments are closely linked (Angulo, Nargund & Chiller 2004). These measures are based on

the assumption that AMR is associated with fitness costs that allow susceptible bacteria to overcome resistant ones, when there is no selective pressure linked to antimicrobial drugs. Yet it appears that these costs are extremely variable (Andersson & Hughes 2010) and can be reduced or even turned into fitness benefits by compensatory mutations (Luo *et al.* 2005). Additionally, the same mechanism or a mechanism found on the same genetic element can confer resistance to both antimicrobial drugs and pollutants (Baker-Austin *et al.* 2006). Thus, the pollution of environmental reservoirs can contribute to the development and maintenance of AMRB. Finally, AMRB are naturally found in soils in the absence of anthropogenic antimicrobial drugs due to the natural production of antibiotic molecules by some bacteria and fungi (Keen & Montforts 2012).

The low reversibility of AMR is all the more worrying in that in some cases resistance is associated with enhanced virulence. As an illustration, the different steps leading to methicillin resistance acquisition in *Staphylococcus aureus* are associated with virulence modifications (Cameron, Howden & Peleg 2011). The methicillin-resistant strain of *S. aureus* (MRSA), USA300, appears to have caused a strong increase in severe cutaneous infections observed in the USA in the 2000s (Chadwick *et al.* 2013). It is an example of bacteria in which AMR and



**Fig. 1.** The complexity of antimicrobial resistance mechanisms (a) and their transmission routes between bacteria (b). (a) Main antimicrobial resistance mechanisms: (1) Decrease in the membrane permeability to a drug. (2) Active extrusion of the drug out of the cell. (3) Modification of the cellular target of a drug. (4) Inactivation of the drug. (b) Mechanisms of antimicrobial resistance acquisition: the resistance to a drug can result from a mutation (1). The mutated bacteria that become resistant will be selected in the presence of the drug. This type of resistance will only be passed to the next generation within a particular strain. In contrast, some resistances can be carried by plasmids that can be transmitted from one bacterial species to another through either cell-to-cell conjugation (2), phage-mediated transduction (3) or transformation by extracellular DNA (4).

virulence are associated. Similarly, the *Escherichia coli* group called ST131 is characterized by both multidrug resistance and high virulence (Da Silva & Mendonça 2012). However, the link between resistance and virulence is highly diverse. Even within a single bacterial species such as *E. coli*, the association between virulence factor and resistance determinant carriage greatly varies according to the resistance mechanisms involved and the vertebrate host species studied (Da Silva & Mendonça 2012).

Considering the significant impact AMR has worldwide on both the economy and human health, it is a matter of urgency to improve our understanding of the role each compartment (i.e. domestic animals, human populations, wildlife and environmental reservoirs) plays in the maintenance and the dispersal of AMR within bacterial populations. However, while considerable efforts have been made to increase our knowledge of AMR dynamics in human populations and domestic animals, much less attention has been paid to wildlife. Yet over the last decade, accumulated evidence has revealed the presence of antibiotics and AMRB in wildlife and natural environments (Allen *et al.* 2010). Studies highlighted the presence of AMR in all ecosystems including the most isolated ones such as Antarctica (Miller, Gammon & Day 2009) and in a wide range of species (Allen *et al.* 2010).

Here, our purpose was to review currently available data concerning AMR in wildlife with the aim of drawing the major lessons that can be inferred from it. To achieve this goal, we carried out searches on the Web of Science (<https://access.webofknowledge.com/>) and PubMed (<http://www.ncbi.nlm.nih.gov/pubmed>) databases using the following terms ('Antimicrobial' OR 'antibiotic') AND 'resistance' AND ('wildlife' OR (('mammal' OR 'bird' OR 'reptile' OR 'amphibian' OR 'fish' OR 'invertebrate') AND ('wild' OR 'free-ranging')))). We ended our research on 20 May 2015. From the resulting list of references, we selected those presenting novel data about any resistant bacteria carried by any wild animal but excluding those living in captivity. We completed the reference selection with additional articles cited in four recent review papers (Guenther, Ewers & Wieler 2011; Wellington *et al.* 2013; Radhouani *et al.* 2014; Sousa *et al.* 2014). This left us with 210 peer-reviewed articles mainly pertaining to birds and mammals in Europe and North America

(Table 1). Only the most relevant are cited in the text body of the present paper. The full list of articles is presented in Appendix S1 (Supporting information).

We investigated the available studies with reference to four questions: (i) Which bacteria species are the most frequently found to be resistant to antimicrobial drugs in wild vertebrates? (ii) How do wildlife species get colonized by AMRB and which exchanges of such bacteria occur between humans, domestic animals and wildlife? (iii) What characterizes the habitats that are the most contaminated by AMR? (iv) What ecological or life-history traits, if any, favour the colonization and potential infection by AMRB in wildlife?

Given the huge heterogeneity of the data reported in the studies we gathered that notably differ by the vertebrate host species they focus on, the bacterial species they search for and the methods they use to isolate bacterial strains and determine their antimicrobial resistance pattern, we decided not to present any meta-analysis. Indeed, considering recent discussions on this issue in the literature (e.g. Kriston 2013; Melsen *et al.* 2014), we felt that in our case discussing the results of primary studies would be more useful than relying on a meta-analysis, which would summarize data that we consider highly inhomogeneous.

To conclude, we underline the gaps that remain in our knowledge of the wildlife compartment and its epidemiological links with human and domestic animal populations, and we suggest important paths to explore to anticipate future resistance transfer between compartments and to avoid human health crises.

### AMR diversity in human pathogens carried by wildlife

In the vast majority of studies focusing on AMR in wildlife, the goal is not to investigate the whole bacterial community present in a host population but rather to assess whether a bacterial species (or strain) can be found in a particular host population. Similarly, not every resistant determinant known in the focus pathogen is systematically searched for, but only a selection of them. It is essential to consider, while trying to obtain information from the currently available data, that they only represent results

**Table 1.** Host groups and regions studied in the 210 articles analysed in our review

Region	Amphibians and reptiles	Birds	Fish	Invertebrates	Mammals	Total
Africa	1	1	1	0	9	12
Asia	1	10	2	1	6	20
Europe	4	72	7	6	53	142
North America	4	33	2	0	24	63
Oceania	0	0	0	0	3	3
Polar regions	0	5	0	0	2	7
South America	4	1	0	0	4	9
Total	14	122	12	7	101	256

The overall total exceeds 210 since some studies concern several groups or regions.

concerning what has been searched for. Generally, the search is driven by human health concerns associated with the focus bacteria or the focus resistance mechanisms. Indeed, only three bacterial groups were studied in more than 10% of the 210 studies we analysed: *E. coli* (115 studies), *Salmonella spp.* (54 studies) and *Enterococcus spp.* (43 studies).

*Escherichia coli* is part of the normal intestinal flora in humans. Nevertheless, it is the most frequent cause of urinary tract and bloodstream infections world-wide. In *E. coli*, the resistance of major concern is resistance to third-generation cephalosporins and carbapenem mainly conferred by enzymes known as extended-spectrum  $\beta$ -lactamases (ESBLs) and carbapenemases (WHO 2014). In wildlife, *E. coli* is the most commonly targeted bacteria in AMR studies. ESBL-producing *E. coli* are now frequently found in wildlife (reviewed in Guenther, Ewers & Wieler 2011), notably in birds and mammals (see for example Costa *et al.* 2006; Literak *et al.* 2010; Silva *et al.* 2011; Gonçalves *et al.* 2013). By contrast, resistance to carbapenems that are now present in *E. coli* in livestock and companion animals (Guerra, Fischer & Helmuth 2014) has not been reported in *E. coli* in wildlife despite searches focused on mammals, birds and reptiles (e.g. Navarro-Gonzalez *et al.* 2013).

Bacteria of the genus *Salmonella* are a major cause of foodborne illness throughout the world. *Salmonella* can be found in the intestines of many animals, including poultry and pigs. Most *Salmonella* strains cause gastroenteritis, while some strains, particularly *Salmonella enterica* serotypes Typhi and Paratyphi, cause enteric fever. During the late 1990s and early 2000s, several clones of multiresistant *Salmonella* emerged, and since then they have expanded world-wide. For example, in *S. enterica* serotype Typhimurium, a genomic element that carries resistance to five antimicrobials may spread horizontally among serotypes. In wildlife, *S. enterica* serotype Typhimurium presenting this pentaresistance has been detected in mammals (Caleja *et al.* 2011) and birds (Čížek *et al.* 2007).

Enterococci are ubiquitous bacteria in various environmental habitats and are commensal of mammals, birds, some reptiles and invertebrates (Aarestrup 2006). Some species have emerged as important causes of nosocomial and community-acquired infections (Van den Bogaard *et al.* 2002; Radhouani *et al.* 2012). They have innate resistance to many antimicrobial agents and they can carry a variety of acquired antibiotic resistance genes, which can be transmitted to other pathogenic bacteria (Murray 1991; Spera & Farber 1994). They are frequently studied in both wild and domestic animals since they are suitable as indicators of antimicrobial resistance in Gram-positive bacteria.

In addition to *E. coli*, *Salmonella spp.* and *Enterococcus spp.*, AMR has been occasionally studied in a few other bacterial groups, among which four were searched for in at least 5% of the articles we considered: *Campylobacter spp.*,

*Enterobacter spp.*, *Klebsiella spp.* and *Staphylococcus spp.* In this article, our aim was to identify the main patterns that can be inferred from the available data. However, we emphasize the fact that those data concern essentially the three pathogens presented above and a few other bacteria, mostly enteric. Future research should aim to widen the range of bacteria groups studied, especially considering that, due to horizontal transfer, groups that are not major human pathogens may, nevertheless, contribute to spreading resistance mechanisms that are clinically relevant. For example, *Kluyvera ascorbata* is a rare bacteria that can occasionally cause severe infections in humans (Ruffini *et al.* 2008). It also has been isolated from wild animals (e.g. Lee *et al.* 2008). Despite the rarity of the severe human cases, *K. ascorbata* is of concern due to its ability to transfer genes encoding for ESBLs to other Enterobacteriaceae. Moreover, as further discussed in our conclusions, culture-independent methods may also help to broaden the range of AMR genes investigated in wildlife. As an illustration, an innovative study recently led on gulls in the USA revealed that the focus population carried a huge variety of AMR genes that were previously unrecognized (Martiny *et al.* 2011).

When considering current knowledge on AMRB found in wildlife, it is also important to keep in mind that the methods usually used to detect AMRB provide information on the presence of resistant strains, but do not assess the proportion of bacteria they represent in the studied population. This quantitative aspect may be an essential component of AMRB dynamics, and methods enabling the investigation of this issue should be used in future studies.

## Exchanges

Mounting evidence attests the occurrence of AMRB exchanges between wildlife, humans and domestic animals. When investigating these exchanges, it is important to note that even bacteria of the same species harbouring the same resistance genes may not have the same origin since they may not belong to the same clonal complex. Yet, more and more studies show that identical or near identical strains, belonging to the same clonal complex, are circulating in wildlife, humans and domestic animals (e.g. Paterson *et al.* 2012; Monecke *et al.* 2013). Nevertheless, identifying similar pathogens in two compartments does not offer a basis for determining how and in which direction the exchanges took place. Thus, it is often very difficult to disentangle the transmission routes of AMRB between two compartments. As an illustration within the references we analysed, we could not draw any statistically based conclusion about the source of AMRB carriage in wildlife since this source could not be precisely determined with certainty in any study. Indeed, several possible transmission routes exist between the focus compartments including direct contact with infected individuals, their tissues or their faeces, water and soil. The

complex network of AMRB transmission routes existing between humans, livestock and the environment has been extensively discussed (Martinez 2009; Allen *et al.* 2010; Davies & Davies 2010); here, we focus on the role of wildlife in this network and rapidly describe the main transmission routes involved.

#### CONTACT

Humans and domestic animals can be in contact with wildlife species that live or feed near habitations and rearing estates. For example, rodent faeces can be touched by humans or their animals and be ingested if they contaminate food. Additionally, rodents living in the vicinity of human habitations and rearing estates can be in direct contact with animal faeces and manure. Moreover, humans can directly touch wild animals they trap, hunt or treat as veterinarians. This transmission route is known to be important for many zoonotic diseases such as tularaemia or brucellosis (e.g. Stewart 1996). There is no reason to think that it might not be important in AMRB exchanges.

Thus, it would be worthwhile to undertake studies aimed at assessing the risk of antimicrobial transmission linked to hunting and trapping practices. Such studies could build on the networks of game meat surveillance that exist in many countries. Wild animals can also ingest contaminated meat from domestic animals. It has been proven that animal products commonly contain AMRB (Overdevest 2011) and wildlife can occasionally feed on dead animals (e.g. stillborn calves or lambs) or on their giblets when they are not collected after home slaughter. Such contamination routes could be important to take into account with a view to controlling the spread of AMR to wildlife.

#### WATER

Water seems to be a major transmission media for AMRB (Taylor, Verner-Jeffreys & Baker-Austin 2011), as suggested by the evidence of the presence of those bacteria in treated water rejected in rivers (Galvin *et al.* 2010), in rivers themselves (Dhanji *et al.* 2011), in lakes (Hamelin *et al.* 2006) and even in sea water (Zhao & Dang 2012). Furthermore, there is clear evidence of the exchanges of resistance genes between environmental bacteria and human pathogens, which can occur in aquatic systems (Wellington *et al.* 2013). AMRB found in water can originate either from human or from domestic animal populations. It should be noted that the four pathogens of major concern cited above (*S. aureus*, *E. coli*, *K. pneumoniae* and *Salmonella spp.*) can persist in water for varying periods according to strains and environmental conditions and have all been recovered from aquatic habitats (Filali *et al.* 2000; Martinez-Urtaza *et al.* 2004; Dolejska *et al.* 2009; Goodwin *et al.* 2012).

First, sewage treatment plants, even the most modern, usually remove neither all antibiotic molecules nor AMRB from the treated sewage (Rizzo *et al.* 2013). The contaminated water is then spread into rivers allowing the dispersal of antibiotics and AMRB downstream. Similarly, livestock effluents containing AMRB and antibiotics can contaminate the aquatic environment. In this case, part of the effluent is not treated at all as contaminated pastures and fields can be directly connected to rivers and groundwater due to run-off and infiltration. Whatever the source of the water contamination, it generally increases downstream of human activity areas (e.g. Pruden, Arabi & Storteboom 2012) and mechanisms of AMR can then be transmitted to wildlife because either they inhabit and/or feed in an aquatic environment or because they drink the water.

In both marine and freshwater habitats, it is a matter of urgency to study the impact of aquaculture on the spread of AMRB. Aquaculture may be the key to success in feeding the growing global human population, but globally it relies on the utilization of large amounts of antibiotics (Cabello *et al.* 2013). The vast majority of aquaculture farms directly discharge both leftover antibiotics and organic matter (faeces and fish alimentation residues) into the surrounding water. Growing evidence shows that these practices are linked to antibiotic accumulation around the farms and favour the development and spread of AMRB below the pans and over distances that may be over one kilometre and probably more, depending on currents (Buschmann *et al.* 2012). Regrettably, few studies focusing on this impact of aquaculture have included research on bacteria infecting wildlife living in aquatic habitats near aquaculture farms. Within our list, only two articles addressed this issue (González *et al.* 1999; Burr *et al.* 2012). Both were led in freshwater. González *et al.* (1999) highlighted antimicrobial resistance in only four AMRB strains: three from water and just one from a wild pike, which did not permit any comparison with wild and farmed trout that were studied in parallel. By contrast, Burr *et al.* (2012) showed that within a single lake, farmed perch *Perca fluviatilis* carried a higher proportion of AMRB than wild individuals of the same species. The diversity of resistance highlighted was also higher in farmed animals and strains did not cluster according to the status (i.e. wild or captive) of the perch. Yet those results were not discussed in the light of the treatments used in farmed perch. Similarly, González *et al.* did not include any information about the treatments used in farmed trout. It is of major importance to include this component in future studies to gain a better understanding of the impact of aquaculture as wild fishes and aquatic mammals can travel long distances and spread AMR. In addition, bivalves inhabiting the surrounding environments could also be contaminated, especially filter feeders, and be at risk if they are eaten by humans (Soonthornchaikul & Garelick 2009).



## SOIL

Antimicrobial resistance exists naturally in soil communities, notably due to the production of antibiotics by some soil bacteria and fungi (Keen & Montforts 2012). But the presence of AMRB in soil can also be the result of direct faeces or urine deposition (e.g. in pastures), of manure use (Heuer, Schmitt & Smalla 2011) or of effluent flows (Wellington *et al.* 2013). This could be an important transmission route with regard to the control of AMR spread (Heuer, Schmitt & Smalla 2011). Aerial transmission has also been suggested as possibly playing a role in AMRB dispersal (Allen *et al.* 2011). Wind could contribute to the spread of small particles of soil contaminated by antibiotics or AMRB. Thus, air should not be excluded from our AMR understanding framework, and studies should be undertaken to investigate its potential role in the spread of AMR. Finally, whatever the source of the soil contamination, wild species feeding in exposed fields could be infected by AMRB and spread them further.

More studies should focus on improving our understanding of the routes that allow AMR exchanges between epidemiological compartments, which is essential to meet the challenge of resistance spread control. AMR associated with drug use in cattle farms may spread both to soil through manure and to neighbouring watersheds through effluents. Targeting these two routes may help to reduce the risk of AMR spread associated with this activity (Pruden *et al.* 2013). Considering AMR genes as environmental contaminants and using methods that allow searching directly for these genes rather than for the bacteria carrying them may help in efficiently following the spread of AMR in all the components of the AMR transmission network. For example, methods involving polymerase chain reaction (PCR) use could permit searching for specific AMR genes whose spread is particularly worrying or that could be used as general AMR contamination markers (Pruden *et al.* 2006; Gillings *et al.* 2015). Overall, it is crucial to raise the awareness of the strong interconnectedness between habitats and compartments induced by multiple exchange routes, which implies that AMR issues must be tackled simultaneously in human populations, domestic animals, wildlife and environmental reservoirs.

### Habitats: where is the resistance?

To understand and control AMR flows, one of the first steps is to determine in which kind of habitat they usually take place and why. It is now established that AMRB are ubiquitous in natural ecosystems. As an illustration, multiresistant *E. coli* have been isolated from water in Antarctica (Miller, Gammon & Day 2009). Spatial analysis is essential to understand AMR dynamics as it allows the consideration of contamination gradients across habitats and the potential consequences of wild species dispersion as well

as human and domestic animal movements (Singer, Ward & Maldonado 2006). However, data allowing comparison of AMRB prevalence across different habitats are scarce, and studies focusing on collecting such spatial data should be encouraged.

Only 10% of the articles we considered allowed us to draw some conclusions concerning the differences of AMR across habitats in terms of prevalence. Indeed, most of the authors either focus on only one kind of habitat or work in different sites but omit to describe them in detail. In certain cases, the study areas are also very large (an administrative region or a state) and include various habitats. Among the 21 suitable articles, we used chi-square tests or Fisher's exact tests when the application conditions of chi-square test were not met to compare the proportions of AMRB carriers between the host groups inhabiting different habitats when such analysis was not initially led by the authors. We chose a *P*-value threshold of 0.05 and applied a Bonferroni correction when several pairwise tests were used to analyse the results of the same study. Eleven studies highlighted a significantly higher prevalence of AMRB within the habitat considered that was the most impacted by human activities compared to more preserved sites, while 10 did not detect any contrast. Resistance mechanism diversity could only be compared in 19 of those articles and was significantly higher in nine of them in the most anthropized places, while no difference was underlined in the remaining 10 papers. Interestingly, none of the articles highlighted a reverse trend neither concerning prevalence nor diversity of AMRB. Thus, available data suggest that the diversity of resistance mechanisms detected, as well as the proportion of individual hosts carrying AMRB, increases with the proximity to human activities. Regrettably in most cases, habitats are just classified according to their main 'function' (e.g. natural reserve, farm, city, sewage plant, etc.). Some authors also use human or livestock density to characterize the study sites (Guenther *et al.* 2010). Such indexes that could be compared across studies should be more widely used. Land-use classification according to satellite data could also be relevant.

As discussed in part II, water seems to play a major role in the dispersal of antibiotics and AMRB to natural ecosystems. As an illustration of this role, studies carried out on marine mammals to date have shown that they carry highly diverse AMRB (Schaefer *et al.* 2009) and that AMRB prevalence has been alarmingly increasing over the last decade (Wallace *et al.* 2013). Furthermore, with the global loss of natural wetlands, waterbirds have become increasingly dependent on alternative and artificial habitats including wastewater treatment wetlands (Murray & Hamilton 2010), which could favour the transmission to wild birds of AMRB of human origin. Thus, aquatic habitats may be more impacted by AMR contamination than terrestrial ones. Yet, comparing AMRB prevalence in aquatic vs. terrestrial host species is challenging since few studies focus on both groups. Among

our references, 68 focus on aquatic species only (we considered as aquatic all species that either live or feed in water), 109 on terrestrial ones, one did not give any detail on the bird species studied and only 32 searched for AMRB in both groups. Among them, only 13 gave enough information to draw some conclusions. In most cases, authors did not investigate this aspect and we used chi-square tests or Fisher's exact tests when the application conditions of chi-square test were not met to compare AMRB distribution between groups. Only three studies gave significant results ( $P < 0.05$ ); they all highlighted a higher AMRB prevalence in aquatic species.

### Host species ecology and contamination risks

To understand the role of wildlife in AMRB dynamics, it is essential to identify among potential host species those ecological traits that favour the carriage of such bacteria since: i) it can help to infer the origin of the habitat contamination by the AMRB and ii) it is crucial to take into account the concerned animal species' characteristics (e.g. diet, life span) in order to predict the role they could play in AMRB evolution, maintenance and dispersal.

A species can be characterized by its habitat, which influences the contamination risk by AMRB. As stated above, the habitats that are the more closely linked to human activities appear to be the most highly contaminated by AMRB (Allen *et al.* 2010). Thus, species inhabiting these habitats appear to be the most strongly impacted. Beyond the habitat occupied by species, two other major ecological traits can influence their role in AMR dynamics, as well as their risk of contamination by any pollutant: i) how they use the habitat resources, notably what they eat and drink, and ii) how they move within their habitat and from this habitat to other places.

Studies offering a basis for comparison of AMRB prevalence in species with different diets within a habitat are scarce. Within our reference list, 101 articles focused on a single species, among which 43 were carnivorous (including piscivores, insectivores and scavengers), 25 were herbivorous and 33 were omnivorous. Among the 109 papers that searched for AMRB in several species, only 35 provided data that allowed comparing AMRB prevalence across species with different diets. The others either studied several species sharing the same diet (34 papers) or did not give enough details to permit any statistical comparison between groups (40 papers). In particular, some authors either do not list the species they sampled (sometimes just referred to as wild birds or wild mammals) or do not give the number of individuals sampled, only referring to those that yielded positive AMRB results. To analyse the results of the 35 studies providing exploitable data, we used chi-square tests or Fisher's exact tests when the application conditions of chi-square test were not met to compare AMRB distribution between the different host species involved grouped into three types of diets: carnivores, omnivores and herbivores. We chose a

$P$ -value threshold of 0.05 and applied a Bonferroni correction when several pairwise tests were used to analyse the results of the same study. In 25 studies, no significant difference was highlighted between the proportions of individuals carrying AMRB across groups. The results of six articles were consistent in showing that carnivorous and omnivorous species were more likely to carry AMRB than herbivorous species (see details in Table 2). By contrast, two papers gave opposite results underlining higher AMRB prevalence in herbivorous than in carnivorous and omnivorous ones (see details in Table 2). Finally, two papers underlined a higher proportion of AMRB carriers in carnivorous than in omnivorous species, while one reported opposite results.

Thus, available data suggest that carnivorous and omnivorous species are generally the most at risk of AMRB carriage. Among bird species, raptors and gulls present high colonization rates with AMRB (Poeta *et al.* 2008; Guenther *et al.* 2010). Similarly, mammalian predators and omnivorous species appear to carry a wide diversity of AMRB (Navarro-Gonzalez *et al.* 2012; Gonçalves *et al.* 2013). Yet, few studies allowed the investigation of this issue and more than two-thirds of them gave non-significant results. Thus, more studies focusing simultaneously on several species living in the same habitat but differing by their diet are needed to be able to draw firm conclusions on this point. Moreover, a direct causal link between these contamination rates and diet remains to be highlighted. The gut microbiota is known to be influenced by diet along with phylogeny and physiology of the host (Muegge *et al.* 2011). At least in mammals, the maximum diversity of bacteria is observed in herbivorous species, an intermediate level of diversity characterizes the predators, while omnivorous species have the lowest bacteria diversity in contrast to what is known regarding AMRB (Ley *et al.* 2008). To understand this contrast, it is important to recall that the data we have concerning the AMRB found in wildlife are based on what has been searched for, meaning that we have information mostly on human pathogens. Available results argue in favour of undertaking research in order to understand the fate of AMRB throughout food chains (Teale 2002).

It is no doubt important to take into account the species that have the highest dispersal capacity due to the role they could play in the spread of AMRB. Thus, the studies reporting AMRB carriage in migratory birds (e.g. Palmgren *et al.* 1997; Middleton & Ambrose 2005; Foti *et al.* 2011) are of particular interest. Similarly, top predators generally forage across large distances (Schoener 1968; Gittleman & Harvey 1982). They could represent a natural reservoir of AMR that could disperse AMRB over large areas. Such species may be key elements of AMR dynamics in natural ecosystems. Omnivorous species often feed on anthropogenic decay and near human habitations and farms, meaning that they could represent a major epidemiological link between domestic animals, humans and wildlife. Furthermore, small anthropophilic

**Table 2.** Studies underlining significant differences in antimicrobial-resistant bacteria (AMRB) carriage across host groups with distinct diets

Host group	Focus bacteria	AMRB carriage across groups	Reference
Mammals	<i>E. coli</i> and <i>Salmonella spp.</i>	Omnivorous > herbivorous	Dias <i>et al.</i> (2015)
Birds	<i>E. coli</i>	Carnivorous > omnivorous	Guenther <i>et al.</i> (2010)
Mammals	<i>Enterococcus spp.</i>	Omnivorous > herbivorous	Mallon <i>et al.</i> (2002)
Mammals	<i>Bacillus spp.</i> , <i>Enterococcus spp.</i> and <i>Staphylococcus spp.</i>	Herbivorous > omnivorous	Meyer <i>et al.</i> (2014)
Mammals	<i>E. coli</i>	No difference in prevalence but more multiresistant strains in carnivorous than in omnivorous	Nhung <i>et al.</i> (2015)
Birds	<i>E. coli</i>	Omnivorous > herbivorous	Sato <i>et al.</i> (1978)
Birds and mammals	<i>E. coli</i>	Carnivorous > herbivorous	Smith <i>et al.</i> (2014)
Birds	<i>E. coli</i>	Omnivorous > carnivorous	Tausova <i>et al.</i> (2012)
Birds, mammals and reptiles	<i>Salmonella spp.</i>	Carnivorous > herbivorous	White & Forrester (1979)
Mammals	<i>E. coli</i>	Omnivorous > herbivorous	Williams <i>et al.</i> (2011)

To analyse the results of the 35 studies providing exploitable data, we used chi-square tests or Fisher's exact tests when the application conditions of chi-square test were not met to compare AMRB distribution between the different host groups. In addition to the 10 papers presented above, 25 studies did not highlight any significant difference between the proportions of individuals carrying AMRB across groups ( $P > 0.05$ , Bonferroni correction was applied when several pairwise tests were used to analyse the results of a single study).

prey species such as rodents could represent a bridge between human/domestic animals and their predators.

Life-history traits are also key to understanding the role of species in pathogen dynamics (Johnson *et al.* 2012) but they have rarely been taken into account in the study of AMR in wildlife. Among our reference list, data were too scarce to lead any analysis focusing on this issue. Yet they could have an important part to play in shaping the role of a species in antimicrobial dynamics. For example, a recent study on two rodent species (bank voles *Myodes glareolus* and wood mice *Apodemus sylvaticus*) in the UK showed that different seasonal population dynamics were associated with different AMRB carriage over time (Williams *et al.* 2011).

## Concluding remarks

Despite growing evidence showing the presence of AMRB in numerous wildlife species living in diverse environments world-wide, studies focusing on this compartment remain scarce. In this article, we used a systematic review to underline key information concerning AMRB carriage in wildlife that can be inferred from the available literature:

- Firstly, the natural habitats that are the most strongly impacted by human activities are the ones in which the highest diversity of AMR is observed in bacteria carried by wildlife, including resistance mechanisms that are of major concern for human health.
- Secondly, ecological characteristics of species as well as their life-history traits can serve to infer their potential role in AMRB epidemiology. Omnivorous, anthropophilic and carnivorous species seem to be at high risk of being potential carriers and potentially spreaders of AMRB.

• Thirdly, AMRB exchanges occur between wildlife, humans and domestic animals but the transmission routes are difficult to disentangle. Direct contacts, soil and water seem to be of major importance in the flows involved.

• Finally, when studying AMRB exchanges, it is crucial to consider that different bacteria may share and exchange resistance genes through horizontal transfer. It may be important to consider bacteria that have never been found to infect humans since the resistance mechanisms they carry could be exchanged with human pathogens.

This work highlights both the important progress that has been made in our understanding of the role played by wildlife in AMRB dynamics and the wide gaps that remain in our understanding of the mechanisms involved.

We recommend that a wider diversity of bacteria should be studied since organisms that are not pathogenic for humans may still carry and spread relevant resistance mechanisms that could be acquired by human/domestic animal pathogenic strains. For instance, while insects may become an important protein source within the next decades, it will be necessary to study the AMRB they may carry when assessing the health risks potentially associated with the development of this new resource. The application of a culture-independent approach, functional metagenomics, allowed the identification of resistance genes in the gut of the gypsy moth *Lymantria dispar*, which proved that insect guts could be a reservoir of antibiotic resistance genes with the potential for dissemination (Allen *et al.* 2009). Culture-independent approaches include PCR-based methods and functional genomics (see Allen *et al.* 2010). Such methods should be developed and used in line with classical culture-based

approaches since they can bring complementary information. Indeed, the results from culture-based methods are highly variable depending on the culture media used (Garcia-Armisen *et al.* 2013) and give no access to the resistance genes carried by uncultivable bacteria.

Furthermore, it is important to stress that when trying to track the fate of AMRB in wildlife, it is essential to access negative results reporting the absence of particular resistance mechanisms in some wild species or some kind of habitats. As illustrated by the low number of articles that allowed statistical analysis of AMRB across habitats and species diets, it is essential to give detailed data concerning the species sampled, to describe focus habitats using comparable indexes such as human density or proportion of different land use and to present results for each species in each habitat. Such improvements seem essential to move forward from successive studies that give information about one particular species in one particular habitat to comparable data that would allow broad comparisons and give way to a better understanding of AMRB dynamics in wildlife.

Finally, data concerning AMRB prevalence in developing countries in wildlife are lacking, while AMR is of major concern in these regions, notably in South-East Asia (Jean & Hsueh 2011). More attention should be paid to this area where the close links existing between wildlife, domestic animals and humans have proved to promote the emergence of pathogens (Chen *et al.* 2013), including AMRB (Hasan *et al.* 2012).

Over the last decade, the One Health approach has been implemented in the study of most emerging diseases, and wildlife has been included in their modelling. It recognizes that the health of humans, animals and ecosystems are interconnected and involves applying a coordinated, collaborative, multidisciplinary and cross-sectoral approach to address potential or existing risks that originate at the animal–human–ecosystems interface. It is urgent to extend this fruitful approach to the study of the emergence and spread of AMR. Otherwise, this missing piece of the puzzle could impair our capacity to limit the emergence, maintenance and spread of resistance.

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## Data accessibility

All data used and discussed in this article were previously published in the papers listed in Appendix S1.

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## Supporting Information

Additional Supporting Information may be found in the online version of this article.

**Appendix S1.** Complete list of the 210 articles gathered through systematic search.

## **Chapitre II**

**Forte prévalence de la céphalosporinase CMY-2 portée  
par des éléments intégratifs et conjugatifs SXT/R391-like  
chez des souches aviaires de *Proteus mirabilis* du sud de la  
France**

## Préambule

L'acquisition et l'hyperproduction de céphalosporinases de type AmpC constitue aujourd'hui une cause non négligeable d'émergence et de diffusion de résistance aux céphalosporines de troisième génération (C3G) en particulier chez les *Enterobacteriaceae*. Les gènes *ampC* codant ces céphalosporinases sont naturellement retrouvés sur le chromosome des entérobactéries dites du groupe 3. Mais depuis quelques années on observe l'émergence de l'acquisition de gènes *ampC* chez des espèces d'entérobactéries qui jusqu'alors en étaient naturellement dépourvues. La mobilisation des gènes *ampC* naturellement présents dans le chromosome de certaines bactéries a été rendue possible grâce à des éléments génétiques mobiles, comme les plasmides ou les éléments intégratifs et conjugatifs (ICEs). Ce phénomène est particulièrement préoccupant pour l'espèce *Proteus mirabilis*, qui est un pathogène fréquemment retrouvé en clinique humaine. L'intégration de ces gènes dans le chromosome des bactéries réceptrices permet d'acquérir et de pérenniser à long terme cette résistance au sein de l'espèce par transmission verticale.

Si de plus en plus d'études rapportent la présence de ces céphalosporinases chez des souches cliniques, il y a cependant peu de données disponibles sur l'existence potentielle d'écosystèmes animaux qui constitueraient des réservoirs primaires ou secondaires d'entérobactéries productrices de ces enzymes.

Dans l'article suivant nous décrivons la présence de *P. mirabilis* producteurs d'AmpC acquises au sein du microbiote des deux espèces de goélands étudiées.

Ces isolats aviaires de *P. mirabilis* producteurs d'AmpC étaient tous porteurs du gène *bla*<sub>CMY-2</sub>. Le support génétique a été identifié comme étant un ICE de la famille SXT/R391-like dans toutes les souches aviaires. Ce support a été retrouvé chez trois souches humaines isolées dans la même région et durant la même période d'étude. Nous avons comparé les différents isolats par rep-PCR. Deux isolats cliniques avaient le même fond génétique que neuf isolats aviaires des deux espèces d'oiseaux.

Nous avons ainsi décrit pour la première fois dans un microbiote aviaire l'existence de souches de *P. mirabilis* productrices de céphalosporinases de type CMY-2 dont le gène est situé sur un ICE de la famille SXT/R391-like.

Article II


**High prevalence of SXT/R391-related integrative and conjugative elements carrying**

***bla*<sub>CMY-2</sub> in *Proteus mirabilis* from gull isolates in the South of France**

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# High Prevalence of SXT/R391-Related Integrative and Conjugative Elements Carrying *bla*<sub>CMY-2</sub> in *Proteus mirabilis* Isolates from Gulls in the South of France

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**The genetic structures involved in the dissemination of *bla*<sub>CMY-2</sub> carried by *Proteus mirabilis* isolates recovered from different gull species in the South of France were characterized and compared to clinical isolates. *bla*<sub>CMY-2</sub> was identified in *P. mirabilis* isolates from 27/93 yellow-legged gulls and from 37/65 slender-billed gulls. It was carried by a conjugative SXT/R391-like integrative and conjugative element (ICE) in all avian strains and in 3/7 human strains. Two clinical isolates had the same genetic background as six avian isolates.**

CMY-2 and its derivatives are the most widespread plasmid-mediated cephalosporinases (AmpC) in *Proteus mirabilis* (1, 2), and they are mainly found in plasmids from incompatibility (Inc) groups A/C and I1 (1, 3). *bla*<sub>CMY-2</sub> mobilization by an integrative and conjugative element (ICE) in *P. mirabilis* was described recently in Japan and Spain (4, 5). Several studies focused on the characterization of AmpC-producing *P. mirabilis* isolates from food-producing animals and pets (6–8). Migratory birds can act as reservoirs and play an important role in the dissemination of these resistance genes (9). This study investigated the occurrence and the molecular structures supporting the spread of *bla*<sub>CMY-2</sub> in *P. mirabilis* isolates from different gull species in the South of France. Human isolates from the same geographical region were used for comparison.

(Preliminary results of this research were presented at the 34th Réunion Interdisciplinaire de Chimiothérapie Anti-Infectieuse [RICAI], Paris, France, 27 to 28 November 2014.)

In April 2012, fecal samples were collected from 93 juvenile nonfledged yellow-legged gulls (*Larus michahellis*) and from 65 slender-billed gulls (*Chroicocephalus genei*) breeding in the island of Carteau in Port-Saint-Louis and in the Giens peninsula (France), respectively. A cotton swab was rotated inside the bird cloacae and was immediately inoculated in tryptic soy broth (Thermo Fisher Scientific). After a 24-h incubation at 37°C, broths were subcultured on chromID ESBL agar biplates (bioMérieux, Marcy l'Etoile, France) and were examined after 24 and 48 h. *P. mirabilis* was identified by matrix-assisted laser desorption ionization–time of flight mass spectrometry (Bruker Daltonics, Bremen, Germany). Susceptibility to amoxicillin, cefalotin, cefoxitin, cefotaxime, ceftazidime, cefepime, and imipenem was tested using the disk diffusion method on Mueller-Hinton agar and was interpreted following the European Committee on Antimicrobial Susceptibility Testing (EUCAST) clinical breakpoints

(version 5.0) ([http://www.eucast.org/clinical\\_breakpoints/](http://www.eucast.org/clinical_breakpoints/)). The criteria used to select suspected AmpC producers were described previously (1). Extended-spectrum beta-lactamase (ESBL) production was excluded using the double-disc synergy test (10). Seven *P. mirabilis* human clinical isolates with AmpC phenotypes collected in 2012 in the same geographical region were used for comparison. Strain typing was performed by repetitive sequence-based PCR (rep-PCR) using the DiversiLab system (bioMérieux, France) as described previously (11).

The presence of genes encoding AmpC was assessed by multiplex PCR as previously described (12). Plasmids were typed using the PCR-based replicon-typing method (PBRT) (13) and the plasmid relaxase gene-typing method (PRaseT) (14). Primers targeting the highly conserved relaxase gene of the ICE belonging to the SXT/R391 family were designed as previously described (14). ICE integration at the 5' end of the chromosomal *prfC* gene was investigated using primers to amplify the *ORF\_96* gene at the ICE 3'

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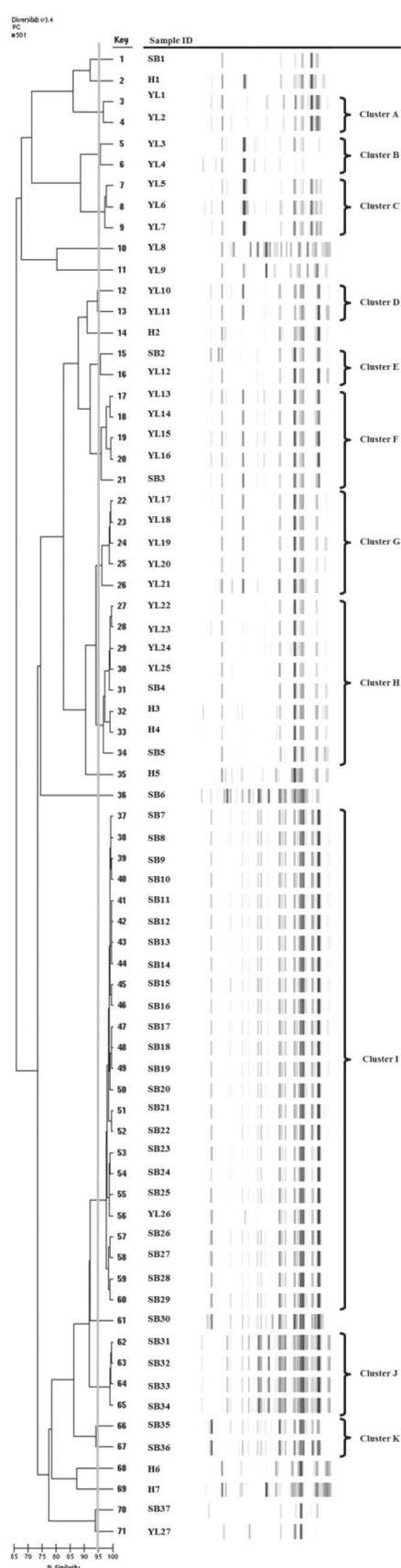
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extremity, degenerate primers to cover *P. mirabilis* and *Escherichia coli* *prfC* gene sequences, and primers that amplify both regions (overlapping PCR) (4, 5) (see Table S1 in the supplemental material). *E. coli* J53 was used as a negative control. All PCR products were sequenced bidirectionally using the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) and an Applied Biosystems 3730xl capillary sequencer. Sequences were identified using the BLAST program and the NCBI database. Mating experiments were performed at 37°C using *P. mirabilis* and azide-resistant *Escherichia coli* J53 strains with a donor to recipient ratio of 1:1. Transconjugants were selected on Drigalski agar (Bio-Rad) containing 200 mg/liter sodium azide and 20 mg/liter cefoxitin. To investigate the possible chromosomal location of *bla*<sub>CMY-2</sub>, I-CeuI-restricted DNA from selected strains and transconjugants was separated by pulsed-field gel electrophoresis (PFGE). Pulse ramps were 90 to 150 s for 24 h and 90 to 120 s for 6 h at 170 V. Southern blotting and hybridization using a *bla*<sub>CMY-2</sub> probe and a probe for the highly specific integrase of the SXT/R391-like ICE were carried out as previously described (15, 16).

TABLE 1 Antibiotic susceptibility testing of *Proteus mirabilis* isolates<sup>a</sup>

Isolate type	rep-PCR cluster	Genetic support of <i>bla</i> <sub>CMY-2</sub>	No. isolates	Selected strains on which Southern blotting was performed <sup>b</sup>	Parental strain or transconjugant <sup>c</sup>	Antibiotic resistance profile (inhibition zone diam, mm <sup>d</sup> )						
						AMX	CTX	CF	FEP	FOX	CAZ	IPM
Avian	A	SXT/R391-like ICE	2	YL11	P	R (6)	S (23)	R (6)	S (29–30)	R (17)	I (19–21)	S (28–30)
	B	SXT/R391-like ICE	2		T	R (6)	R (13)	R (6)	S (30)	R (6)	R (9)	S (32)
					P	R (6)	I (19)	R (6)	S (24–27)	R (14–17)	I (19–20)	S (25–27)
	T	R (6)	R (10)		R (6)	S (33)	R (6)	R (6)	S (30)			
	C	SXT/R391-like ICE	3		P	R (6)	S (23–26)	R (6)	S (27–34)	S (19–21)	I (19–21)	S (25–28)
					T	R (6)	R (10)	R (6)	S (32)	R (6)	R (6)	S (30)
	D	SXT/R391-like ICE	2		P	R (6)	S (23–26)	R (6)	S (29–34)	R (17–18)	I (20–21)	S (26)
					T	R (6)	R (11)	R (6)	S (32)	R (6)	R (6)	S (31)
	E	SXT/R391-like ICE	2		P	R (6–12)	S (23–26)	R (6)	S (26–39)	R (18)	S/I (19–27)	S (24–29)
					T	R (6)	R (16)	R (6)	S (34)	R (8)	R (12)	S (30)
	F	SXT/R391-like ICE	5		P	R (6)	S/I (19–26)	R (6)	S (27–34)	R (15–18)	S/I (19–24)	S (25–29)
					T	R (6)	R (8)	R (6)	S (33)	R (6)	R (6)	S (32)
	G	SXT/R391-like ICE	5		P	R (6)	S/I (19–25)	R (6)	S (25–33)	R (10–18)	S/I (19–27)	S (27–30)
					T	R (6)	R (6)	R (6)	S (32)	R (6)	R (6)	S (30)
	H	SXT/R391-like ICE	6		P	R (6–7)	S (23–28)	R (6)	S (28–39)	R (13–18)	S/I (19–30)	S (26–33)
T				R (6)	R (15)	R (6)	S (34)	R (9)	R (10)	S (32)		
I	SXT/R391-like ICE	24	P	R (6–10)	S (23–32)	R (6)	S (29–41)	R (8–18)	S/I (19–31)	S (24–31)		
			T	R (6)	R (9)	R (6)	S (37)	R (6)	R (8)	S (29)		
J	SXT/R391-like ICE	4	P	R (6–8)	S (25–32)	R (6)	S (32–41)	R (18)	S/I (21–32)	S (24–31)		
			T	R (6)	R (6)	R (6)	S (34)	R (6)	R (6)	S (30)		
K	SXT/R391-like ICE	2	P	R (6)	S (28–30)	R (6)	S (34–39)	RI (18)	S (28–29)	S (30–31)		
			T	R (6)	R (9)	R (6)	S (33)	R (6)	R (10)	S (32)		
Singletons	SXT/R391-like ICE	7	P	R (6)	S/R (16–29)	R (6)	S (27–37)	S/R (18–19)	S/I (19–27)	S (23–29)		
			T	R (6)	R (11–15)	R (6)	S (33–38)	R (6–8)	R (11–17)	S (31–33)		
Human clinical	H	IncA/C plasmid <sup>e</sup>	1	H4	P	R (6)	S (25)	R (6)	S (27)	R (18)	I (20)	S (25)
					T	R (6)	R (6)	R (6)	S (33)	R (6)	R (6)	S (31)
		SXT/R391-like ICE	1	H3	P	R (6)	S (23)	R (6)	S (32)	R (15)	I (20)	S (25)
					T	R (6)	R (8)	R (6)	S (34)	R (6)	R (7)	S (32)
	Singletons	SXT/R391-like ICE	2	P	R (6)	S/I (19–23)	R (6)	S (25–35)	R (17–18)	I (19–21)	S (22–26)	
				T	R (6)	R (9–11)	R (6)	S (28–33)	R (6)	R (6–13)	S (29–32)	
	Singleton	IncA/C plasmid <sup>e</sup>	1	P	R (6)	I (19)	R (6)	S (25)	R (17)	I (19)	S (23)	
				T	R (6)	R (12)	R (6)	S (38)	R (6)	R (8)	S (32)	
	Singleton	IncA/C plasmid	2	H7	P	R (6)	S (21–23)	R (6)	S (34–37)	R (18)	I (20)	S (27–30)
					T	R (6)	R (15–16)	R (6)	S (32–35)	R (6)	R (6–10)	S (33)

<sup>a</sup> Antibiotic susceptibility testing was performed and interpreted according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) clinical breakpoints (version 5.0) ([http://www.eucast.org/clinical\\_breakpoints/](http://www.eucast.org/clinical_breakpoints/)). Mating experiments were performed using one randomly chosen representative *P. mirabilis* isolate for each clone defined by rep-PCR and all singletons.

<sup>b</sup> Southern blotting with *bla*<sub>CMY-2</sub> and ICE-specific integrase probes was performed after PFGE following DNA macrorestriction using the intronic endonuclease I-CeuI.

<sup>c</sup> P, parental strain; T, transconjugant.

<sup>d</sup> The range of the inhibition zone diameter for clustered isolates is indicated. AMX, amoxicillin; CF, cefalotin; CTX, cefotaxime; CAZ, ceftazidime; FEP, cefepime; FOX, ceftoxitin; IPM, imipenem; R, resistant; S, susceptible; I, intermediate.

<sup>e</sup> One strain also harbored a SXT/R391-like ICE that did not carry *bla*<sub>CMY-2</sub>.



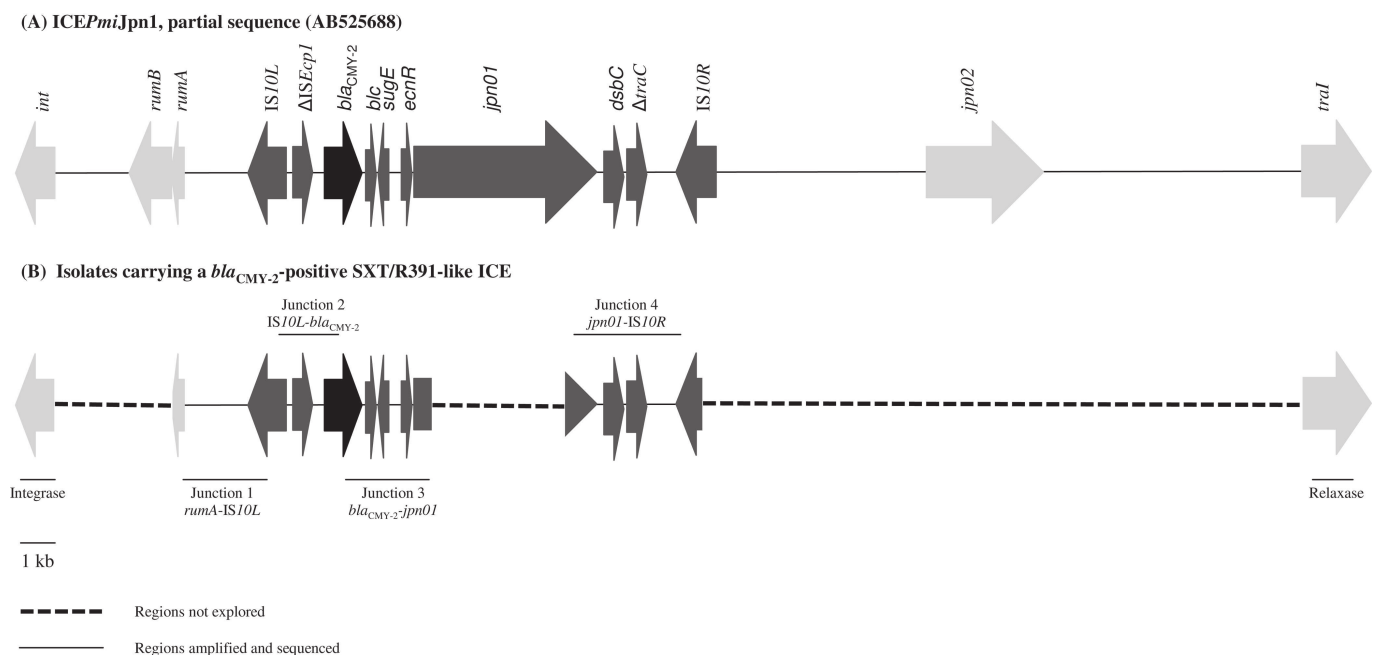


FIG 2 Genetic organization of the SXT/R391-like ICE carrying bla<sub>CMY-2</sub> in *P. mirabilis*. The same molecular structure was observed in all 64 bla<sub>CMY-2</sub>-positive avian strains and in 3/7 (42.9%) human strains. This structure was similar to that of ICE*PmiJpn1* (GenBank accession no. AB525688). Light gray arrows represent the conserved genes of the ICE. Dark gray arrows represent genes carried by the Tn10 composite transposon. Black arrows represent the bla<sub>CMY-2</sub> gene. Thin black lines represent the different primers used to explore this region. (A) Partial sequence of ICE*PmiJpn1* used as a template for PCR mapping. (B) Schematic representation of the regions amplified and sequenced in this study.

Positive overlapping PCR amplification demonstrated that the ICE was integrated in the chromosome of each *P. mirabilis* isolate and the corresponding transconjugant. These data suggest that the ICE is involved in bla<sub>CMY-2</sub> dissemination in all avian and in three human isolates. PCR analysis of ICE insertion sites in randomly chosen isolates for each clone of our collection and of their respective transconjugants gave the same result. As expected, (i) ORF<sub>96</sub> was present in *P. mirabilis* and its transconjugant but was absent in *E. coli* J53, (ii) *prfC* was present in *P. mirabilis* and *E. coli* J53 but could not be amplified in the transconjugant (due to disruption caused by the ICE insertion), and (iii) the overlapping PCR covering the ORF<sub>96</sub> and *prfC* genes was positive in *P. mirabilis* and its transconjugant but negative in *E. coli* J53. This confirmed the insertion of the bla<sub>CMY-2</sub>-carrying ICE at the 5' end of *prfC*.

*P. mirabilis* isolates carrying bla<sub>CMY-2</sub> were described in dogs (6), cats (18), and chickens (19). Mata et al. recently highlighted the increasing prevalence of bla<sub>CMY-2</sub>-carrying SXT/R391-like ICEs in *P. mirabilis* human isolates in Spain between 1999 and 2007 (5). In their study, the bla<sub>CMY-2</sub> genetic environment was similar to the one observed in our avian *P. mirabilis* isolates. A similar structure was described in the ICE*PmiJpn1* element recovered from a *P. mirabilis* clinical isolate in Japan in 2006 (Fig. 2). Our study is the first description of such a structure in *P. mirabilis* isolates in France and tends to confirm the hypothesis about the role of ICEs in bla<sub>CMY-2</sub> dissemination worldwide (5). The finding that the ICE was the exclusive genetic support of the resistance gene in all avian isolates, which were classified in different clusters, suggests that it may play an important role in the spread of antibiotic resistance genes among these birds and in marine ecosystems. Although the biotic/abiotic reservoirs of resistant strains of *P. mirabilis* continue to be poorly known, the presence of similar

clones in avian and human isolates in our study may suggest exchanges between different ecosystems (wildlife versus human). Moreover, *P. mirabilis* has already been described as a shuttle species between human and animal guts and water bodies (20).

As ICEs from the SXT/R391-like family are widespread in environmental strains of *Proteus* spp., *Vibrio* spp., *Photobacterium* spp., and *Shewanella* spp. (21), the insertion of beta-lactam resistance genes in this structure is worrying and should be monitored. Further studies are needed to assess the presence of beta-lactam-resistant ICE-positive strains (*P. mirabilis* and other clinically relevant bacteria) in the environment and in wildlife microbiota.

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### **Chapitre III**

**Persistance de souches de *P. mirabilis* porteuses du gène *bla*<sub>CMY-2</sub> sur un élément intégratif et conjugatif SXT/R391-like au sein de goélands du sud de la France**

## Préambule

Les entérobactéries résistantes aux  $\beta$ -lactamines ont été décrites dans le tube digestif des oiseaux sauvages dans plusieurs pays à travers le monde, y compris les régions les plus reculées. Cependant, la plupart des études sont transversales ou de prévalence, qui décrivent le portage de bactéries à Gram-négatif antibiorésistantes à un moment précis. La plupart des études longitudinales portant sur la persistance des gènes codant des BLSE ou des AmpC chez l'animal ont été menées chez les animaux d'élevage et producteurs de denrées alimentaires. A notre connaissance, il n'y a eu que peu d'études longitudinales portant sur des bactéries à Gram négatif multirésistantes chez les oiseaux sauvages.

L'étude suivante, dont l'échantillonnage a été effectué en 2013, est un suivi de celle de 2012 dans laquelle nous avons décrit une prévalence élevée de *Proteus mirabilis* producteurs de CMY-2 chez les deux espèces de goélands étudiées. Comme le gène *bla*<sub>CMY-2</sub> était situé dans un ICE de la famille SXT/R391-like, une structure génétique qui n'avait été décrite jusqu'alors qu'au Japon et en Espagne, nous avons étudié la persistance et la stabilité de ce déterminant de résistance chez ces deux espèces de goélands à un an d'intervalle.

Nous avons isolé des souches de *P. mirabilis* résistantes aux C3G lors de la campagne de 2013. Tous les isolats étaient porteurs du gène *bla*<sub>CMY-2</sub> situé sur un ICE de la famille SXT/R391-like. La présence de ce gène de résistance sur ce même support génétique à un an d'intervalle chez les mêmes colonies de goélands témoigne de sa persistance dans la faune sauvage. Des clones similaires de *P. mirabilis* ont été identifiés dans les deux campagnes d'échantillonnage chez les deux espèces de goélands et chez les isolats humains utilisés lors de la campagne de 2012.

Cette étude met l'accent sur la persistance de déterminants d'antibiorésistance chez les goélands dans le sud de la France, soutenant notre hypothèse que ces oiseaux sauvages peuvent représenter un réservoir zoonotique pouvant persister plusieurs années. L'analyse clonale prouve une transmission interespèce avec les risques zoonotiques qui en découlent.

Article III

**Persistence of *bla*<sub>CMY-2</sub>-producing *Proteus mirabilis* in two gull colonies at a one-year interval in Southern France**

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Soumis dans *Veterinary Microbiology*

**Persistence of *bla*<sub>CMY-2</sub>-producing *Proteus mirabilis* in two gull colonies at a one-year interval in Southern France**

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**Running Title:** Persistence of *bla*<sub>CMY-2</sub>-carrying ICEs in *Proteus mirabilis* from gulls

**Keywords:** persistence, *bla*<sub>CMY-2</sub>, SXT/R391 integrative and conjugative element, ICE, *Proteus mirabilis*, gulls, France

**Figures:** 1 in supplementary data

**Tables:** 1 in text

**Abstract:** 60 words

**Text:** 938 words

41    **Abstract**

42    We describe the persistence of *bla*<sub>CMY-2</sub>-producing *Proteus mirabilis* isolated from two gull  
43    colonies in Southern France sampled in 2012 and 2013. PCR-mapping showed that *bla*<sub>CMY-2</sub>  
44    was located on a similar genetic background which was an SXT/R391-like integrative and  
45    conjugative element. Rep-PCR (Diversilab) analysis showed similar clones between the avian  
46    isolates from the two years of study and some human isolates.



Resistance to broad-spectrum cephalosporins in *Enterobacteriaceae* from animal origin has been increasingly reported in the last 15 years (Ewers et al., 2012; Guenther et al., 2011). Companion animals (Carattoli et al., 2005; Costa et al., 2008), food-producing animals (Huijbers et al., 2014; Michael et al., 2015) and wildlife (Alcalá et al., 2015; Costa et al., 2006; Guenther et al., 2011) have been identified as carriers of  $\beta$ -lactam-resistant *Enterobacteriaceae*. This presence can become a major health problem since similar bacterial clones have been recovered from animal and human clinical isolates (Bonnedahl et al., 2009; Hernandez et al., 2013; Valentin et al., 2014), which suggests that animals can act as a potential reservoirs of multidrug resistance (MDR) genes.

$\beta$ -lactam resistant *Enterobacteriaceae* isolates have been extensively described in the digestive tract of wild birds in several countries worldwide (Alcalá et al., 2015; Bonnedahl and Järhult, 2014; Hernandez et al., 2013), including regions remote from human habitation (Sjölund et al., 2008). However, most studies are cross-sectional or prevalence studies, which describe the carriage of MDR Gram-negative bacteria at a precise moment of time. Most longitudinal studies focusing on the persistence of ESBL and pAmpC-encoding genes in animals have been carried out in livestock and food-producing animals (Giovanardi et al., 2013; Hansen et al., 2013; Keelara et al., 2013; Laube et al., 2013; Liebana et al., 2006; Von Salviati et al., 2014). To our knowledge, there are only few longitudinal studies focusing on MDR Gram-negative bacteria carriage in wild birds (Bonnedahl et al., 2014).

The present study is a follow-up to our 2012 study in which we reported the high prevalence of *bla*<sub>CMY-2</sub>-harboring *Proteus mirabilis* in two gull colonies in Southern France (Aberkane et al., 2015), a region where birds have close contact to humans. Moreover, two human clinical isolates had the same genetic background as six avian isolates. As *bla*<sub>CMY-2</sub> was located in a SXT/R391-like integrative and conjugative element (ICE), a genetic structure which had only been described in Japan and Spain (Harada et al., 2010; Mata et al., 2011), we aimed to study

the persistence and steadiness of this particular resistance determinant in the gull population at a one-year interval.

In April 2013, we collected 193 cloacal samples from two juvenile gull colonies in Southern France. The species involved included yellow-legged gulls (YLG) (*Larus michahellis*, 101 samples) breeding in the island of Carteau in Port-Saint-Louis and slender-billed gulls (SBG) (*Chroicocephalus genei*, 92 samples) breeding in Giens peninsula. The same sampling method was used as the previous study (Aberkane et al., 2015). Screening was performed in the same gull colonies (i.e., same species and same locations) as in our 2012 study but on different bird individuals since rectal swabbing can be performed only for juvenile gulls. Screening for *P. mirabilis* resistant to third-generation cephalosporins (3GC) was carried out using the ChromID ESBL agar biplates (bioMérieux, Marcy-l'Etoile, France) selective media. Bacterial identification and antibiotic susceptibility testing was performed as described in the preceding study (Aberkane et al., 2015).

In the 2013 screening campaign, 34/193 (17.6%) samples were positive for cephalosporin-resistant *P. mirabilis* isolates. Ten isolates came from YLG (10/101, 9.9%) and 24 isolates came from SBG (24/92, 26.1%). All isolates showed resistance to amoxicillin, co-amoxiclav, cefalotin and cefoxitin and were positive for the CMY-2-encoding gene (Table 1). For comparison, the 2012 campaign retrieved 64/158 (40.5%) CMY-2-producing *P. mirabilis* isolates with YLG and SBG yielding 27 and 37 isolates, respectively (Aberkane et al., 2015). Although the 2013 campaign could show a slow decline in resistance rates when compared to the 2012 positivity rate (40.5%), we show here the persistence of this resistance determinant in two gull populations.

The genetic backgrounds of all 98 *bla*<sub>CMY-2</sub>-carrying *P. mirabilis* avian isolates from the 2012 and 2013 campaigns were analysed by rep-PCR using the DiversiLab system (bio-Mérieux,

France), as well as seven human clinical isolates collected in 2012 (Supplementary Figure 1). Fourteen clusters (named A-N) and 12 singletons were found. Seven clusters included isolates from both species coming from the 2012 and 2013 campaigns (clusters B, D, E, F, I, M and N). Two clusters (clusters A and L) included only YLG isolates from the 2012 campaign and two other clusters (clusters H and J) included only SBG isolates from the 2012 campaign. Three clusters included avian and human clinical isolates (clusters C, G and K). PCR-mapping of the genetic environment of *bla*<sub>CMY-2</sub> was performed as previously described (Aberkane et al., 2015) and showed that *bla*<sub>CMY-2</sub> was part of a SXT/R391-like ICE in all 34 *P. mirabilis* isolates from 2013. This ICE was successfully transferred by conjugation to *E. coli* J53 for a representative isolate of each rep-PCR clone. As for the avian isolates of the 2012 campaign, no plasmid encoding *bla*<sub>CMY-2</sub> was recovered in 2013.

Similar clones were recovered in the two sampling campaigns (Supplemental Figure 1) while avian isolates which presented the same genetic background as some human clinical isolates of 2012 were recovered in 2013. The study design does not allow us to know if the gulls are intermittent or persistent carriers of CMY-2-producing *P. mirabilis* since a same individual was only sampled once during the entire study. However, the presence of this resistance gene on similar genetic structures at a one-year interval in the same colonies witnesses its establishment in the wild fauna. Strain fitness and stability of the ICE in the chromosome of *P. mirabilis* could explain this persistence. Longitudinal surveys of the carriage of antibiotics resistance determinants have been performed scarcely in animals (Giovanardi et al., 2013; Hansen et al., 2013; Keelara et al., 2013; Laube et al., 2013; Liebana et al., 2006; Persoons et al., 2010; Raufu et al., 2014; Von Salviati et al., 2014). Most studies were carried out in livestock and food-producing animals, which are generally submitted to various antibiotics to treat clinical disease problems and to serve as growth promoters. Nonetheless, these studies showed that resistance determinants could persist from months to several years. Moreover,

Liebana et al. showed that despite the discontinuation of antibiotics use at the beginning of their study, CTX-M enzymes could persist for more than six months amongst calves and their environment (Liebana et al., 2006). This is concordant with the present study showing the persistence of the *bla*<sub>CMY-2</sub> resistance determinant although a potential environmental exposure to antibiotics cannot be excluded.

This study emphasises the persistence of MDR determinants in wild gulls from Southern France, supporting our hypothesis that wild birds may represent a zoonotic reservoir which may persist for several years without any known antibiotic selection pressure. Clonal analysis proves interspecies transmission with arising zoonotic risks in this highly crowded area.

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## **Competing interests:**

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## **Ethical approval:**

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234



235 **Table.** Characterization of *bla*<sub>CMY-2</sub>-positive *P. mirabilis* isolates from the 2012 and 2013  
236 screening campaigns.

237 YLG, yellow-legged gull isolates; SBG, slender-billed gull isolates.

238 **Supplementary Figure.**

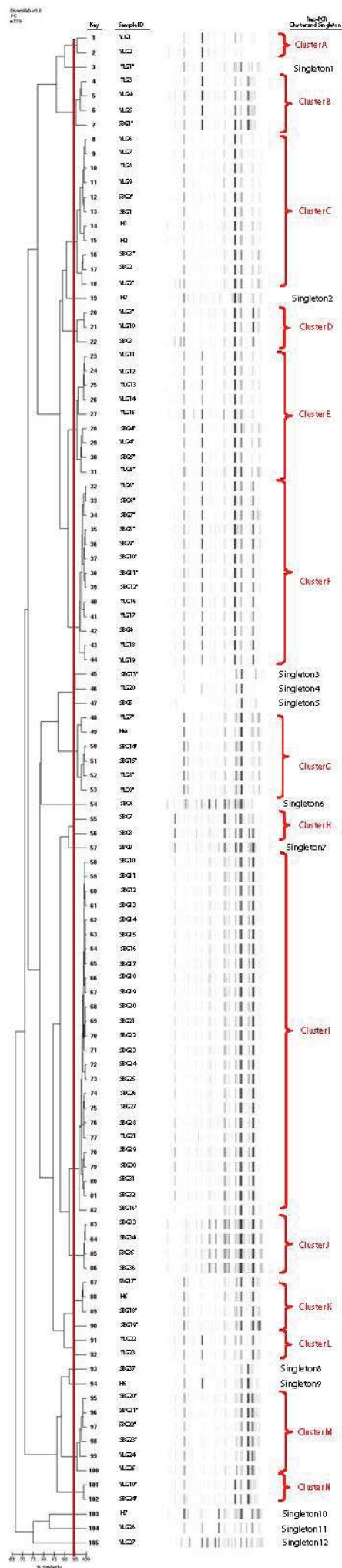
239 Dendrogram of *bla*<sub>CMY-2</sub>-positive *P. mirabilis* isolates from the 2012 and 2013 screening  
240 campaigns.

241 YLG, yellow-legged gull isolates; SBG, slender-billed gull isolates; H, human isolates; \*,  
242 strains of the 2013 campaign.

243

Table

Collection date (No. isolates)	Source (No.)	Cephalosporinase-producing samples			
		No. CMY-2-carrying isolates	Genetic support of CMY-2 (No.)	No. Of Diversilab clusters	No. Singletons
2012 (165)	YLG (93)	27	SXT/R391-like ICE (27)	9	3
	SBG (65)	37	SXT/R391-like ICE (37)	6	4
	Human (7)	7	SXT/R391-like ICE (3), IncA/C plasmid (4)	3	3
2013 (193)	YLG (101)	10	SXT/R391-like ICE (10)	6	1
	SBG (92)	24	SXT/R391-like ICE (24)	9	1



## **Chapitre IV**

**Présence d'*Escherichia coli* résistants aux carbapénèmes  
au sein du microbiote cloacal de goélands leucophées dans  
le sud de la France**

## Préambule

Les carbapénèmases acquises constituent actuellement l'une des menaces les plus inquiétantes pour la santé publique en terme d'antibiorésistance. En 2013, une *Salmonella corvalis* produisant une carbapénémase de type NDM-1 a été décrite chez un rapace sauvage (un milan noir, *Milvus migrans*). Ainsi, d'autres études sont nécessaires pour comprendre le rôle des oiseaux sauvages dans la transmission de bactéries résistantes aux carbapénèmes. L'objectif de notre étude a été d'enquêter sur la présence d'*Escherichia coli* résistants aux carbapénèmes chez les deux espèces de goélands étudiées. En 2012, nous avons recueilli 158 échantillons cloacaux provenant de ces deux espèces.

Nous avons pu isoler sur milieux sélectifs 22 souches d'*E. coli* résistantes aux carbapénèmes à partir de goélands leucophées, alors qu'aucune n'a été isolée de goélands railleurs. Toutes étaient porteuses du gène *bla<sub>VIM-1</sub>*. Nous les avons comparées au niveau moléculaire avec des isolats sensibles provenant des deux espèces de goélands et des souches humaines sensibles provenant de patients hospitalisés dans la même région. Les souches résistantes étaient reliées génétiquement aux souches sensibles isolées des deux espèces de goélands et avec les souches humaines.

Ces résultats sont suffisamment alarmants pour essayer d'identifier la source de contamination de ces oiseaux. Ils soulignent également la complexité de la circulation de l'antibiorésistance dans cette région. En effet, contrairement aux souches de *P. mirabilis* productrices de CMY-2, ces souches d'*E. coli* productrices de VIM-1 n'ont été isolées que chez les goélands leucophées. De plus, ce type de carbapénèmases n'est que très rarement identifié en France. On pourrait alors se poser la question sur l'origine de ces souches.

Article IV

**VIM-1 carbapenemase-producing *Escherichia coli* in gulls from southern France**

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## VIM-1 carbapenemase-producing *Escherichia coli* in gulls from southern France

### Running title: Carbapenemase-producing *Escherichia coli* in gulls

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¶Co-managed work

### Abstract:

Acquired carbapenemases currently pose one of the most worrying public health threats related to antimicrobial resistance. A NDM-1-producing *Salmonella* Corvallis was reported in 2013 in a wild raptor. Further research was needed to understand the role of wild birds in the transmission of bacteria resistant to carbapenems. Our aim was to investigate the presence of carbapenem-resistant *Escherichia coli* in gulls from southern France. In 2012, we collected



158 cloacal swabs samples from two gull species: Yellow-legged gulls (*Larus michahellis*) that live in close contact with humans and Slender-billed gulls (*Chroicocephalus genei*) that feed at sea. We molecularly compared the carbapenem-resistant bacteria we isolated through culture on selective media with the carbapenem susceptible strains sampled from both gull species and from stool samples of humans hospitalized in the study area. The genes coding for carbapenemases were tested by multiplex PCR.

We isolated 22 carbapenem-resistant *E. coli* strains from Yellow-legged gulls while none were isolated from Slender-billed gulls. All carbapenem-resistant isolates were positive for *bla<sub>VIM-1</sub>* gene. VIM-1 producing *E. coli* were closely related to carbapenem susceptible strains isolated from the two gull species but also to human strains.

Our results are alarming enough to make it urgently necessary to determine the contamination source of the bacteria we identified. More generally, our work highlights the need to develop more bridges between studies focusing on wildlife and humans in order to improve our knowledge of resistant bacteria transmission routes.

**Keywords:** antimicrobial resistance, enterobacteria, humans, *Larus*, molecular characterization, phylogenetic analyses, wild birds

## Introduction

Among the antimicrobial resistant bacteria (AMRB) of concern recently isolated from birds, a NDM-1 carbapenem-resistant *Salmonella* (*S. enterica* subsp. *enterica* serovar Corvallis) was reported in 2013 in a wild raptor (a black kite, *Milvus migrans*) in Germany (Woodford *et al.* 2014).

This detection raised questions about the potential risk of the spread of resistance potentially associated with this wild reservoir. Acquired carbapenemases currently pose one of the most worrying public health threats related to antibiotic resistance (Gupta *et al.* 2011; Poirel, Potron & Nordmann 2012). They confer resistance to carbapenems, but also to almost all  $\beta$ -lactams, the most widely used class of antibiotic, and are encoded by genetic elements that are transferable between bacteria (Potron, Poirel & Nordmann 2014; Krahn *et al.* 2016; Luca *et al.* 2016). The actual number of carbapenemase producing bacterial isolates is rising and the epidemiological status of these bacteria (sporadic versus local spread versus national endemicity) is progressively worsening worldwide (Glasner *et al.* 2013; WHO 2014).

Carbapenems represent the latest therapeutic innovation for  $\beta$ -lactams, but this innovation is old, the latest group of molecules having been approved for clinical use more than a decade ago. Yet they are currently our last effective defence against multiresistant Gram-negative bacteria (Woodford *et al.* 2014).

Our ability to limit the rise and spread of carbapenemase producers, which occur only at basal levels in many countries at present, should serve as a key performance indicator for the success or failure of the efforts that have been called for by international organizations and governments to reduce the impact of antibiotic resistance (Woodford *et al.* 2014). To meet

this challenge, we need to investigate the role of any non-human reservoirs of carbapenem-resistant bacteria, which could favor their further spread in human populations (Woolhouse *et al.* 2015). To date, carbapenem-resistant bacteria have been isolated from water in some rivers and sewage plants as well as in a few pets and food animals (reviewed in Woodford *et al.* 2014). The resistant bacteria isolated from a black kite is so far the sole evidence of the presence of carbapenem-resistant bacteria in a wild species without any direct link with domestic animals or humans. The only other evidence of carbapenem resistance in wildlife is from a pig farm in Germany where a VIM-1 carbapenem-resistant *Salmonella* serovar Infantis was isolated in a mouse (*Mus musculus*) (Fischer *et al.* 2012). Verona integron-encoded metallo- $\beta$ -lactamases (VIM) belong to class B carbapenemase and were first described in Italy in 1999 (Lauretti *et al.* 1999). Greece is now considered to be the epicenter of the spread of VIM-producing *Enterobacteriaceae* to other European countries where they have been punctually detected including Spain, Italy and France (Canton *et al.* 2012; Mathlouthi *et al.* 2016). In the light of these data, further research is clearly needed to understand the potential role of some wild species in the spread of carbapenem-resistant bacteria.

To contribute to the development of this research, we chose to focus on *Escherichia coli* for three reasons: i) It is an ubiquitous bacteria that can be carried by a wide range of species including humans, other mammals and birds. ii) It is the most frequent cause of urinary tract and bloodstream infections worldwide. iii) It is a major cause of carbapenem-resistant infection, accounting for 25% of the episodes reported in France during the last decade (INVS 2014). Hence, our aim was to investigate the presence of carbapenem-resistant *E. coli* in a species that lives in close contact with humans following its recent colonization of urban habitats and that has subsequently experienced a strong demographic increase: the Yellow-legged gull (YLG, *Larus michahellis*; Duhem *et al.* 2008). The focal YLG population was previously reported to carry high loads of extended-spectrum  $\beta$ -lactamase (ESBL) producing

*E. coli* (Bonnedahl *et al.* 2009). We also investigated the *E. coli* strains found in Slender-billed gulls (SBG, *Chroicocephalus genei*) living in the same area. We chose to study both species since they share the same environment but their feeding habits differ. YLG are opportunistic, feeding on fresh fish, but, like the black kite, they also feed on refuse and carcasses, whereas SBG mainly feed on marine fishes (Flitti *et al.* 2009). We investigated *E. coli* carried by chicks since, within the colonies we studied, they had no contact with humans and they could only be contaminated by bacteria brought by adults or already present in the colony. Thus, finding AMRB in those chicks would mean that these bacteria have either been transmitted from adults to chicks or that the environment (surrounding water or soil) is contaminated by them.

In France, as in most Western European countries, carbapenemase-producing bacteria infections have so far been limited to hospital settings and represent only a few cases per year. Yet the number of those cases has significantly increased in recent years in many European countries, including France (ECDC 2013; INVS 2014). In all, 913 infectious episodes associated with carbapenem-resistant enterobacteria were reported nationwide from January 2004 to March 2014, 25% of which were due to *E. coli* strains (INVS 2014). Through the investigation of *E. coli* strains carried by gull chicks in two colonies in south-eastern France, our aim was to elucidate four questions: i) Are the focus populations harboring some carbapenem-resistant bacteria while those bacteria are still rare in the neighboring human population? ii) Is the proportion of individuals carrying these AMRB similar in the two target species, despite their contrasting feeding habits? iii) Are the *E. coli* genotypes carried by gulls closely related to those recently isolated in humans in the study region? iv) Which resistance mechanisms are involved in the potentially detected resistances?

## **Materials and Methods**

### **Ethics statement**

The study has been approved by the Scientific and Ethical Council of the Tour du Valat Foundation, which is in charge of the ethical issues in our research centre, on October 5, 2011. The results have subsequently been reviewed by this council. Birds were handled and sampled under the supervision of two registered bird ringers of the « Museum National d'Histoire Naturelle » of Paris (Thomas Blanchon & Yves Kayser) who made every effort to avoid any animal suffering. Permits for fieldwork were issued by the municipality of Port-Saint-Louis and the Communauté d'Agglomération Toulon Provence Méditerranée.

### **Sampling, bacterial strains and antibiotic susceptibility testing.**

Cloacal swabs were collected from gull chicks, aged from 1 to 4 weeks, in two colonies. The Yellow-legged gull colony was situated on an islet near the village of Port-Saint-Louis (4°51'26.50"E. 43°22'39.93"N), where 93 swabs were sampled on chicks on May 23, 2012. The colony of Slender-billed gulls was located in the Giens peninsula (6°08'20.10"E. 43°03'01.14 N.). 65 samples were collected there on July 23, 2012.

Immediately after sampling, the swabs collected from gulls were placed in Oxoid Tryptone Soya Broth (Thermo Scientific™). They were then transported to the laboratory and incubated at 37C° overnight. Following incubation, a loopful was streaked on plates of Hektoen (BioMérieux, Marcy-l'Etoile, France) and of a selective chromogenic medium for the detection of carbapenem-resistant bacteria (Oxoid Brilliance CRE; Thermo Scientific™). The bacteria were incubated for 24 to 48h on these media depending on observation of growth of bacterial colonies. *E. coli* identity was confirmed by matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry (Bruker Daltonik, Bremen, Germany). Antimicrobial susceptibility testing was performed by disk diffusion assay on

Mueller-Hinton agar (Bio-Rad, Marne-la-coquette, France) and interpreted according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST 2012, version 2.0) clinical breakpoints ([http://www.eucast.org/clinical\\_breakpoints/](http://www.eucast.org/clinical_breakpoints/)). Antibiotic agents tested were: amoxicillin, amoxicillin-clavulanic acid, ticarcillin, ticarcillin-clavulanic acid, piperacillin, piperacillin-tazobactam, imipenem, cephalotin, cefoxitin, cefotaxime, ceftazidime, cefpodoxime, cefpirome, cefepime, moxalactam, aztreonam. Metallo- $\beta$ -lactamase production was investigated in *E. coli* isolated from the selective medium by the Carbapenemase/Metallo- $\beta$ -Lactamase Confirmation Identification Pack (Rosco Diagnostic Neo-Sensitabs<sup>TM</sup>, Eurobio, Courtaboeuf, France).

Twenty-five clinical isolates of carbapenem-susceptible non-pathogenic *E. coli*, recovered from human patient's stools in the same geographical area (Montpellier hospital) and during the study period, were used for phylogenetic comparison.

In addition, four *E. coli* strains belonging to reference phylogroups (A1, B1, B2<sub>3</sub>, D1) were kindly provided by *Prof. Richard Bonnet*.

### **Detection and identification of carbapenem-resistance genes**

The *E. coli* strains that were isolated from the media containing carbapenem were further analyzed to determine the mechanisms involved in resistance to carbapenems using a multiplex PCR (Dallenne *et al.* 2010; Hornsey, Phee & Wareham 2011). Briefly, the presence of the most prevalent carbapenemase genes (including *bla*<sub>KPC</sub>, *bla*<sub>VIM</sub>, *bla*<sub>OXA-48</sub> group and *bla*<sub>IMP</sub>) was assessed by multiplex PCR, as previously described (Dallenne *et al.* 2010) and *bla*<sub>NDM</sub> gene presence was investigated by PCR assay (Hornsey, Phee & Wareham 2011). Metallo- $\beta$ -lactamase production was assessed by the Carbapenemase/Metallo- $\beta$ -Lactamase Confirmation Identification Pack (Rosco Diagnostic Neo-Sensitabs<sup>TM</sup>, Eurobio, Courtaboeuf, France).

All amplified PCR products were purified using the ExoSap purification kit (ExoSap-it, GE Healthcare, Piscataway, NJ, USA) and bidirectional sequencing was performed using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and an Applied Biosystems 3730 XL capillary sequencer. Each sequence was then compared with already known carbapenemase genes available in the NCBI database using the BLAST program. The detection of *bla*<sub>VIM-1</sub> gene was confirmed by simplex PCR assay and sequencing using the following primers: VIM\_F (5'- AGTGGTGAGTATCCGACAG-3') and VIM\_R (5'-TGCAACTTCATGTTATGCCG-3').

### **Molecular analyses**

To determine the relatedness between the *E. coli* isolates sampled from YLG, SLG and humans, we performed PCR phylotyping, discriminant single nucleotide polymorphism (SNP), multi locus sequence typing (MLST) and polymorphic variable-number of tandem repeats loci (VNTR).

All isolates were assigned to the eight *E. coli* phylogenetic groups and subgroups (A0, A1, B1, A1xB1, B2<sub>2</sub>, B2<sub>3</sub>, D1, and D2) using PCR on the basis of the presence or absence of three DNA fragments (*chuA*, *yjaA* and *tspE4.C2*)(Clermont, Bonacorsi & Bingen 2000) and on the comparison with four reference strains (A1, B1, B2<sub>3</sub>, D1).

Allele-specific real-time PCR was used to screen seven SNPs (*fadD234*, *clpX267*, *uidA138*, *clpX177*, *clpX234*, *lysP198*, *icdA177*) on seven housekeeping genes (Sheludchenko, Huygens & Hargreaves 2010) using an Applied Biosystems 7300.

MLST procedures were performed using Wirth *et al.* MLST scheme (Wirth *et al.* 2006). Seven housekeeping genes (*Adk*, *FumC*, *GyrB*, *Icd*, *Mdh*, *PurA*, *RecA*) were amplified by PCR and sequenced by Eurofins (Germany). The sequences were assembled and trimmed (3,423 nucleotides in total) using the MLST Databases at the University of Warwick (freely



available at <http://mlst.warwick.ac.uk/mlst/>). Alleles and sequence type (ST) numbers were assigned de novo in Bionumerics (Applied-Maths, Sint Maartens-Latem, Belgium).

The genotyping assay of VNTR was performed on six polymorphic loci (CVN15, CVN7, CVN4, CVN1, CVN2, CVN14) (Lindstedt *et al.* 2007) using a genetic analyser Applied Biosystems 7330xl.

### **Phylogenetic and statistical analyses**

Exact Fisher tests were used to test for differences in *E. coli* phylogroup distributions among host populations.

The multiple alignments of all complete MLST sequences were conducted using ClustalW in BioEdit v.7.0.9.0. software. Maximum-likelihood (ML) tree construction was based on the MLST sequences and the best-fitting ML model under the Akaike Information Criterion was GTR (General Time Reversible) +  $\Gamma$  (gamma distribution) for nucleotides as identified by ModelTest (Posada & Crandall 1998). The most likely DNA tree and corresponding bootstrap support values were obtained by PhyML using Mega 5.0 software (Tamura *et al.* 2011) with nearest neighbor interchange branch swapping and 100 bootstrap replicates. All positions containing gaps and missing data were eliminated.

Genetic polymorphism of VNTR data was measured in terms of allelic richness per locus and sample ( $R_s$ ) (Mousadik & Petit 1996), gene diversity was measured in terms of expected heterozygosity per locus and sample ( $H_s$ ) using the unbiased estimator adapted to haploid data (Nei & Chesser 1983) and the genotypic linkage disequilibrium was tested by the log-likelihood ratio G-statistic after Bonferroni's correction using FSTAT v 2.9.4 software (Goudet 2003).

MLST, SNP and VNTR data were analyzed with Bionumerics software v7.0 (Applied-Maths, Sint Maartens-Latem, Belgium). Based on allelic profiles, the evolutionary

relationship between isolates was assessed by a minimal spanning tree (MST) implemented in Bionumerics. The MST is a graphical tool that links the nodes by unique minimal paths in a given dataset: the total summed distance of all branches is minimized. The Prim's algorithm calculated a standard MST with single and double locus variance priority rules.

## **Results**

### **Detection of carbapenem-resistant *E. coli***

Twenty-two *E. coli* strains isolated from Yellow-legged gulls (Table 1) were resistant to most of the  $\beta$ -lactam antibiotics, except aztreonam, and were metallo- $\beta$ -lactamase producers. Those carbapenemase producing *E. coli* originated from 18 of the 93 YLG chicks on which we sampled cloacal swabs. Two birds carried two carbapenemase producing isolates and one gull carried three. All 22 carbapenemase-producing *E. coli* isolates were positive for *bla*<sub>VIM-1</sub> gene. Conversely, no carbapenem-resistant bacteria were isolated from the samples collected on 65 Slender-billed gulls. Table 1 indicates the number of isolates sampled from each host group that were used for each type of analysis (Phylogroup, SNP, MLST and VNTR).

### **Phylogroups**

Table 2 shows the prevalence of phylogenetic groups for the entire set of isolates from the different host samples. Seven phylogroups were determined in susceptible *E. coli* samples and only three phylogroups (A1, A1xB1, B2<sub>2</sub>) in VIM-1 resistant *E. coli* isolates. The distribution of phylogroups in resistant isolates was significantly different from that observed in susceptible samples ( $P < 0.001$  Fisher's exact test). Among the 22 carbapenem-resistant isolates, 18 belonged to the phylogroup A1 (81.8%). This proportion was significantly higher than that found in the susceptible isolates as a whole ( $P < 0.001$  Fisher's exact test).

### **Multilocus sequence typing (MLST)**

The MLST analysis involved 92 nucleotide sequences (Table 1) with 7 partial housekeeping gene sequences (i.e. 4,963 nucleotides in total). Among the 360 variable sites, 255 were informative sites over the 4,963 bases. The number of unique haplotypes was 68 out of 88 in total, excluding the four reference strains. The PhyML tree (Figure 1) with bootstraps based on concatenated 7 fragments of sequences which totalized 4,963 nucleotides showed that the resistant isolates (orange symbols) were grouped in five clusters grouping 4 (phylogroup A), 6 (phylogroup A), 2 (phylogroup AxB), 8 (phylogroup A) and 2 (phylogroup B) isolates. We noticed that these five clusters were scattered in the phylogenetic tree (Figure 1) with blue node bootstrap of 62, 95, 86, 69 and 82 respectively. The genetic diversity is more extended in susceptible strains due to the number of clusters grouping together.

### **Single nucleotide polymorphism (SNP)**

In the SNP analysis, 26 distinct haplotypes were detected among the 88 *E. coli* isolates studied (reference strains were not included, see Table 1). The number of SNP haplotype combinations was as follows: 3 SNP haplotypes out of 22 isolates in resistant *E. coli* from Yellow-legged gulls, 11 out of 26 isolates in susceptible *E. coli* from Yellow-legged gulls, 11 out of 15 isolates in susceptible *E. coli* from Slender-billed gulls and 14 out of 25 isolates in susceptible *E. coli* from humans. The diversity of SNP found in the carbapenem-resistant isolates (3 out of 27 haplotype combinations) was lower than in other strains, but this difference was not significant ( $P = 0.07$  Fisher's exact test). Elsewhere, the CCCGCCT (*fadD234*, *clpX267*, *uidA138*, *clpX177*, *clpX234*, *lysP198*, *icdA177*) SNP combination was significantly more frequent in VIM-1 strains (18 out of 22) than the others ( $P < 0.001$ , Fisher's exact test) and absent in the *E. coli* isolates sampled in Slender-billed gulls.

### **Variable number tandem repeat (VNTR)**

The VNTR gave complete results for 79 isolates only (Table 1). All the loci were polymorphic, showing from 3 (CVN7 and CVN15) to 14 alleles (CVN14). There was lower

genetic variability in the sample of resistant isolates as shown by the allelic richness ( $R_s = 2.3 \pm 1.2$ ) vs. other samples (susceptible isolates from Yellow-legged gulls  $R_s = 4.5 \pm 3.6$ ; Susceptible isolates from Slender-billed gulls  $R_s = 3.0 \pm 2.2$ , Susceptible isolates from humans  $R_s = 4.3 \pm 2.9$ ). However the difference of  $R_s$  between each pair of samples was not significant (Wilcoxon test, p-values range between 0.574 and 0.936).

Elsewhere, the gene diversities per locus were similar in all samples except for locus CVN2 which showed a higher value for resistant YLG strains ( $H_s = 0.69$ ) compared to the three other sample groups (Susceptible strains from YLG:  $H_s = 0.22$ , SBG:  $H_s = 0.26$ , humans:  $H_s = 0.17$ ). However the difference of  $H_s$  between each pair of samples was not significant (Wilcoxon test, p-values range between 0.261 and 0.936).

A significant linkage disequilibrium ( $P < 0.001$ ) was underscored at 3 loci pairs: (CVN1 x CVN2, CVN1 x CVN14, CVN2 x CVN14) in the resistant YLG sample group, whereas no significant linkage disequilibrium was detected in any loci combination in other samples, even in the susceptible *E. coli* isolated from YLG.

### **Phylogenetic analysis based on MLST, SNP and VNTR**

The minimum spanning tree presented in figure 2 is based on genetic sequence similarity according to MLST (7 fragments of gene sequences shortened and aligned to the reference sequences in the MLST Databases at the University of Warwick (freely available at <http://mlst.warwick.ac.uk/mlst/>) (i.e. 3,423 nucleotides in total)), SNP and VNTR analyses. The phylogenetic structure highlighted is similar to that underlined in figure 1. Isolates of the same phylogroup tend to cluster together. Carbapenem-resistant isolates do not form a distinct cluster. Conversely, they are grouped in 3 clusters that also include carbapenem susceptible strains isolated from the different host species (YLG, SLG and humans). In addition, only one resistant isolate presents a unique sequence, the others are grouped in five clonal complexes including 2 to 6 strains.

## Discussion

We highlighted the presence of VIM-1 carbapenem-resistant *E. coli* strains in Yellow-legged gulls in southern France. Our results confirm that gulls represent a bird group that frequently carries antimicrobial resistant bacteria, as was previously shown in several studies (e.g. Čížek *et al.* 2007; Poirel *et al.* 2012; Hasan *et al.* 2014) led in particular in southern Europe (Stedt *et al.* 2014).

Alarmingly, while the previous report of carbapenem-resistant bacteria in a wild bird was a single case in a raptor (Fischer *et al.* 2013), we detected VIM-1 bearing *E. coli* carriage in 18 different chicks, which raises the question of the extent of wildlife contamination in the study region. Interestingly, we identified 5 clonal complexes and one unique genotype within the VIM-1 containing bacteria we detected (Figure 2). This suggests that several distinct introductions of carbapenem-resistant *E. coli* occurred on the islet. Further studies are needed to investigate the extent of the circulation of VIM-1 containing bacteria within gull populations in Southern France.

Our findings are all the more worrisome if we consider that gulls live in close contact with human populations since they feed on waste matter, notably in cities, and thus represent a bridge species for pathogens between wildlife and humans. Moreover Yellow-legged gulls are not migratory birds; yet, young individuals can fly large distances from their native colony to their wintering sites that include the whole of the Rhone Valley and the French Atlantic coast (Sadoul & Pin 2009). Thus, this species could favor the spread of carbapenem-resistant bacteria, at least within France.

By contrast, we did not detect any carbapenem-resistant *E. coli* in Slender-billed gulls. This species does not breed in the same colonies as YLG, but SBG and YLG can share resting sites and are thus frequently in contact. Furthermore the two colonies we studied are located

only 110km apart. This distance can easily be covered by a gull within a day, meaning that these sites are not isolated from one another in terms of potential bacteria exchanges between the species we studied. As stated above, the two species differ by their diet, which suggests that the carriage of carbapenem-resistant bacteria by YLG may be associated with their feeding habits and/or the habitat they visit to feed. In France, from January 2004 to March 2014, 913 infectious episodes associated with carbapenem-resistant enterobacteria were reported, including 233 episodes due to *E. coli*, all of which were detected in hospitals (INVS 2014). Many of those episodes (481 out of 913) were linked to a previous stay of the patient in a foreign country (INVS 2014, Cuzon *et al.* 2010; Crémet *et al.* 2012), which underscores the rarity of carbapenem-resistant infection originating in France. Furthermore, the resistance gene we detected (*bla*<sub>VIM-1</sub>) is uncommon in France, where it caused only 5% of the reported infections due to carbapenem-resistant enterobacteria, the OXA-48 and OXA-48-like genes being the most frequent in the country (74%)(INVS 2014). *bla*<sub>VIM-1</sub> is an integron-borne metallo-β-lactamase gene which was first reported in *Pseudomonas aeruginosa* in Italy in 1996 (Lauretti *et al.* 1999). It encodes for a class B carbapenemase which also hydrolyses all β-lactams except monobactams, and evades all β-lactamase inhibitors. VIM-1 bearing bacteria have been reported from clinical samples in Greece although they are beginning to spread in southwestern Europe, notably in Spain and Italy, while France seems, for now, to be less affected (Canton *et al.* 2012; Mathlouthi *et al.* 2016).

The phylogenetic analyses performed using phylotyping and three types of genetic markers (SNP, MLST and VNTR) clearly showed that Yellow-legged gulls, Slender-billed gulls and humans share the same pool of *E. coli* strains. Our results confirm that *E. coli* exchanges are frequent between gulls and humans, as was previously demonstrated in the region (Bonnedahl *et al.* 2009). The occurrence of such exchanges highlights the potential risk of resistance spreading from gulls to humans (Stedt *et al.* 2014).

VIM-1 containing *E. coli* are closely related to carbapenem susceptible strains isolated from the two gull species and humans. Nevertheless, their group can be distinguished from the susceptible group through two genetic traits. First, PCR phylotyping showed that the 92 strains we studied included bacteria belonging to 8 phylogroups. No phylogroup was significantly more present than others in susceptible strains. By contrast, phylogroup A, to which some susceptible strains also belong, represented 81.8% of the VIM-1 bearing *E. coli*. The association between some phylogroups and antimicrobial resistance patterns is for now poorly understood. Nevertheless, several studies have already highlighted that phylogroup A *E. coli* are over-represented within resistant strains isolated in France (Smati *et al.* 2013), including chromosomal AmpC  $\beta$ -lactamase overproducers carried by humans (Corvec *et al.* 2007) as well as ESBL *E. coli* detected in cattle (Valat *et al.* 2012) and ampicillin-resistant isolates from pigs (Bibbal *et al.* 2009). Further studies are still needed to determine if *E. coli* belonging to phylogroup A are more likely to acquire antimicrobial resistances and why. Besides carbapenem resistant strains tended to be less diverse than susceptible ones according to VNTR and SNP analysis. This lower diversity is consistent with the higher selection pressure, potentially linked with antimicrobial molecule presence, resulting in strong bottlenecks that is expected to have contributed to the emergence of resistant strains. This suggests that the resistance was recently acquired by the bacteria we isolated or that a selection pressure favored the expansion of a pre-existent clone.

We report here the second isolation worldwide of carbapenem-resistant enterobacteria from wild birds and the first detection in gulls. In depth molecular analysis of blaVIM genetic surroundings and features will be necessary in the future to fully understand the downstream impacts of our findings. Yet, here our aim was not to fully characterize the genetic background of carbapenem resistant *E. coli* carried by wildlife but rather to warn over the potential role of wild birds as carbapenem-resistant bacteria carriers and spreaders. Our results are alarming enough to justify an urgent call for further studies. They also make it a matter of urgency to determine the



contamination source of the bacteria we identified. The next step could be to extend our work to several other YLG and SBG colonies, including some in which the two species are breeding in neighboring islets, as well as to repeat sampling other time to improve our knowledge of VIM-1 carrying bacteria circulation in French gull populations. Such extended sampling could also help in disentangling the respective roles of feeding habits and environmental pollution in carbapenem-resistant bacteria contamination. To complete this analysis, it would also be necessary to search for resistant bacteria within the environment, especially in water, since it has previously been shown that rivers and coastal areas can contain carbapenem-resistant bacteria even when those pathogens are rare in the surrounding human populations (Aubron *et al.* 2005; Montezzi *et al.* 2015). More generally, our work highlights the urgent need to develop more bridges between studies focusing on wildlife and humans in order to improve our knowledge of resistant bacteria dynamics. Such bridges will be a key factor in enabling us to efficiently face the challenge of antimicrobial resistance in the future.

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## **Data Accessibility**

Molecular data will be archived in the following database:  
<http://mlst.warwick.ac.uk/mlst/>

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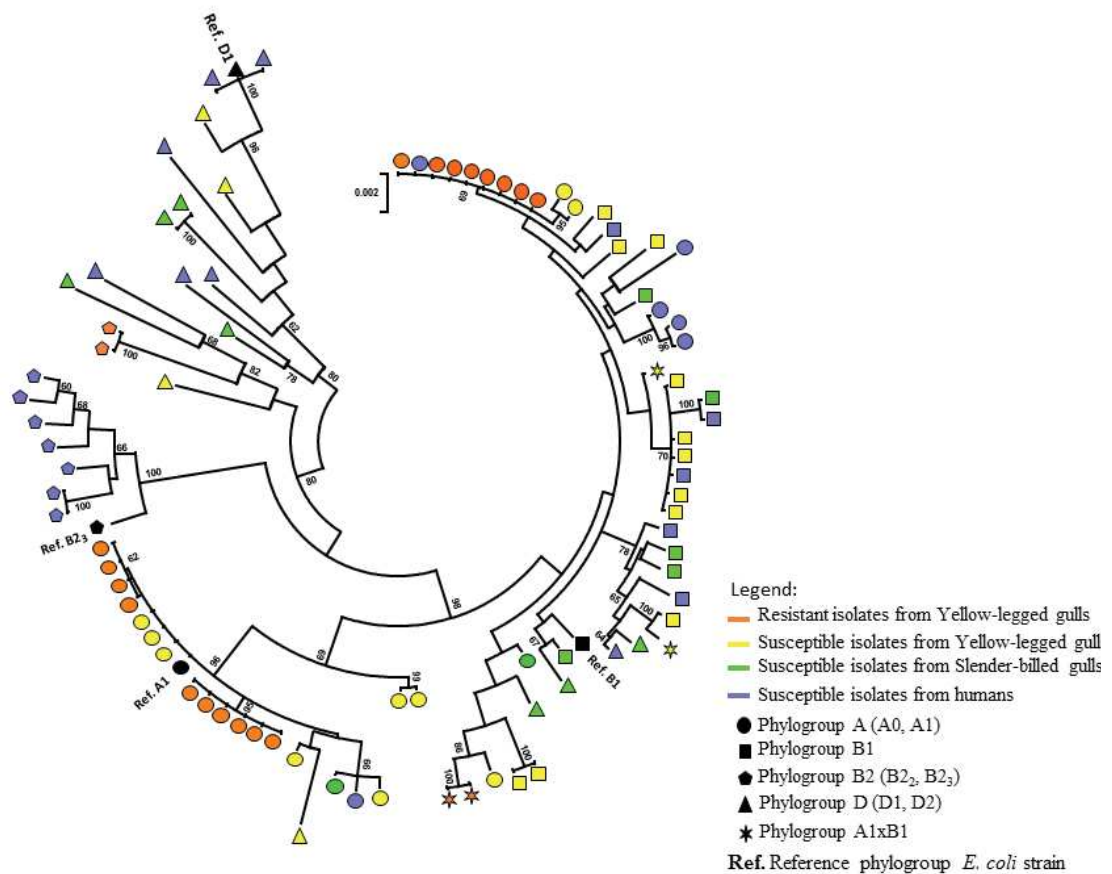
**Table 1. Summary of the strains studied and the analyses in which they were included.**

Host species	Resistant to carbapenem	Number of strains	Strains for which complete data are available			
			Phylogroup	MLST	SNP	VNTR
Yellow-legged gulls	Yes	22	22	22	22	20
Slender-billed gulls	No	26	26	26	26	25
Humans	No	15	15	15	15	14
Phylogroup reference strains (humans)	No	25	25	25	25	18
	No	4	4	4	4	2
<b>Total</b>		<b>92</b>	<b>92</b>	<b>92</b>	<b>92</b>	<b>79</b>

**Table 2. Prevalence of eight *E. coli* phylogroups in the strains isolated from Yellow-legged gulls, Slender-billed gulls and human patients.**

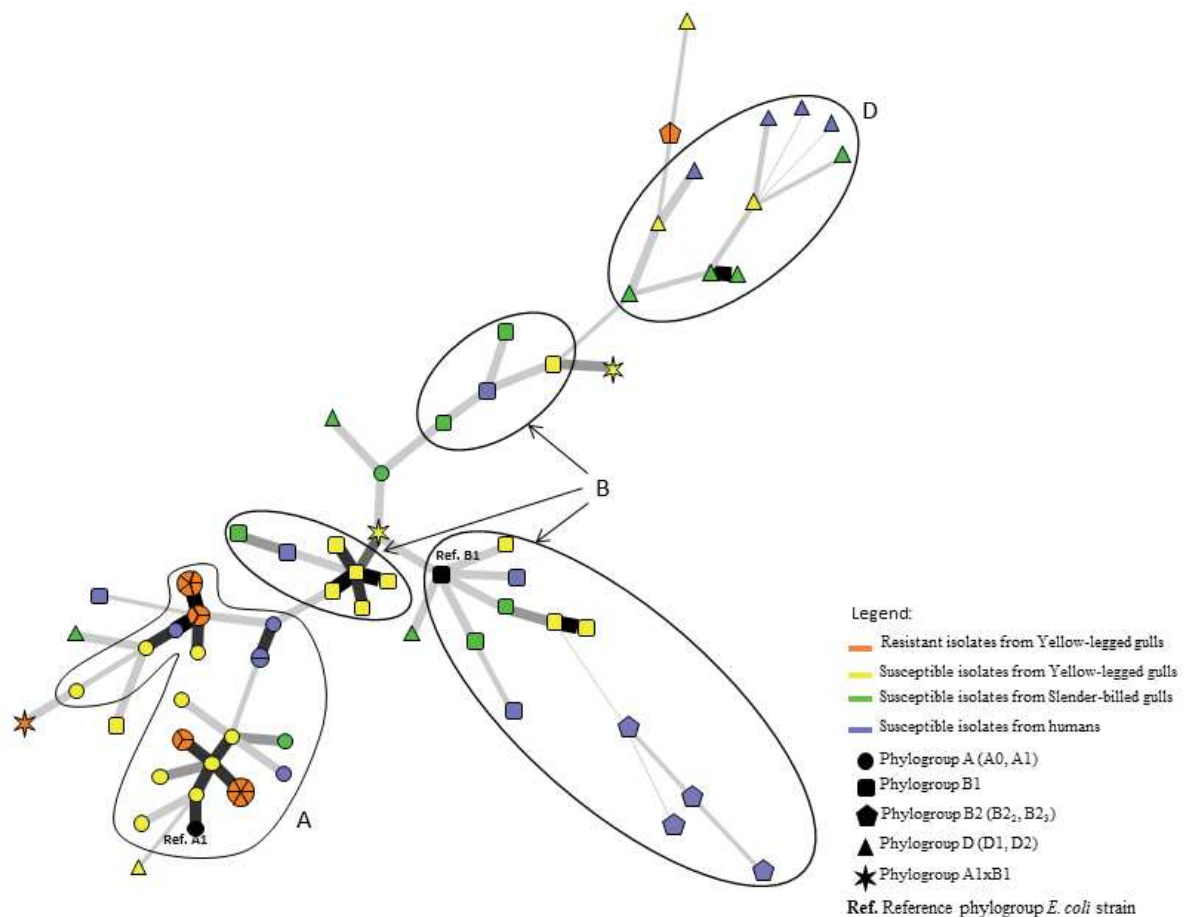
Sample group	n	Number of strains of each phylogenetic groups (% in the sample group)							
		A0	A1	A1xB1	B1	B2 <sub>2</sub>	B2 <sub>3</sub>	D1	D2
Resistant <i>E. coli</i> from Yellow-legged gulls	22	0	18 (81.8%)	2 (9.1%)	0	2 (9.1%)	0	0	0
Susceptible <i>E. coli</i> from Yellow-legged gulls	26	6 (23.1 %)	4 (15.4%)	2 (7.7%)	10 (38.4%)	0	0	4 (15.4%)	0
Susceptible <i>E. coli</i> from Slender-billed gulls	15	1 (6.7%)	1 (6.7%)	0	6 (40.0%)	0	0	4 (26.6%)	3 (20.0%)
Susceptible <i>E. coli</i> from humans	25	4 (16.0%)	2 (8.0%)	0	6 (24.0%)	0	7 (28.0%)	4 (16.0%)	2 (8.0%)

**Figure 1. Phylogenetic relationships among the 92 *Escherichia coli* isolates studied based on concatenated MLST sequences.** The circle tree was constructed using maximum likelihood methods. Bootstrap values greater than or equal to 60% are indicated at the nodes, those relating to the five clusters containing carbapenem-resistant isolates are shown in blue. The *E. coli* strains were isolated from Yellow-legged gulls (YLG), Slender-billed gulls (SBG) and humans.





**Figure 2. Minimum spanning tree of 79 of the studied *Escherichia coli* strains based on MLST, SNPs and VNTR.** The 13 strains for which part of the VNTR data was missing were excluded. The phylogroups are shown as ovals. Clonal complexes are indicated by symbols proportional in size to the number of strains within them. Black lines connecting strains indicate that they differ at least by one VNTR (bold thick lines) to two VNTR, seven MLST genes and two SNPs (the thinnest lines).



## **Chapitre V**

### **Présence de *Vibrio cholerae* résistant aux carbapénèmes dans le microbiote cloacal des goélands**

## Préambule

Il y a actuellement une émergence de souches de *Vibrio cholerae* antibiorésistantes qui sont de plus en plus décrites dans le monde. Cependant, la résistance de cette espèce aux céphalosporines de troisième génération, la plupart du temps par l'intermédiaire de gènes de résistance codant des BLSE ou des céphalosporinase, reste rarement décrite. A notre connaissance, la résistance de *Vibrio* spp. conférée par des carbapénèmases n'a été que très rarement rapportée, essentiellement en Asie du sud-est, où plusieurs études ont décrit des souches cliniques et environnementales productrices de métalloenzymes de type NDM-1. En outre, en 2015, une étude a décrit un nouveau type de carbapénèmases, VCC-1, isolé de crevettes au Canada. Une autre étude a rapporté la présence de souches de *V. cholerae* résistantes aux carbapénèmes dans des échantillons environnementaux collectés sur les côtes allemandes de la mer baltique et de la mer du nord. Cependant, aucun gène de résistance n'a été retrouvé chez ces souches.

Dans l'article suivant nous décrivons une souche de *V. cholerae* non-O1/non-O139 productrice de deux carbapénèmases de type VIM-1 et VIM-4. Elle a été isolée d'un échantillon cloacal d'un goéland leucophée. Les gènes *bla*<sub>VIM</sub> faisaient partie d'un intégron de classe 1, situé sur un plasmide de type IncA/C. Cette étude souligne une fois de plus la présence de gènes codant des carbapénèmases dans le microbiote de la faune sauvage.

Article V

**A non-O1/non-O139 *Vibrio cholerae* avian isolate co-carrying the *bla*<sub>VIM-1</sub> and *bla*<sub>VIM-4</sub> genes, France**

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# Non-O1/Non-O139 *Vibrio cholerae* Avian Isolate from France Cocarrying the *bla*<sub>VIM-1</sub> and *bla*<sub>VIM-4</sub> Genes

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**We describe here a non-O1/non-O139 *Vibrio cholerae* isolate producing both VIM-1 and VIM-4 carbapenemases. It was isolated from a yellow-legged gull in southern France. The *bla*<sub>VIM</sub> genes were part of a class 1 integron structure located in an IncA/C plasmid. This study emphasizes the presence of carbapenemase genes in wildlife microbiota.**

Multidrug-resistant *Vibrio cholerae* strains have been increasingly reported worldwide (1). However, data on resistance to third-generation cephalosporins, mostly via genes encoding extended-spectrum  $\beta$ -lactamase (ESBL) (2, 3) or cephalosporinase determinants (4) are limited. Carbapenemase-mediated resistance in *Vibrio* spp. has been reported only in India, where clinical and environmental *V. cholerae* isolates carrying the NDM-1 metalloenzyme were described in several studies (4–6). We describe here an avian strain of *V. cholerae* that was isolated in southern France and that coharbors the *bla*<sub>VIM-1</sub> and *bla*<sub>VIM-4</sub> carbapenemase genes.

In April 2013, 93 cloacal swab samples from juvenile unfledged yellow-legged gulls (*Larus michahellis*) breeding on the island of Carteau, Port-Saint-Louis, France, were screened for bacteria resistant to broad-spectrum  $\beta$ -lactam antibiotics. Briefly, swab samples were inoculated in Trypticase soy broth (Thermo Fisher Scientific) and grown at 37°C for 24 h. Samples were then subcultured in ESBL agar plates (bioMérieux, Marcy l'Etoile, France) and were examined after 24 and 48 h of incubation. *Enterobacteriaceae* resistant to multiple drugs via different resistance mechanisms (e.g., third-generation cephalosporin-resistant *Escherichia coli* and *Proteus mirabilis* harboring plasmid-mediated cephalosporinase genes or ESBL genes) were recovered (7; our unpublished data). In addition, a *V. cholerae* strain showing resistance to third-generation cephalosporins was detected. No other multidrug-resistant *V. cholerae* strain was recovered. Species identification was performed by matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry (Bruker Daltonics, Bremen, Germany). Moreover, PCR analysis of the *rfb* gene cluster (8), the cholera toxin *ctxA* gene (9), and the colonization factor *tcpA* gene (9) revealed a nontoxicogenic, non-O1/non-O139 isolate.

Susceptibility testing was performed using the disk diffusion method on Mueller-Hinton agar and was interpreted according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) clinical breakpoints (version 5.0) ([http://www.eucast.org/clinical\\_breakpoints/](http://www.eucast.org/clinical_breakpoints/)) (10). The strain was intermediate or resistant to most  $\beta$ -lactam antibiotics, except aztreonam. The MICs for amoxicillin, cefotaxime, ceftazidime, imipenem, ertap-

enem, doripenem, and meropenem were determined in the parental strain and the transconjugant with the Etest method (bioMérieux, Marcy l'Etoile, France) (Table 1). Metallo- $\beta$ -lactamase production was observed by using the carbapenemase/metallo- $\beta$ -lactamase confirmative identification pack (Rosco Diagnostica NeoSensitabs, Eurobio, Courtaboeuf, France). Specifically, reduced susceptibility to meropenem was corrected by the addition of dipicolinic acid, while the addition of cloxacillin and boronic acid had no effect. ESBL production was excluded with the double-disk synergy test (11), while culture in Mueller-Hinton agar impregnated with 2 ml of  $5 \times 10^{-3}$  M EDTA restored the activity of all  $\beta$ -lactam antibiotics, as previously described (12). Susceptibility testing using the disk diffusion method showed that fluoroquinolones, chloramphenicol, cotrimoxazole, and tetracycline remained active. Only tobramycin showed intermediate susceptibility among the aminoglycosides, while amikacin, isepamicin, netilmicin, and gentamicin remained active.

Detection of the most prevalent carbapenemase genes (including *bla*<sub>KPC</sub>, *bla*<sub>VIM</sub>, *bla*<sub>OXA-48</sub>, and *bla*<sub>IMP-1</sub>), assessed by multiplex PCR as previously described (13), and of the *bla*<sub>NDM</sub> gene (14), assessed by PCR assay, gave a positive result for the *bla*<sub>VIM</sub> gene. This was confirmed by simplex PCR assay using the primers VIM\_F (5'-AGTGGTGAGTATCCGACAG-3') and VIM\_R (5'-TGCAACTTCATGTTATGCCG-3'). Bidirectional sequencing performed using the BigDye Terminator v3.1 cycle sequencing kit

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TABLE 1 Susceptibility of parental and recipient strains

Antibiotic	MIC (mg/liter) for:		
	Parental strain ( <i>V. cholerae</i> )	Recipient strain (J53 <i>E. coli</i> )	J53 <i>E. coli</i>
Amoxicillin	>256	>256	2
Cefotaxime	4	8	0.064
Ceftazidime	6	24	0.064
Aztreonam	0.38	0.032	0.064
Imipenem	3	4	0.25
Ertapenem	0.19	0.064	0.064
Meropenem	0.5	0.75	0.064
Doripenem	0.75	0.75	0.064
Amikacin	2	0.5	0.5
Gentamicin	1.5	0.25	0.25
Tobramycin	2	0.75	0.5

(Applied Biosystems, Foster City, CA, USA) and an Applied Biosystems 3730 XL capillary sequencer identified both *bla*<sub>VIM-1</sub> and *bla*<sub>VIM-4</sub> genes.

To characterize the genetic environment of the *bla*<sub>VIM</sub> genes, the amplicons of parental and recipient strains were analyzed by PCR mapping and sequencing using specific primers (Table 2) (GenBank accession number KR262557). Both *bla*<sub>VIM-1</sub> and *bla*<sub>VIM-4</sub> genes were part of the same class 1 integron (Fig. 1), located in the IncA/C plasmid. These two carbapenemase gene cassettes flanked an *aac*(6')-IIc gene cassette that confers resistance to aminoglycosides. A PcS (strong) promoter variant, divergent to the integrase gene, was identified in the class 1 integron, with a functional P2 promoter located downstream of the PcS in the *attI1* site. It resulted from the insertion of three G residues. The PcS-P2 association has rarely been described in class 1 integrons and might confer high-level gene cassette expression (15). A similar integron containing the *bla*<sub>VIM-1</sub> and *aac*(6')-IIc genes was previously described in an *Enterobacter cloacae* clinical isolate from Greece (GenBank accession number AY648125) (16), but this is the first description of a class 1 integron with two *bla*<sub>VIM</sub> variants. It may be hypothesized that the presence of two *bla*<sub>VIM</sub> genes in a single integron might enable better plasticity in the case of rearrangements of the cassette network under selective pressure caused, for instance, by antibiotics.

Mating experiments were performed on agar plates, as previously described (6), at 25°C and 37°C using the rifampin-resistant *E. coli* J53 strain as the recipient, with a donor-to-recipient ratio of 4:1. Transconjugants were selected on Drigalski agar (Bio-Rad) containing 250 mg/liter rifampin and 4 mg/liter cefotaxime. A transconjugant that coharbored the *bla*<sub>VIM-1</sub> and *bla*<sub>VIM-4</sub> genes was obtained from the *V. cholerae* isolate at 25°C, with a transfer frequency of  $3 \times 10^{-6}$  transconjugants per recipient. PCR mapping showed that the genetic structure that harbored both *bla*<sub>VIM</sub> genes was the same in the parental and recipient strains. No transfer was obtained at 37°C, despite repeated attempts. The plasmid relaxase gene typing (PRaseT) method, which allows detection of the major replicon groups, and a PCR-based replicon typing method revealed the presence of an IncA/C plasmid in the parental and recipient strains (17, 18). Conversely, the SXT integrative and conjugative element, which is a major resistance determinant in *V. cholerae* (1), was detected in only the parental strain by PRaseT. No other typeable mobile genetic element was found. Plasmid content analysis using the method of Kado and Liu (19)

TABLE 2 Primers used for PCR mapping of the cassette network

Primer name	Primer sequence (5' to 3')
VIM-L1	TCATTGTCCGTGATGGTGATGA
VIM-L2	CCGGGCGGTCTAGACTTGCT
VIM-R1	CGATATGCGACCAACACCATC
VIM-R2	GCCATTACGCCAGATCG
attI1	GGCATCCAAGCAGCAAGCGCGTT
sul1	GTCCGACATCCACGACGTCTGATC

revealed only one plasmid of ~150 kb in both the parental and recipient strains (see Fig. S1 in the supplemental material). These results suggest that the *bla*<sub>VIM-1</sub> and *bla*<sub>VIM-4</sub> genes were carried by the broad-host-range IncA/C transmissible plasmid, a widespread *bla*<sub>VIM</sub>-carrying genetic element (20) and a common resistance determinant in *V. cholerae* (21).

Although nonepidemic, non-O1/non-O139 *V. cholerae* isolates are human pathogens that may cause diarrhea and extraintestinal infections (21). Fluid resuscitation remains the first-line therapy, but the use of antibiotics allows decreased symptom duration and pathogen dissemination. This is, to our knowledge, the first description of a non-O1/non-O139 *V. cholerae* isolate harboring *bla*<sub>VIM</sub> carbapenemase genes worldwide and the first description of a carbapenemase-producing *V. cholerae* in Western countries.

Here, its identification in an animal microbiota brings new insights into the presence of such strains in wildlife ecosystems. Walsh et al. (6) previously described NDM-1-carrying *V. cholerae* in seepage and tap water samples collected in New Delhi, India, emphasizing the transfer frequency of this metalloenzyme in various recipient species at environmentally relevant temperatures. In their study, conjugative transfer of IncA/C plasmids harboring *bla*<sub>NDM-1</sub> in *V. cholerae* did not happen at 37°C, and average transfer frequencies of  $10^{-6}$  and  $10^{-4}$  were observed at 25°C and 30°C, respectively. Similarly, our study found that the plasmid could transfer at 25°C with comparable frequencies, whereas conjugation attempts at 37°C were unsuccessful. This suggests that optimal transfer conditions would mainly occur in the environment rather than in the gut. The presence of metalloenzymes on different genetic locations (IncA/C and nontypeable plasmids [4, 6], chromosome [5, 6]) in *V. cholerae* is of concern because it indicates that they can spread easily on various mobile genetic elements.

Yellow-legged gulls inhabit mostly coastal regions across the Mediterranean, where they breed in dense colonies. As they can fly long distances across the European and northern African borders, especially during their first year of life, they could play a role in the dissemination of *bla*<sub>VIM</sub>-harboring *V. cholerae*. Based on the feeding habits of yellow-legged gulls (they mainly rely on anthropogenic resources) and their microbiota diversity (including *E. coli*), it is reasonable to think that they are an important zoonotic reservoir of multidrug-resistant organisms, including *bla*<sub>VIM</sub>-carrying bacteria. This is consistent with reports highlighting the increasing prevalence of carbapenemase-producing microorganisms in wildlife microbiota (22). Moreover, their direct exposure to human activities might play a role in the spread of antibiotic resistance, as previously illustrated by ESBL-positive *E. coli* isolates with similar genetic background recovered among gulls and humans in several countries, including southern France (23). Further studies are



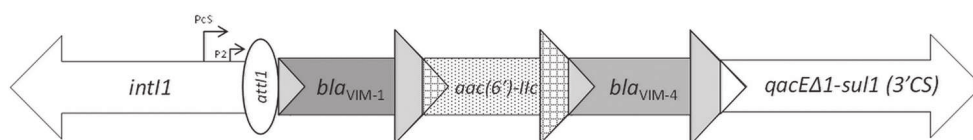


FIG 1 Linear map of the class 1 integron harboring both *bla*<sub>VIM-1</sub> and *bla*<sub>VIM-4</sub> genes. Arrows, relative gene size and direction of transcription; black arrows, gene cassette promoters PcS and P2.

needed to assess the prevalence of carbapenemase genes and their genetic background in wildlife microbiota.

**Nucleotide sequence accession number.** The sequence of the class 1 integron harboring both *bla*<sub>VIM-1</sub> and *bla*<sub>VIM-4</sub> genes has been submitted to GenBank under accession number [KR262557](#).

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## IX. Conclusions – Discussion

Ce travail de thèse a permis d'identifier et de caractériser l'existence d'un réservoir de bactéries multirésistantes aux antibiotiques au sein du microbiote cloacal de goélands méditerranéens en France, puis de comparer ces BMR aviaires aux isolats bactériens retrouvés chez l'homme dans la même région d'étude en colonisation ou impliqués dans des processus infectieux.

Plus particulièrement, cette étude a révélé l'existence d'isolats de *P. mirabilis* producteurs de CMY-2. Le fait que l'ICE soit le support génétique exclusif de cette résistance indique qu'il pourrait jouer un rôle important dans la dissémination de ce gène de résistance chez ces goélands et dans l'écosystème marin. Ce qui est en adéquation avec la forte prévalence de ces éléments génétiques mobiles retrouvés chez des bactéries ubiquitaires de l'environnement aquatique, notamment celles du genre *Vibrio* (24). C'est aussi la première description de ce gène de résistance sur un ICE chez des souches humaines en France, cela a été décrit dans trois autres régions dans le monde (25-27), ce qui pourrait confirmer l'hypothèse de Mata sur l'importance de ces éléments génétiques dans l'émergence de ces résistances chez l'homme, mais aussi dans la faune sauvage, d'autant plus que nous avons montré qu'il y avait une persistance dans le temps de ces gènes de résistances portés par ces ICEs chez ces deux espèces de goélands.

Cependant, lors de notre échantillonnage nous avons aussi isolé des souches d'*E. coli* productrices de céphalosporinases à haut niveau. Plusieurs plasmides ont été détectés chez ces souches qui possèdent le même gène de résistance que les *P. mirabilis* mais n'ont cependant pas le même support génétique. Ces ICEs de la famille SXT/R391-like n'ont été retrouvés chez aucun *E. coli* producteurs de céphalosporinase, de même que les *P. mirabilis* possédant le gène *bla*<sub>CMY-2</sub> ne possédaient aucun plasmide détectable avec les techniques utilisées. Cela pourrait être dû à une spécificité d'hôte de ces ICEs de la famille SXT/R391-like porteurs du gène *bla*<sub>CMY-2</sub>. En effet, n'ayant été retrouvée que chez *P. mirabilis* (25-27), cette structure génétique chromosomique pourrait conférer un avantage sélectif à cette espèce bactérienne lorsque soumise à une pression de sélection antibiotique constante dans le temps.



Un deuxième résultat très important et très intéressant est la présence d'espèces bactériennes productrices de carbapénèmases au sein du même microbiote. Il s'agit de la deuxième description d'*E. coli* productrices de carbapénèmases chez des oiseaux sauvages, et la première chez des goélands. Ceci est particulièrement préoccupant en raison des situations d'impasses thérapeutiques et des problèmes de santé publique que peut causer ce type de souches. Il s'agit aussi de la première description d'une souche de *V. cholerae* produisant une carbapénémase de type VIM, et la première description d'une souche de cette espèce résistante aux carbapénèmes dans les pays occidentaux.

Cependant et comme développé en préambule, les bactéries productrices de carbapénèmases de type VIM sont rares en France, responsable de seulement 5% des infections signalées (28). Elles sont plus souvent retrouvées en Grèce, bien qu'elles commencent à se répandre en Europe du sud-ouest, notamment en Espagne et en Italie (29, 30). La France en revanche semble pour le moment moins touchée, où les gènes *bla<sub>OXA-48</sub>* sont les plus fréquemment retrouvés (28). En outre, le fait que ces souches soient reliées génétiquement aux souches sensibles humaines et aviaires isolées de la même région d'étude, confirme l'existence d'échanges entre les goélands et les humains, comme cela avait été démontré précédemment dans la région avec des *E. coli* producteurs de BLSE (21). De plus, la présence de ces souches dans une seule espèce de goélands nous oriente vers une sélection de la résistance qui a eu lieu dans l'environnement et / ou au sein de la faune. Cette sélection peut avoir conduit soit à la circulation soutenue d'une souche amenée de l'étranger, à l'émergence de souches résistantes indépendante directement liées à la pollution locale ou d'une contamination d'origine humaine locale suivie d'une amplification liée aux conditions environnementales de la colonie. Le fait de retrouver le même gène de résistance chez une souche de *V. cholerae* suggère un éventuel transfert génétique entre ces deux espèces. Les plasmides de type IncA/C ayant un large spectre d'hôte (31), ils pourraient jouer un rôle important dans la transmission et la dissémination de ces résistances.

De plus, *V. cholerae* étant une bactérie ubiquitaire dans l'environnement (24), on pourrait se poser la question sur la diffusion et la persistance de ces gènes de résistance dans ce dernier. Une étude rapporte la présence de souches environnementales d'*E. coli* productrices de ce même type de carbapénèmases sur différents types de plasmides (32). L'environnement pourrait ainsi jouer le rôle

non seulement de réservoir de ces gènes mais aussi d'intermédiaire entre les différents compartiments étudiés.

Avec cette étude nous démontrons la complexité de la circulation de l'antibiorésistance au sein du microbiote étudié. Nous ne possédons actuellement pas assez d'informations pour bien comprendre ce phénomène. Cependant, ce travail ouvre de nombreuses perspectives d'un point de vue épidémiologique mais également fondamental sur les mécanismes et les supports génétique de cette antibiorésistance. En effet, il illustre bien les apports importants des outils d'épidémiologie moléculaire dans la surveillance de l'émergence et la compréhension de la dynamique de transmission et de diffusion des BMR dans la faune sauvage.

## X. Perspectives

Notre étude permet d'envisager d'explorer différentes perspectives :

1. L'une des premières perspectives sera de caractériser les autres BMR isolées dans notre échantillonnage aviaire. En particulier, d'étudier les supports génétiques des céphalosporinases de haut niveau retrouvées chez les isolats d'*E. coli* et les comparer avec ceux retrouvés chez les patients du CHU de Montpellier et les *P. mirabilis*. Nous investiguerons aussi les souches d'*E.coli* résistants aux carbapénèmes isolées lors de la deuxième année d'étude afin de confirmer leur persistance dans le temps. Ces dernières sont porteuses du même gène de résistance *bla<sub>VIM</sub>*. Il serait aussi pertinent de caractériser les souches de *P. mirabilis*, *E. coli* et *Klebsiella pneumoniae* productrices de BLSE isolées de ce même microbiote, et réaliser un suivi longitudinal de ces BMR au sein de cet écosystème aviaire afin d'étudier la dynamique temporelle de ce phénomène et de comparer nos résultats avec ceux de l'étude de Bonnedahl *et al* (21).

2. Il serait aussi intéressant d'explorer les habitats visités par ces goélands en les équipant de balises GPS. Ces derniers peuvent être amenés à prospecter d'autres sites à des fins alimentaires. Ceci pourrait aider à expliquer leur contamination par des BMR

3. Dans notre étude nous ne nous sommes intéressés qu'à un seul écosystème animal sauvage que sont les goélands. Nous souhaiterions donc aussi inclure un hôte qui vit en contact plus étroit avec l'homme que les goélands comme le rat surmulot (*Rattus norvegicus*). Les données disponibles montrent que cet espèce peut être porteuse de BMR (18). Elle diffère des goélands par sa capacité de dispersion, les oiseaux parcourant des distances beaucoup plus longues que les rongeurs, ce qui nous permettra d'évaluer les conséquences de ces contrastes sur la dynamique des bactéries antibiorésistantes.

4. Afin de compléter ce travail, il serait nécessaire d'explorer le compartiment manquant à notre étude, à savoir l'environnement, en particulier dans l'écosystème aquatique qui a déjà été décrit comme étant un réservoir de ce type de souches BMR, et de les comparer avec nos souches aviaires afin de mieux comprendre la dynamique de transmission de ces résistances. En effet, l'eau pourrait représenter un intermédiaire entre les compartiments étudiés, ce qui expliquerait la présence de mêmes clones chez

l'homme et les deux espèces d'oiseaux. L'analyse des propriétés physico-chimiques de cette eau serait aussi très pertinente. Il a été démontré que la présence de facteurs extrinsèques comme les métaux lourds ou des métabolites d'antibiotiques favorisaient l'émergence et le maintien des gènes de résistance aux antibiotiques chez les bactéries (6, 33, 34).

5. En plus de la recherche de BMR directement dans l'eau, une recherche de ces dernières pourrait être réalisée dans la même région aux sein d'organismes filtreurs aquatiques (e.g. *Mytilus galloprovincialis*) (35), qui pourraient jouer le rôle de sentinelle pour les BMR. En effet, ce sont de véritables concentrateurs de bactéries circulantes et peuvent donc constituer des modèles biologiques très intéressants pour avoir une image de la diversité bactérienne aquatique. Par ailleurs, au sein de notre région d'étude, la Camargue, ces organismes sont ubiquitaires. Il serait intéressant d'échantillonner ces derniers et d'étudier leur portage bactérien dans différents habitats le long d'un continuum entre zones potentiellement sources de souches antibiorésistantes (e.g. hôpitaux, stations d'épuration) et zones moins touchées par les activités humaines (e.g. réserves naturelles). En effet, des études ayant porté sur la présence de bactéries antibiorésistantes dans l'eau, ont montré qu'il existait des gradients de densité et de diversité de ces bactéries le long de cours d'eau en aval de sources potentielles de souches antibiorésistantes telles que des hôpitaux et des stations d'épuration (36, 37). Ainsi, en prenant l'hypothèse qu'il existe des zones sources de contamination par les bactéries antibiorésistantes, nous pouvons supposer que la quantité et la diversité des souches résistantes devrait diminuer au sein de la faune sauvage, comme dans l'eau, à mesure que l'on s'éloigne de ces sources.

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## **Annexes**



# First Description of IncX3 Plasmids Carrying *bla*<sub>OXA-181</sub> in *Escherichia coli* Clinical Isolates in Burkina Faso

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Carbapenemase-producing *Enterobacteriaceae* (CPE) have been increasingly reported worldwide. The few studies available on CPE epidemiology in West and East Africa highlight the identification of carbapenemases in Cameroon (NDM-4), Kenya (NDM-1), Sierra Leone (VIM and DIM-1), Senegal (OXA-48), and Tanzania (KPC, IMP, OXA-48, VIM, and NDM) (1). Although *bla*<sub>OXA-48</sub> genes are widely spread in North Africa, *bla*<sub>OXA-48</sub> derivatives have been rarely reported in Africa. Indeed, *bla*<sub>OXA-163</sub> was detected only twice in Egypt and *bla*<sub>OXA-181</sub> (a point mutant analogue of OXA-48) only once in South Africa (1). Here, we describe the first four cases of *Escherichia coli* carrying the *bla*<sub>OXA-181</sub> gene in Burkina Faso.

Four *E. coli* strains (Table 1) were isolated from four patients in two hospitals in Ouagadougou, Burkina Faso. Carbapenem MICs, determined using the Etest (bioMérieux), were 1 to 1.5 mg/liter, 0.125 to 0.75 mg/liter, and 0.25 to 0.5 mg/liter for ertapenem, doripenem, and imipenem, respectively (Table 1). Three patients received antibiotics before strain isolation (Table 1). None of the patients reported recent travel outside Burkina Faso. Multiplex PCR and DNA sequencing targeting the most prevalent extended-spectrum- $\beta$ -lactamase (ESBL)- and carbapenemase-encoding genes (2, 3) revealed the presence of *bla*<sub>CTX-M-15</sub> and of *bla*<sub>OXA-181</sub> in the four isolates. No other carbapenemase-encoding gene (corresponding to NDM, VIM, IMP, and KPC) was detected. Multilocus sequence typing (MLST) (<http://bigsd.bweb.pasteur.fr/>) showed that the four strains belonged to new sequence type (ST) ST692, which is described here for the first time. Enterobacterial repetitive intergenic consensus sequence PCR (ERIC-PCR) (4) patterns (see Fig. S1 in the supplemental material) and the variable-number tandem-repeat (VNTR) (5) profile determined on the basis of analysis of 7 polymorphic loci (6-1-5-8-3-5-1; see Table 1) confirmed the genetic links among the four *E. coli* strains. However, the review of medical records indicated that the four patients were hospitalized in different structures (hospitals and wards). Although there was no relationship or housing shared between the patients, these data support the hypothesis of infections by the same multidrug-resistant clone circulating in these hospitals or in the general community.

Plasmid DNA was extracted by alkaline lysis and subsequently analyzed by gel electrophoresis as previously described (6). Comparative analysis was carried out using reference plasmids RP4 (54 kb), pCFF04 (85 kb), and pIP173 (126.8 kb) and showed two

different plasmids of ca. 120 kb and ca. 54 kb, respectively, in each strain.

Mating experiments performed using azide-resistant *E. coli* strain J53 as a recipient under various conditions were unsuccessful. Plasmid DNA was extracted using a QIAprep Spin Miniprep kit (Qiagen) and transferred by electroporation into *E. coli* DH10B (Invitrogen, Cergy-Pontoise, France). The *bla*<sub>OXA-181</sub>-carrying transformants showed ertapenem and imipenem MICs of 0.38 to 0.5 mg/liter and 0.5 to 0.75 mg/liter, respectively. Analysis by plasmid relaxase gene typing (7) and PCR-based replicon typing (8) identified IncX3-type relaxase and ColE-type replication initiation genes, respectively, in the transformants. Alkaline lysis of transformants and subsequent electrophoresis showed that these genes were carried by the ca. 54-kb plasmid. As a *bla*<sub>OXA-181</sub>-carrying IncX3 plasmid was recently identified in *E. coli* in China (9), PCR mapping was carried out in the four strains and their respective transformants with primers designed using plasmid pOXA181\_EC14828 as the template (GenBank accession number KP400525). All primers used for PCR mapping are reported in Table S1 in the supplemental material. PCR mapping gave similar results in all four *E. coli* strains. DNA regions surrounding the *bla*<sub>OXA-181</sub> gene are detailed in Fig. 1 and showed that *bla*<sub>OXA-181</sub> was part of the Tn2013 transposon, as previously described (10). The same genetic context was recovered in all six *bla*<sub>OXA-181</sub>-surrounding sequences available in the GenBank database (GenBank accession numbers KP400525, AB972272, JN205800, NZ\_JRKW01000020, JQ996150, and KT005457) (11, 12). The *repA1* gene (encoding a ColE-type replication initiation protein) was downstream of Tn2013. This replicase gene was also found on plasmids pKP3-A (JN205800) and pMR3-OXA181 (NZ\_JRKW01000020) that belong to the ColE and IncN incom-

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**TABLE 1** Clinical and microbiological characteristics of the four *bla*<sub>OXA-181</sub>-producing *E. coli* isolates and their respective transformants and of *E. coli* J53<sup>a</sup>

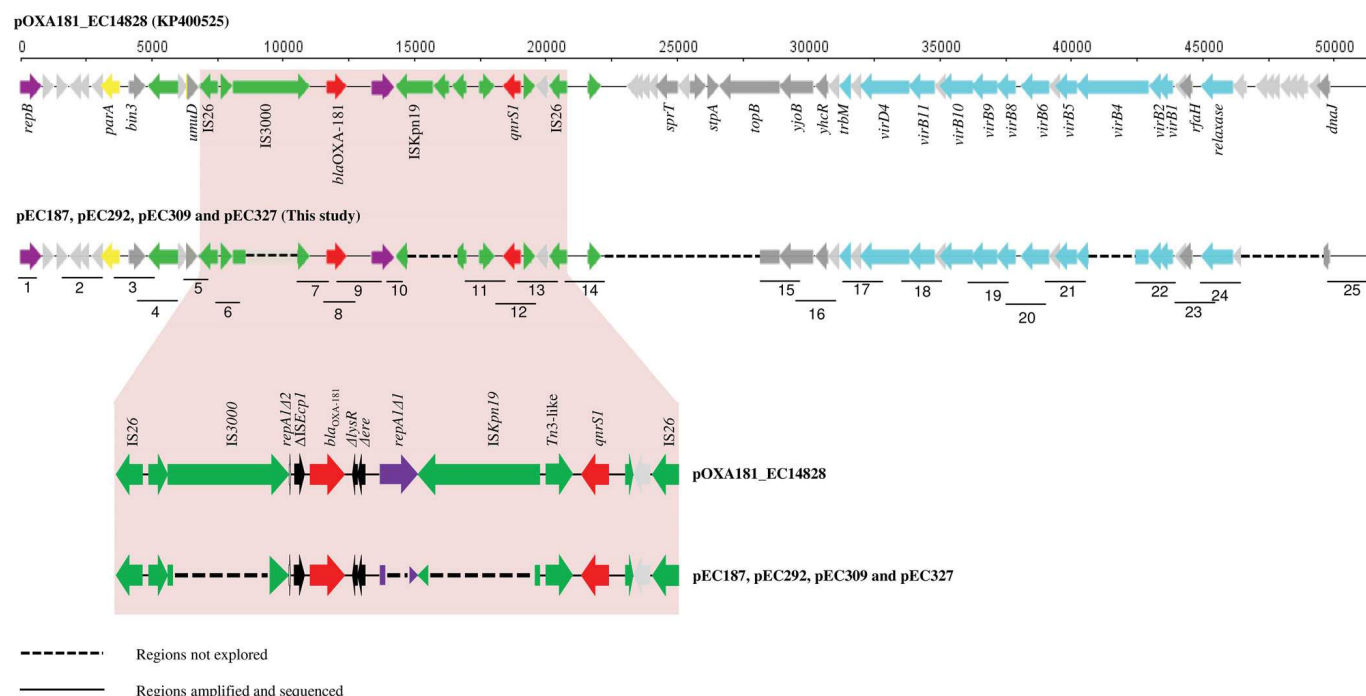
Characteristic	Result								
	EC187	EC187 (T)	EC292	EC292 (T)	EC309	EC309 (T)	EC327	EC327 (T)	<i>E. coli</i> J53
Patient	F, 12 yrs old		M, 2 yrs old		F, 65 yrs old		F, 21 yrs old		
Origin	Urine		Suppuration		Suppuration		Urine		
Clinical symptom or diagnosis	Dysuria		Abdominal pain		Peritonitis		Unknown		
Use of antibiotics in the previous 3 mo	None reported		CFM, CRO, GE		AMC, GE		CRO, GE		
MLST	ST692		ST692		ST692		ST692		
VNTR <sup>b</sup>	6-1-5-8-3-5-1		6-1-5-8-3-5-1		6-1-5-8-3-5-1		6-1-5-8-3-5-1		
MIC (mg/liter)									
Ertapenem	1	0.5	1.5	0.5	1.5	0.5	1.5	0.38	0.06
Doripenem	0.125	ND	0.25	ND	0.25	ND	0.75	ND	ND
Imipenem	0.25	0.5	0.5	0.75	0.38	0.5	0.38	0.5	0.25
Associated resistance									
ESBL	CTX-M-15	None	CTX-M-15	None	CTX-M-15	None	CTX-M-15	None	
Non-β-lactam resistance	CIP, GE, SXT, TE	ND	CIP, GE, SXT, TE	ND	CIP, GE, SXT, TE	ND	CIP, GE, SXT, TE	ND	

<sup>a</sup> (T), transformant; F, female; M, male; ND, not determined; AMC, amoxicillin; AMC, amoxicillin-clavulanic acid (co-amoxiclav); CFM, cefixime; CIP, ciprofloxacin; CRO, ceftriaxone; GE, gentamicin; SXT, sulfamethoxazole-trimethoprim; TE, tetracycline.

<sup>b</sup> Data represent CNV1, CNV2, CNV3, CNV4, CNV7, CNV14, and CNV15.

patibility groups, respectively. This suggests that *bla*<sub>OXA-181</sub> might have come from a ColE-type scaffold. Fluoroquinolone resistance gene *qnrS1* was also detected downstream of *bla*<sub>OXA-181</sub> (Fig. 1). An IncX3-specific backbone was recovered at the 5' extremity of

*bla*<sub>OXA-181</sub>-surrounding regions and included, in addition to the *repB* replicase gene, the *parA* partition gene (13) and the *umuD* gene involved in SOS mutagenesis (14). Large-scale PCR mapping targeting various plasmid regions, including transfer, replication,



**FIG 1** Genetic map of the four plasmids harboring *bla*<sub>OXA-181</sub> described in this report. Purple arrows represent the replicase genes. Light-gray arrows represent genes encoding hypothetical proteins. Yellow arrows represent genes encoding partition systems. Dark-gray arrows represent accessory genes. Green arrows represent transposase-encoding genes and insertion sequences. Red arrows represent antimicrobial resistance genes. Blue arrows represent genes implicated in plasmid transfer. The genetic context of *bla*<sub>OXA-181</sub> is visually extended at the bottom. Plasmid pOXA181\_EC14828 was harbored by an *E. coli* isolate in China (GenBank accession no. KP400525) and was used as a model to map the four *bla*<sub>OXA-181</sub>-carrying plasmids described in this report. Thin black lines represent the 25 oligonucleotide pairs used for PCR mapping in all four plasmids. All amplicons were fully sequenced and displayed 100% identity to those of plasmid pOXA181\_EC14828.

and partition systems, was also performed and covered a total of 29,569 bp, which amounts to ca. 55% coverage compared to the estimated size of the plasmid (Fig. 1; see also Table S1 in the supplemental material). All PCR products displayed 100% identity to those encoded by the respective regions of plasmid pOXA181\_EC14828 (Fig. 1).

Since the first description in Indian hospitals in 2011, OXA-181-positive *Enterobacteriaceae* have been reported worldwide (1, 11). Their emergence in West Africa in IncX3 plasmids is of particular concern because these plasmids mediate the spread of carbapenemases in *Enterobacteriaceae* (15, 16). Moreover, a recent study found an IncX3 plasmid harboring *bla*<sub>OXA-181</sub> in a *Klebsiella variicola* isolate in fresh vegetables imported to Switzerland from Asia (12). This plasmid, named pKS22 (KT005457), is highly similar to pOXA181\_EC14828 (100% coverage and 99% identity) and therefore to the four IncX3 plasmids described in our report. The presence of highly similar IncX3 plasmids in Asia, Africa, and Europe might suggest the epidemic potential of the members of this plasmid lineage and their role in worldwide dissemination of OXA-181.

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RESEARCH ARTICLE

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# High prevalence of extended-spectrum $\beta$ -lactamase producing *enterobacteriaceae* among clinical isolates in Burkina Faso

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## Abstract

**Background:** Nothing is known about the epidemiology and resistance mechanisms of extended-spectrum  $\beta$ -lactamase-producing *Enterobacteriaceae* (ESBL-PE) in Burkina Faso. The objective of this study was to determine ESBL-PE prevalence and to characterize ESBL genes in Burkina Faso.

**Methods:** During 2 months (June-July 2014), 1602 clinical samples were sent for bacteriologic investigations to the microbiology laboratories of the three main hospitals of Burkina Faso. Isolates were identified by mass spectrometry using a matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) BioTyper. Antibiotic susceptibility was tested using the disk diffusion method on Müller-Hinton agar. The different ESBL genes in potential ESBL-producing isolates were detected by PCR and double stranded DNA sequencing. *Escherichia coli* phylogenetic groups were determined using a PCR-based method.

**Results:** ESBL-PE frequency was 58 % (179 strains among the 308 *Enterobacteriaceae* isolates identified in the collected samples; 45 % in outpatients and 70 % in hospitalized patients). The CTX-M-1 group was dominant (94 %, CTX-M-15 enzyme), followed by the CTX-M-9 group (4 %). ESBL producers were more often found in *E. coli* (67.5 %) and *Klebsiella pneumoniae* (26 %) isolates. *E. coli* isolates ( $n = 202$ ; 60 % of all *Enterobacteriaceae* samples) were distributed in eight phylogenetic groups (A = 49, B1 = 15, B2 = 43, C = 22, Clade I = 7, D = 37, F = 13 and 16 unknown); 22 strains belonged to the sequence type ST131. No association between a specific strain and ESBL production was detected.

**Conclusions:** This report shows the alarming spread of ESBL genes in Burkina Faso. Public health efforts should focus on education (population and healthcare professionals), surveillance and promotion of correct and restricted antibiotic use to limit their dissemination.

**Keywords:** *Enterobacteriaceae*, ESBL, Clinical samples, Inpatient and outpatient, Burkina Faso

## Background

The emergence and spread of Multidrug Resistant (MDR) bacteria are major public health threats. Particularly, bacteria that produce extended-spectrum  $\beta$ -lactamases (ESBL) are of great concern because their resistance to

penicillins and narrow- and extended-spectrum cephalosporins reduces considerably the treatment options [1]. ESBL genes originally evolved from the  $\beta$ -lactamase *TEM-1*, *TEM-2* and *SHV-1* genes through mutations of the amino acids surrounding the active site and were mainly detected in nosocomial pathogens [2]. However, during the past decade, the rapid and massive spread of CTX-M-type ESBLs has been described worldwide. This has considerably changed their epidemiology because they combine the expansion of mobile genetic elements with specific clonal dissemination [3]. Furthermore, such

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plasmids typically carry resistance genes also to other drugs, such as aminoglycosides and fluoroquinolones [2]. Recent studies suggest that CTX-M-type ESBL-producing *Enterobacteriaceae* (ESBL-PE) are endemic in most countries of Europe, Asia and South America, with high rates of CTX-M-type ESBL-producers particularly among *Escherichia coli* (30 to 90 %) and *Klebsiella pneumoniae* (10 to 60 %) [4, 5]. Despite these public health concerns, little is known about ESBL diffusion in Africa. ESBL-PE rates between 8.8 and 13.1 % were reported in South Africa [6] and an alarmingly high proportion of ESBL-PE (49.3 %) was found among clinical isolates from Ghana [7]. Conversely, no information is available on the epidemiology of ESBL-producing pathogens in hospital or community settings in Burkina Faso, a low-income country close to Ghana. Therefore, the aim of the present study was to estimate ESBL occurrence in clinical samples from hospitalized and non-hospitalized patients and to characterize the ESBL genes as well as the genetic background of the identified *E. coli* strains.

## Methods

### Study setting

During 2 months (June–July 2014), all consecutive clinical samples sent to the microbiology laboratories of the three main hospitals of Burkina were investigated. Specifically:

1. Yalgado Ouedraogo Teaching Hospital (CHU-YO) is the largest medical institution located in Ouagadougou, the capital city with a population of about 2 million inhabitants. This hospital has 716 beds and intensive care units that are used for surgical, medical and trauma emergencies. Annually, more than 20,000 inpatients (children and adults) are admitted among 126,000 consultations.
2. Sourou Sanou Teaching Hospital (CHU-SS) is the major healthcare and referral centre for the southern and western regions of Burkina Faso. It has 521 beds distributed in different specialized (medicine, surgery, gynaecology obstetric and paediatric) acute care units. The annual number of hospitalizations ranges from 15,000 to 20,000 patients among 108,000 consultations.
3. Charles de Gaulle Paediatric Teaching Hospital (CHUP-CDG) is the referral paediatric hospital in Ouagadougou with 120 beds. About 6000 children are seen each year and 5000 are hospitalized. The microbiology laboratory also receives samples from adult outpatients.

### Specimen collection, identification and antimicrobial susceptibility testing

In June and July 2014 (CHU-SS and CHUP-CDG) and July 2014 (CHU-YO), 1602 clinical samples were sent to

the three microbiology laboratories for bacteriologic investigations (CHU-YO:  $n = 521$ , CHU-SS:  $n = 528$  and CHUP-CDG:  $n = 553$ ). Bacterial cultures could be obtained only from 584 of these samples (mainly because of poor pre-analytical sample handling) and they included 308 *Enterobacteriaceae* isolates. *Enterobacteriaceae* isolates were recovered from urine ( $n = 185$ ), pus ( $n = 56$ ), aspirates from various anatomic sites ( $n = 38$ ), stool ( $n = 16$ ), blood ( $n = 8$ ), vaginal swabs ( $n = 3$ ) and cerebrospinal fluid samples ( $n = 2$ ). The remaining 276 isolates included Gram positive cocci (*Staphylococcus* spp and *Streptococcus* spp) and Gram negative bacilli (e.g., *Pseudomonas aeruginosa* and *Acinetobacter baumannii*). Species identification was performed by matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry (Bruker Daltonics, Bremen, Germany). Antimicrobial susceptibility was tested with the disk diffusion method on Müller-Hinton agar. The following antibiotics were tested: penicillins (amoxicillin, amoxicillin-clavulanic acid, piperacillin, piperacillin-tazobactam, ticarcillin, ticarcillin-clavulanic acid), monobactam (aztreonam), oxacephem (moxalactam), extended-spectrum cephalosporins (cefepime, cefotaxime, ceftazidime, cefpodoxime, cefoxitin, ceftazidime, cephalotin), carbapenems (imipenem), aminoglycosides (amikacin, gentamicin, netilmicin and tobramycin), quinolones (nalidixic acid, ciprofloxacin, levofloxacin, ofloxacin) fosfomycin, chloramphenicol, tetracycline and trimethoprim-sulfamethoxazole. Results were interpreted according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) clinical breakpoints (Version 5.0) ([http://www.eucast.org/clinical\\_breakpoints/](http://www.eucast.org/clinical_breakpoints/)). ESBL production was detected by using the combined double-disk synergy method [8]. In case of high-level cephalosporinase production, the combined double-disk synergy test was performed using cloxacillin-supplemented medium. Ertapenem minimal inhibitory concentrations (MIC), determined using the Etest (bioMérieux), were determined for all isolates.

### Molecular identification of ESBL genes

DNA was extracted from one single colony for each isolate in a final volume of 100  $\mu$ L of distilled water by incubation at 95 °C for 10 min followed by a centrifugation step. The presence of *bla*<sub>CTX-M</sub> (CTX-M group 1, 2, 8, 9 and 25), *bla*<sub>TEM</sub>, *bla*<sub>SHV</sub> and *bla*<sub>OXA-like</sub> genes was assessed by multiplex PCR according to a previously published method [9]. DNA from reference *bla*<sub>CTX-M</sub>, *bla*<sub>TEM</sub>, *bla*<sub>SHV</sub> and *bla*<sub>OXA-like</sub>-positive strains was used as positive control. PCR products were visualized after electrophoresis on 1.5 % agarose gels containing ethidium bromide at 100 V for 80 min. A 100 bp DNA ladder (Promega, USA) was used as a marker size. PCR products were purified using the ExoSAP-IT

purification kit (GE Healthcare, Piscataway, NJ, USA) and sequenced bidirectionally on a 3100 ABI Prism Genetic Analyzer (Applied Biosystems). Nucleotide sequence alignment and analyses were performed online using the BLAST program available at the National Center for Biotechnology Information web page <http://www.ncbi.nlm.nih.gov>.

#### PCR detection of *Escherichia coli* phylogroups and ST131

*E. coli* phylogenetic grouping was performed using the PCR-based method described by Clermont et al. [10]. For strains assigned to the B2 phylogenetic group, the sequence type (ST) 131 was determined using a PCR method specific for the O25-b serotype with primers that target the *pabB* and *trpA* genes, as previously described [11].

#### Statistical analysis

Statistical analysis was performed with Epi Info, version 3.5.3 [Centers for Disease Control and Prevention (CDC), Atlanta, GA, USA]. Associations between demographic variables (sex, site of infection and age) and infection by ESBL-PEs were analysed by using odds ratio and a multinomial logistic regression model, when appropriate. A value of  $p < 0.05$  was considered to be statistically significant.

## Results

### Occurrence of ESBL-producing enterobacteriaceae

During the study period, 308 different enterobacterial isolates were recovered from 158 hospitalized and 150 non-hospitalized patients (Table 1). The mean age of these patients was  $29.7 \pm 24.6$  years and the sex ratio 1.4; 118 patients (38 %) were younger than 15. Among these 308 isolates, 179 (58 %) were identified as potential ESBL-PEs by antimicrobial susceptibility testing. PCR analysis confirmed that they all carried ESBL genes (Table 1). Considering the isolate origin, ESBL-PE prevalence was of 65 % (42/65) at CHU-YO, 59 % (84/142) at CHU-SS and 52 % (53/101) at CHUP-CDG. Moreover, ESBL-PEs were found in 45 % of outpatients and 70 % of hospitalized patients ( $p < 0.001$ ). In hospitalized patients, no demographic factor was significantly associated with ESBL-PE occurrence ( $p > 0.05$ ) (Table 1). Conversely in outpatients, the ESBL-PE prevalence was significantly higher among patients older than 65 years of age (Odd Ratio [OR] = 6.4, 95 % CI = 0.47–86.34;  $p < 0.001$ ). ESBL-PE rate was also significantly higher in male than female outpatients (OR = 4.59) and in urinary samples (59 of 119; 50 %) (Table 1). Species identification showed that the 179 ESBL-PEs included 121 (67.5 %) *E. coli*, 46 (26 %) *K. pneumoniae*, 7 (4 %) *Enterobacter cloacae*, 2 (1 %) *Providencia stuartii*, 1 (0.5 %) *Enterobacter aerogenes*, 1 (0.5 %) *Citrobacter freundii* and 1 (0.5 %) *Morganella morganii* species (Table 2). The highest proportion of ESBL-PEs was found

**Table 1** Demographic characteristics and source of the bacterial isolates

Variable	Outpatients (n = 150)				Inpatients (n = 158)			
	ESBL-positive (n = 68)	ESBL-negative (n = 82)	Odds Ratio (95 % CI)	P-value	ESBL-positive (n = 111)	ESBL-negative (n = 47)	Odds Ratio (95 % CI)	P-value
Sex				<0.001				0.724
F (n = 129)	16	48	1		47	18	1	
M (n = 179)	52	34	4.59 (2.14–9.84)		64	29	0.85 (0.42–1.70)	
Source of isolates				0.019				0.139
Urine sample (n = 185)	59	60	1		48	18	1	
Pus (n = 56)	06	07	0.87 (0.28–2.75)		32	11	1.09 (0.46–2.61)	
Aspirate (n = 38)	02	03	0.68 (0.11–4.20)		24	09	1.00 (0.39–2.56)	
Other <sup>a</sup> (n = 29)	01	12	0.08 (0.01–0.67)		07	09	0.29 (0.09–0.90)	
Age				<0.001				0.607
≤28 days	01	02	1		09	04	1	
>28 days–1 year	01	08	0.20 (0.01–4.72)		07	04	0.78 (0.14–4.27)	
>1–5 years	02	15	0.31 (0.02–5.19)		06	06	0.44 (0.09–2.28)	
>5–15 years	04	07	1.14 (0.08–16.95)		30	12	1.11 (0.29–4.31)	
>15–65 years	44	45	1.96 (0.17–22.35)		49	19	1.15 (0.32–4.17)	
>65 years	16	05	6.40 (0.47–86.34)		10	02	2.22 (0.33–15.18)	

**Abbreviations:** CI confidence interval, ESBL extended-spectrum beta-lactamase, F females, M males, n number

<sup>a</sup>Other: stool, cerebrospinal fluid, blood samples and high vaginal swabs

**Table 2** Prevalence of ESBL-producing isolates among the different Enterobacteriaceae species identified in our samples

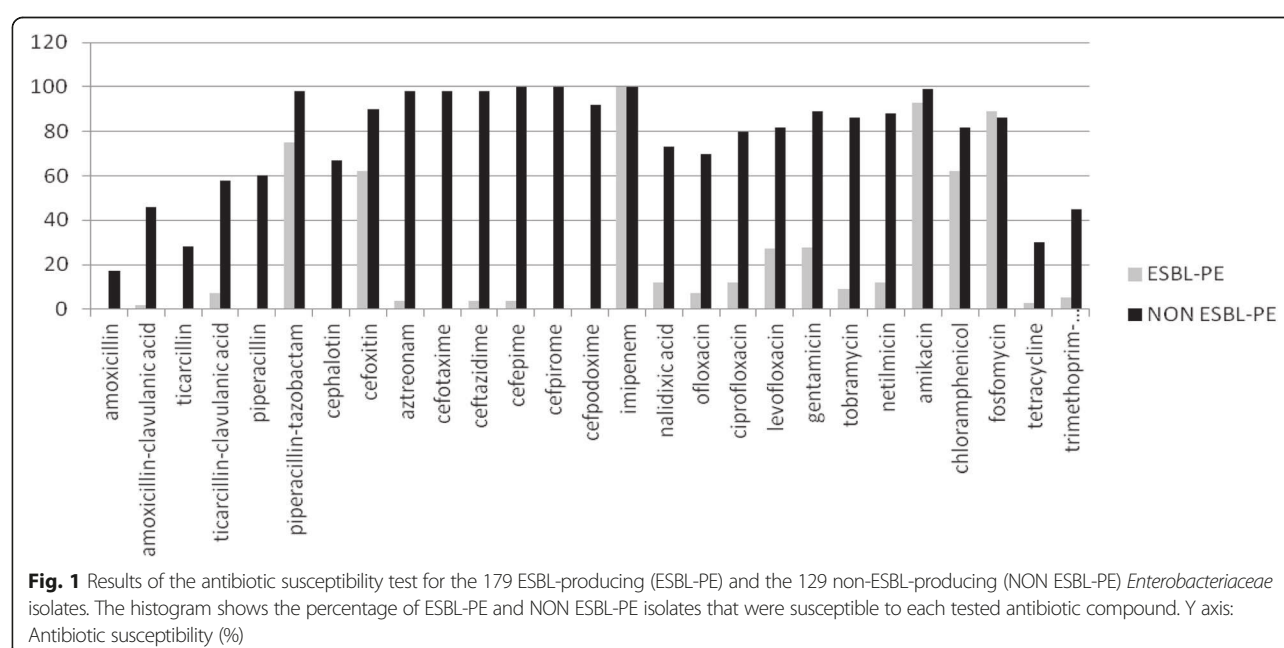
Species	Distribution of ESBL-producing isolates in samples (%)								
	Within species	Total (%)	Urine (n = 185)	Stool (n = 16)	Blood (n = 18)	Pus (n = 56)	Aspirates (n = 38)	HVS (n = 3)	CSF (n = 2)
<i>Escherichia coli</i>	121/202 (60 %)	67.5 %	67/114	0/15	0	29/39	23/31	2/2	0/1
<i>Klebsiella pneumoniae</i>	46/70 (66 %)	26 %	32/51	0	5/6	6/8	3/4	0/1	0
<i>Enterobacter cloacae</i>	7/13 (54 %)	4 %	6/10	0	0	1/3	0	0	0
<i>Enterobacter aerogenes</i>	1/3 (33 %)	0.5 %	1/2	0	0	0	0/1	0	0
<i>Citrobacter koseri</i>	0/1	–	0	0	0	0	0/1	0	0
<i>Citrobacter freundii</i>	1/3 (33 %)	0.5 %	1/2	0	0	0/1	0	0	0
<i>Proteus mirabilis</i>	0/5	–	0/4	0	0	0/1	0	0	0
<i>Providencia stuartii</i>	2/6 (33 %)	1 %	0/1	0	1/1	1/3	0/1	0	0
<i>Salmonella spp</i>	0/3	–	0	0/1	0/1	0	0	0	0/1
<i>Morganella morganii</i>	1/1 (100 %)	0.5 %	0	0	0	1/1	0	0	0
<i>Leclercia adecarboxylata</i>	0/1	–	0/1	0	0	0	0	0	0
Total (%)	179/308 (58 %)	100 %	107/185	0/16	6/8	38/56	26/38	2/3	0/2

Abbreviations: CSF cerebrospinal fluid, ESBL extended-spectrum beta-lactamase, HVS high vaginal swab, n = number

in blood samples (6/8, 75 %). Moreover, within each species, the fraction of ESBL producers was highest among *Morganella morganii* isolates (100 %), followed by *K. pneumoniae* (66 %) and *E. coli* (60 %) (Table 2). The 129 non-ESBL-PEs included *E. coli* (81/2002, 40 %), *K. pneumoniae* (24/70, 34 %) *Enterobacter cloacae* (6/13, 46 %), *Providencia stuartii* (4/6, 66 %) *Enterobacter aerogenes* (2/3, 77 %) *Citrobacter freundii* (2/3, 66 %), *Salmonella spp* (3/3, 100 %), *Proteus mirabilis* (5/5, 100 %) and *Leclercia adecarboxylata* (1/1) species.

### Antibiotic susceptibility patterns

The susceptibility pattern of ESBL producing ( $n = 179$ ) and non-producing ( $n = 129$ ) *Enterobacteriaceae* isolates is shown in Fig. 1. ESBL-PE isolates were more resistant to the other tested antibiotics than non-producers: cotrimoxazole (45 % vs 5 %), gentamicin (89 % vs 27.5 %), tobramycin (86 % vs 9 %), netilmicin (88 % vs 12 %), ciprofloxacin (80 % vs 12 %), ofloxacin (70 % vs 7 %) and levofloxacin (82 % vs 27 %) ( $p < 0.05$ ). None of the collected *Enterobacteriaceae* isolates was resistant to



imipenem. Four isolates had high ertapenem MCI. Additional investigations showed that these four isolates carried blaOXA181 (47).

#### Molecular characterization of ESBL and other $\beta$ -lactamase genes

Most ESBL-PE isolates (94 %) were identified as CTX-M group 1 producers because all of them carried the bla<sub>CTX-M-15</sub> gene. CTX-M group 9 producers represented only 4 % of all ESBL-PEs (bla<sub>CTX-M-14</sub> was

detected in three isolates and bla<sub>CTX-M-27</sub> in five samples (Table 3). The bla<sub>SHV-12</sub> gene was detected in two isolates. The ESBL genes were detected alone or in association with one to three other  $\beta$ -lactamase genes: bla<sub>OXA-1</sub>, bla<sub>SHV-1</sub> and bla<sub>TEM-1</sub>. While bla<sub>CTX-M-15</sub> was found in all the different enterobacterial species, bla<sub>CTX-M-14</sub> was detected only in *E. coli* samples ( $n = 3$ ), bla<sub>CTX-M-27</sub> in *E. coli* ( $n = 2$ ), *K. pneumoniae* ( $n = 1$ ) and *E. cloacae* ( $n = 1$ ) isolates, and bla<sub>SHV-12</sub> in one *E. coli* and one *K. pneumoniae* sample (Table 3).

**Table 3** Characterization of genes encoding beta lactamases in the 179 ESBL-producer isolates

Isolates (n)	Outpatients (n = 68)			Inpatients (n = 111)	
	Hospital (n)	ESBL Type	Other $\beta$ -lactamases	ESBL Types	Other $\beta$ -lactamases
<i>Escherichia coli</i> (121)	CHU-YO (37)	CTX-M-15 (1)	OXA-1(1)	CTX-M-14 (1)	SHV-1, TEM -1 (1)
		CTX-M-15 (2)	SHV-1 (2)	CTX-M-14 (1)	TEM-1 (1)
		CTX-M-15 (1)	TEM-1 (1)	CTX-M-15 (3)	TEM-1, OXA-1 (3)
		CTX-M-15 (1)	–	CTX-M-15 (7)	TEM-1(7)
		–	–	CTX-M-15 (3)	SHV-1 (3)
		–	–	CTX-M-15 (12)	OXA-1 (12)
		–	–	CTX-M-15 (3)	–
		–	–	CTX-M-27 (2)	TEM-1 (2)
		–	–	–	–
	CHUSS (52)	CTX-M-15 (3)	SHV-1, OXA-1 (3)	CTX-M-15 (1)	SHV-1, OXA-1 (1)
		CTX-M-15 (1)	TEM-1, OXA-1 (1)	CTX-M-15 (1)	TEM-1, OXA-1 (1)
		CTX-M-15 (2)	TEM-1 (2)	CTX-M-15 (1)	TEM-1 (1)
		CTX-M-15 (27)	OXA-1 (27)	CTX-M-15 (15)	OXA-1 (15)
		SHV-12 (1)	–	–	–
	CHUP-CDG (32)	CTX-M-15 (1)	OXA-1 (1)	CTX-M-14 (2)	TEM-1, OXA-1 (2)
		CTX-M-15 (4)	TEM-1(4)	CTX-M-15 (8)	TEM-1, OXA-1 (8)
		CTX-M-27 (1)	OXA-1, TEM-1(1)	CTX-M-15 (10)	TEM-1(10)
		–	–	CTX-M-15 (6)	OXA-1 (6)
<i>Klebsiella pneumonia</i> (46)	CHU-YO (5)	CTX-M-15(1)	TEM-1, OXA-1(1)	CTX-M-15 (1)	SHV-1, OXA-1, TEM-1 (1)
		SHV-12 (1)	–	CTX-M-15 (2)	OXA-1 (2)
	CHUSS (24)	CTX-M-15(1)	SHV-1, OXA-1, TEM-1(1)	CTX-M-15(3)	SHV-1, OXA-1, TEM-1(3)
		CTX-M-15(2)	SHV-1, OXA-1, (2)	CTX-M-15(2)	SHV-1, OXA-1 (2)
		CTX-M-15(3)	SHV-1, TEM-1 (3)	CTX-M-15(2)	OXA-1, TEM-1 (2)
		CTX-M-15(5)	OXA-1 (5)	CTX-M15 (2)	OXA-1 (2)
		CTX-M-15(1)	TEM-1(1)	CTX-M-15(1)	–
		CTX-M-15(2)	SHV-1 (2)	–	–
		–	–	–	–
	CHUP-CDG (17)	CTX-M-15 (2)	OXA-1 (2)	CTX-M-15 (3)	OXA-1(3)
		–	–	CTX-M-15 (5)	EM-1, OXA-1 (5)
		–	–	CTX-M-15(4)	SHV-1, TEM-1(4)
		–	–	CTX-M-15(2)	SHV-11
		–	–	CTX-M-27(1)	SHV-1(1)
		–	–	–	–
Other strains (12)		CTX-M-15(2)	OXA-1, TEM-1 (2)	CTX-M-15 (5)	OXA-1, TEM-1
		CTX-M-15 (2)	TEM-1, SHV-1 (2)	CTX-M-15 (1)	SHV-11(1)
		CTX-M-27 (1)	OXA-1 (1)	CTX-M-15 (1)	–

Abbreviations: N number



### ***Escherichia coli* phylogenetic groups and sequence type 131**

The phylogenetic group analysis revealed diversity in both ESBL-producing and non-producing *E. coli* isolates ( $n = 202$ ). Specifically, *E. coli* isolates belonged to eight different phylogenetic groups (A = 49, B1 = 15, B2 = 43, C = 22, Clade I = 7, D = 37 F = 13) and 16 could not be classified according to Clermont *and al.* method [10]. These 16 isolates might represent a new phylogenetic group. Phylogenetic group A was more represented among ESBL-producers (31 of 121; 26 %), followed by group D and B2 (for both: 26 of 121; 21.5 %). Non-ESBL producers belonged mainly to the phylogenetic groups A and B2 (18 and 17 of 81, respectively; 21 %). Moreover, the ST131 sequence type was detected in 16 ESBL-producers and in six non-producers (Table 4).

### **Discussion**

In this study, we investigated the frequency of ESBL production by *Enterobacteriaceae* isolates from clinical samples sent to the three main hospitals of Burkina Faso in June and July 2014. Overall, 58 % of these isolates were ESBL-PEs. This is much higher than the rates reported in Europe [12, 13] and in other African countries: Algeria (17.7–31.4 %), Egypt (42.9 %) [14] and Ghana (49.4 %) [7]. Lack of antibiotic surveillance may have contributed to increasing the ESBL-PE problem that certainly has been present in Burkina Faso for a long time. Indeed, it has been shown that in countries with limited resources where hygiene is poor and antibiotics are misused, the absence of anti-microbial surveillance programmes increases the risk of multi-resistance development by bacteria in hospitals and in the community [15–17]. We found that blood cultures had the highest proportion of ESBL-PE isolates. This differs from the results of a recent literature review on ESBL-PE prevalence in Africa [18] showing a significantly lower proportion of ESBL-PE in blood cultures than in other specimens. This discrepancy is certainly explained by the

small number of enterobacterial strains (eight of which six were ESBL-PEs) recovered from blood samples. Indeed, 107 ESBL-PEs were identified in urine samples (107/185, 58 %), a prevalence similar to what reported in previous studies [7, 19–21]. ESBL producers were more often found in *E. coli* (67 %) and *K. pneumoniae* (26 %) isolates, in agreement with previous works showing that these two species are the predominant ESBL-producers worldwide [2, 22]. ESBL-producing *E. coli* is considered to be responsible for hospital- and community-acquired infections, while ESBL-producing *K. pneumoniae* is considered mainly a nosocomial pathogen [2, 22]. In agreement, we identified ESBL-producing *K. pneumoniae* most frequently in samples from hospitalized patients. ESBL-PE prevalence differed considerably between outpatients and inpatients (45 % vs. 70 %:  $p < 0.001$ ). More than two thirds of enterobacterial infections in hospitalized patients were thus caused by an ESBL-PE. In Burkina Faso, patients are usually hospitalized only in the case of very severe symptoms and after a long and empiric antibiotic therapy. These factors could explain this alarmingly high resistance level in hospitalized patients and also in outpatients (45 % compared with 7.5 % of community-acquired infections in Morocco [23] and 11.7 % in Nigeria) [24]. In outpatients, ESBL-PE frequency was significantly higher in isolates from older patients (more than 65 years of age, [OR] = 6.4, 95 % CI = 0.47–86.34). These results are in agreement with the study by Colodner and al. [3] showing that elderly patients present a higher antibiotic pressure and more underlying diseases, two significant risk factors for infection by ESBL producers [25]. In addition, ESBL-PE rate was significantly higher in male outpatients (OR = 4.59, 95 % CI = 2.14–9.84) and the urinary tract was the most frequent source (59 of 119, 50 %). The possible explanation may be that complicated urinary tract infections are more frequent in elderly men than elderly women [26].

**Table 4** Phylogenetic group assignment of the 202 *E. coli* strains subdivided based on the detection or not of ESBL genes

	ESBL-Positive ( $n = 121$ )										ESBL-Negative ( $n = 81$ )									
	A	B1	B2	C	Clade I	D	F	Unknown	ST131		A	B1	B2	C	Clade I	D	F	Unknown	ST131	
Hospital (number of samples)																				
CHU-YO (55)																				
Outpatients (16)	0	0	3	0	0	2	0	0	1		0	0	1	6	0	0	0	4	0	
Inpatients (39)	9	2	4	6	0	5	6	0	3		0	0	3	0	0	2	0	2	1	
CHU-SS (90)																				
Outpatients (59)	6	1	11	4	2	8	1	0	8		7	2	7	4	1	3	2	0	4	
Inpatients (31)	7	1	5	1	0	3	1	1	4		2	2	1	1	1	0	1	3	0	
CHUP-CDG (57)																				
Outpatients (20)	0	1	0	0	0	2	2	1	0		7	2	3	0	0	1	0	1	1	
Inpatients (37)	9	2	3	0	3	6	0	3	0		2	2	2	0	0	5	0	1	0	

In this study most ESBL-PEs were resistant to multiple drugs, especially to fluoroquinolones, aminoglycosides, cotrimoxazole and tetracycline, as described in previous studies [27–29]. This level of multi-resistance could lead to potential therapeutic impasses. Indeed, more than three quarters of ESBL-PE isolates were resistant to fluoroquinolones and aminoglycosides (but for amikacin), thus compromising the choice of antibiotic treatment, especially for outpatients with urinary tract infections. Moreover alternative antimicrobial agents, such as amikacin, fosfomycin and imipenem, are very expensive and difficult to obtain in Burkina Faso. These alarming results should act as an impetus for the establishment of antibiotic control policies. Indeed, currently, there is no restriction in the use of antibiotics in Burkina Faso. Antibiotics can be purchased over the counter without medical prescription. Patients may buy only a few tablets of an antibiotic because of limited money availability. Moreover, patients may begin an antimicrobial regimen and stop it when they feel better, before the end of the treatment, to save the remaining tablets for another time.

Finally, we found that 94 % of ESBL-PEs carried the *bla*<sub>CTX-M-15</sub> gene. In the last decade, CTX-M enzymes, particularly CTX-M-15, have emerged worldwide and are the most prevalent in Europe, America and Asia [30–36]. Moreover, eight strains were CTX-M group 9 producers (*bla*<sub>CTX-M-27</sub> in five and *bla*<sub>CTX-M-14</sub> in three). These genes have been previously detected in *E. coli* isolates in Kenya [37] and in Egypt [38]. Nevertheless, the *bla*<sub>CTX-M-15</sub> gene remains dominant in the African continent: 59 % of ESBL-PE in South Africa [34], 83 % in Mali [39], 91 % in Tunisia [40] and 96 % in Cameroon [41]. The *bla*<sub>SHV-12</sub> gene (detected in one *E. coli* and one *K. pneumoniae* sample) has emerged in recent years and has been also detected in different *Enterobacteriaceae* isolates in the previously quoted studies in African countries [34, 38–41].

The phylogenetic group assignment of the 202 *E. coli* isolated showed a high diversity in both populations (outpatients and in-patients) without any association between a specific strain and ESBL production. This indicates that the high frequency of ESBL carriage is not caused by the epidemic spread of a single resistant clone. This contrasts with previous studies in which the dissemination of CTX-M-15-producing isolates was associated with the spread of the ST131 *E. coli* strain belonging to phylogenetic group B2 [42–44]. Indeed, in the present study, most isolates were assigned to the commensal groups A (49/202, 24 %) and B2 (43/202, 21 %). Only 13 % (16/121) of ESBL-producers and 7 % (6/81) of non ESBL-producers belonged to the ST131 clone. Moreover, some ESBL-producing *E. coli* isolated from urine, pus and blood samples belonged to three phylogenetic groups associated with CTX-M-15 dissemination: the virulent extra-intestinal group D (26/121) [45]

and groups C (11/121) and F (10/121), usually detected in urinary tract infections [46]. This important genetic diversity among isolates suggests that the high rate of ESBL production and associated resistance are more likely caused by the diffusion of plasmids carrying antibiotic resistance genes than to cross-transmission between patients. The maintenance of these plasmids was probably favoured by antibiotic pressure. Further investigations, including multilocus sequence typing and plasmid characterization, are needed to complete this study.

## Conclusions

In summary, this first survey shows an alarmingly high frequency of multi-resistant ESBL-PEs among clinical isolates in Burkina Faso. The analysis of the resistance genes highlighted an important dissemination of *bla*<sub>CTX-M-15</sub> without clonal dissemination, suggesting a strong antibiotic selection pressure in hospital and community settings. Public health efforts should focus on educating the population and healthcare professionals about the proper use of antibiotics to halt/limit the spread of multi-resistant bacteria.

## Abbreviations

CHUP-CDG, Charles de Gaulle Paediatric Teaching Hospital; CHU-SS, Soro Sanou Teaching Hospital; CHU-YO, Yalgado Ouedraogo Teaching Hospital; ESBL, extended-spectrum  $\beta$ -lactamases; ESBL-PE, extended-spectrum  $\beta$ -lactamase-producing *Enterobacteriaceae*; MALDI-TOF, matrix-assisted laser desorption ionization-time of flight; MDR, multidrug resistant; OR, odd ratio

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## Authors' contributions

Conceived and designed the experiments: OAS, CC, JPH, GS, SI, NB, SL and OR. Performed the experiments: OAS, KF and PA, AS, BN. Contributed reagents/materials/analysis tools: OAS, KF and PA, AS, BN. Contributed to the writing of the manuscript: OAS, CC, JPH, GS, DD. All authors read and approved the final manuscript.

## Competing interests

The authors declare that they have no competing interests.

## Consent for publication

Not applicable.

## Ethics approval and consent to participate

Arbitrary numbers were assigned to the isolates recovered from patient specimens. The study was approved by the ethics authorities of each hospital: MS/SG/CHUSS/DG/DL 2014–171 July 2, 2014. This allowed us to conduct our study. Informed written consent was obtained from subjects and at least one parent of each child before enrollment.

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# Fecal Carriage of *Enterobacteriaceae* Producing Extended-Spectrum Beta-Lactamases in Hospitalized Patients and Healthy Community Volunteers in Burkina Faso

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Extended-spectrum  $\beta$ -lactamase-producing *Enterobacteriaceae* (ESBL-PE) have been described worldwide, but few reports focused on Burkina Faso. To assess the prevalence of digestive carriage of such bacteria in the community and in the hospital, 214 fecal samples, 101 from healthy volunteers and 113 from hospitalized patients without digestive pathology, were collected in Bobo Dioulasso, Burkina Faso economic capital, during July and August 2014. Stool samples were screened using ESBL agar plates. Strains were identified by mass spectrometry using the Biotyper MALDI-TOF. ESBL production was confirmed with the double-disc synergy test. Susceptibility was tested using the disk diffusion method on Müller-Hinton agar. The main ESBL genes were detected using multiplex PCR and bidirectional gene sequencing. *Escherichia coli* phylogenetic groups were identified using a PCR-based method. During the study period, prevalence of subjects with fecal ESBL-PE was 32% (69/214), 22% among healthy volunteers and 42% among inpatients. All but two ESBL, CTX-M-15 and ESBL-PE, were mostly *E. coli* (78%). Among the 60 ESBL-producing *E. coli* strains, 26% belonged to phylogenetic group D, 23.3% to group A, 20% to group B1, 6.6% to group B2, and 3.3% to the ST131 clone. Univariate analysis showed that history of hospitalization and previous antibiotic use were risk factors associated with ESBL-PE fecal carriage. In Burkina Faso, the prevalence of both healthy subjects from the community and hospitalized patients with fecal ESBL-PE is alarmingly high. This feature should be taken into consideration by both general practitioners and hospital doctors with regard to empirical treatments of infections, notably urinary tract infections.

## Introduction

**E**XTENDED-SPECTRUM  $\beta$ -LACTAMASES (ESBL) hydrolyze a wide range of  $\beta$ -lactam antibiotics, including second- and third-generation cephalosporins.<sup>1,2</sup> Furthermore, ESBL-producing enterobacterial (ESBL-PE) isolates that produce new ESBL families (especially CTX-M enzymes) are associated with resistance to fluoroquinolones and aminoglycosides and have been detected worldwide. This might limit the available treatment options and increase the likelihood of empiric treatment failure.<sup>3,4</sup>

Digestive tract is the main reservoir of *Enterobacteriaceae* causing infections whatever their onset (community or hospital).<sup>5</sup> Therefore, knowing the prevalence of subjects with digestive tract carriage of ESBL-PE in the community and among hospitalized patients is a manner to predict the involvement of such bacteria in infections and their level of transmission. From this point of view, the situation is particularly dramatic in Africa where subjects with ESBL-PE digestive tract carriage range from 10% to 50% and is higher than 60% in the case of ESBL-producing *Escherichia coli*.<sup>6,7</sup> In Burkina Faso, a low-income country of West Africa, nothing is

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known about the digestive tract carriage of ESBL-producing *Enterobacteriaceae* both among inpatients and healthy subjects. The objective of the present study was to assess the prevalence of patients hospitalized at the Sourou Sanou University Hospital and healthy volunteers living in the city of Bobo Dioulasso with digestive tract carriage of ESBL-PE, and to characterize the ESBL produced as well as the genetic background of the *E. coli* producing these enzymes.

## Materials and Methods

### *Patients, specimen collection, and ethical clearance*

This study was conducted in Bobo Dioulasso, Burkina Faso economical capital with a population of about 1 million inhabitants. During July and August 2014, 214 fecal samples were collected from 101 healthy volunteers in the community and 113 patients who were hospitalized at the Sourou Sanou University Hospital for more than 48 hr (people with digestive pathologies were excluded from the study). Sourou Sanou University Hospital is the major healthcare and referral center for Burkina Faso southern and western regions. It has 521 beds distributed in different specialized (medicine, surgery, gynecology, obstetrics, and pediatrics) acute care units. Each participant was interviewed by health professionals using a home-made standardized questionnaire to record the following data: age, gender, antibiotic treatment during the past 3 months, and any hospital stays in the previous year. The study was approved by the Sourou Sanou University Hospital board (Authorization No. MS/SG/CHUSS/DG/DL 2014-171, July 2, 2014). Informed written consent was obtained from all subjects and at least one parent for each child before enrollment.

### *Detection of ESBL-PE isolates and antibiotic susceptibility testing*

Briefly, 0.5 g of each fresh stool sample was suspended in 5 ml of sterile saline and 100  $\mu$ l aliquots were plated on ESBL agar plates (bioMérieux, Marcy-l'Etoile, France) and were examined after 24 and 48 hr of incubation at 37°C. Each distinct morphotype of colonies that grew on ESBL agar plates was studied. Species identification was performed by Matrix-Assisted Laser Desorption-Ionization Time-of-Flight (MALDI-TOF) Mass Spectrometry (Bruker Daltonics, Bremen, Germany). All *Enterobacteriaceae* isolates were screened for ESBL production using the double-disc synergy test.<sup>8</sup> In all positive isolates, antimicrobial susceptibility was tested by using the disk diffusion method on Müller-Hinton agar for the following antibiotics: amoxicillin, amoxicillin-clavulanic acid, aztreonam, cefepime, cefotaxime, cefpirime, cefpodoxime, cefoxitin, ceftazidime, cephalothin, moxalactam, piperacillin, piperacillin-tazobactam, ticarcillin, ticarcillin-clavulanic acid, imipenem, nalidixic acid, ciprofloxacin, levofloxacin, ofloxacin, amikacin, gentamicin, netilmicin, tobramycin, fosfomycin, chloramphenicol, tetracycline, and trimethoprim-sulfamethoxazole. Results were interpreted following the European Committee on Antimicrobial Susceptibility Testing (EUCAST) clinical breakpoints (Version 5.0) ([www.eucast.org/clinical\\_breakpoints/](http://www.eucast.org/clinical_breakpoints/)).

### *Molecular identification of ESBL genes*

DNA was extracted from single colonies in a final volume of 100  $\mu$ l of distilled water by incubation at 95°C for 10 min,

followed by a centrifugation step. Multiplex PCR was used to identify strains harboring *bla*<sub>CTX-M</sub> genes that encode different CTX-M variants (*i.e.*, CTX-M group 1, 2, 8, 9, and 25) as well as the *bla*<sub>TEM</sub>, *bla*<sub>SHV</sub>, and *bla*<sub>OXA-like</sub> genes, as previously described.<sup>9</sup> DNA samples from reference strains carrying *bla*<sub>CTX-M</sub>, *bla*<sub>TEM</sub>, *bla*<sub>SHV</sub>, or *bla*<sub>OXA-like</sub> were used as positive controls. PCR products were separated by electrophoresis on 1.5% agarose gels containing ethidium bromide at 100 V for 80 min. A 100 bp DNA ladder (Promega, Fitchburg, WI) was used for size estimation. All specific PCR products were purified using the ExoSAP-IT PCR Clean-up Kit (GE Healthcare, Piscataway, NJ) and bidirectional sequencing was performed on a 3100 ABI Prism Genetic Analyzer (Applied Biosystems, Foster City, CA). Sequence alignment and analysis were performed using the BLAST program available at the National Center for Biotechnology Information ([www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)).

### *Detection of E. coli phylogenetic groups and sequence type 131 clone*

To determine the phylogenetic group of the ESBL-producing *E. coli* isolates, we used the PCR-based method described by Clermont *et al.*<sup>10</sup> For strains assigned to the B2 phylogenetic group, the presence of *E. coli* sequence type (ST) 131 was determined using an O25b-specific PCR method with *pabB* and *trpA* (control) allele-specific primers, as previously described.<sup>11</sup>

### *Statistical analysis*

Data were analyzed with Epi Info version 3.5.3 (Centers for Disease Control and Prevention, Atlanta, GA). Conditional logistic regression analysis was used for the univariate analysis of risk factors and odds ratio with 95% confidence intervals. A  $p < 0.05$  was considered to be statistically significant.

## Results

### *Prevalence and risk factors of ESBL-PE fecal carriage*

During the study period, 214 subjects (101 healthy volunteers and 113 hospitalized patients) with a mean age of  $21.9 \pm 7.1$  years were enrolled (Table 1). Among the healthy volunteers, 6 (6%) were hospitalized in the previous year and 22 (22%) received antibiotics, within the 3 months before inclusion in the study, such as  $\beta$ -Lactams ( $n = 9/22$ , 41%), fluoroquinolones ( $n = 7/22$ , 32%), or other unspecified antibiotics ( $n = 6/22$ , 27%). Among the hospitalized patients, 21 (18.5%) were also hospitalized in the previous year and 58 (51%) received antibiotics, within the 3 months before sampling, such as  $\beta$ -Lactams ( $n = 36/58$ , 62%), fluoroquinolones ( $n = 13/58$ , 22%), or other antibiotics ( $n = 9/58$ , 16%) (Table 1). The previous use of antibiotics and prior hospitalization were different for both populations ( $p < 0.005$ ) (Table 2). Among the 214 stool samples, 69 (32% [26–39%]) grew on selective ESBL agar; 47 of these samples were from inpatients (42% [33–51%] of 113) and 22 from healthy volunteers (22% [15–31%] of 101) ( $p = 0.002$ ). ESBL production by these 69 samples was confirmed with the double-disc synergy test and by PCR. When taking into account the unit where the enrolled patients were hospitalized, we found that 65% (19/29) of patients enrolled from medicine wards were ESBL-PE carriers, 47% (14/30)

TABLE 1. DEMOGRAPHIC CHARACTERISTICS, PREVALENCE OF ESBL-PE FECAL CARRIAGE IN THE STUDY POPULATION

Variables	Healthy volunteers (n = 101)	Inpatients (n = 113)	Total (n = 214)
Age, years, mean (SD)	18.5 (12.5)	25.9 (18.8)	21.9 (7.1)
Males, n (%)	47 (46)	46 (40.7)	93 (43)
ESBL-PE carriers, n (%)	22 (22)	47 (42)	69 (32)
Hospitalization duration, days, mean (SD)	—	10.3 (11.2)	—
Previous hospitalization (in the last year), n (%)			
Yes	6 (6)	21 (18.5)	27 (12.6)
No	95 (94)	92 (81.5)	187 (87.4)
Antibiotic treatment (in last 3 months), n (%)			
Yes	22 (22)	58 (51)	80 (37.4)
No	45 (44)	37 (33)	82 (38.3)
Not known	34 (34)	18 (16)	52 (24.3)
Used antibiotics, n (%)			
$\beta$ -Lactams	9 (41)	36 (62)	45 (21)
Fluoroquinolones	7 (32)	13 (22)	20 (9.3)
Other antibiotics <sup>a</sup>	6 (27)	9 (16)	15 (7)

<sup>a</sup>Tetracycline, doxycycline, trimethoprim-sulfamethoxazole, erythromycin, clindamycin, and gentamicin.  
SD, standard deviation.

from surgery wards, 28% (8/29) from gynecology/obstetrics ward, and 24% (6/25) from the pediatric department. Univariate analysis did not identify any specific factor associated with ESBL-PE fecal carriage in the hospitalized population (Table 3). Conversely, in the healthy controls, previous use of antibiotics and anterior hospitalization were risk factors for ESBL-PE carriage ( $p < 0.05$ ) (Table 3).

#### Characterization of and associated resistance in ESBL-PE isolates

From the 69 ESBL-positive samples, 77 different ESBL-PE isolates were identified by MALDI-TOF Mass Spectrometry: *E. coli* was the main enterobacterial species (60/77: 78%), followed by *Klebsiella pneumoniae* (16/77: 21%) and *Enterobacter cloacae* (1/77: 1%). Among the healthy volunteers, 21/101 had an ESBL-producing *E. coli* among whom three had also an ESBL-producing *K. pneumoniae* isolate, and one had an ESBL-producing *K. pneumoniae*. The three healthy volunteers who carried both ESBL-producing *E. coli* and *K. pneumoniae* isolates were hospitalized during the previous year. Among the hospitalized patients, 47/113 had ESBL-producing isolates: 34 an *E. coli* isolate, 7 a *K. pneumoniae* isolate, 1 an *E. cloacae* isolate, and 5 both an *E. coli* isolate and a *K. pneumoniae* isolate.

The susceptibility pattern of the ESBL-producing strains is summarized in Figure 1. ESBL-producing isolates from healthy volunteers were more frequently resistant to chloramphenicol and fluoroquinolones, while isolates from inpatients showed higher resistance rates to tetracyclines and

aminoglycosides. Most samples from both populations were also resistant to sulfamethoxazole-trimethoprim. All isolates were susceptible to imipenem.

#### $\beta$ -Lactamase production characterization

ESBL-positive strains harbored genes encoding CTX-M group 1 enzymes [CTX-M-15: 75/77 (98%)], CTX-M group 9 enzymes [CTX-M-14: 1/77 (1%)], or SHV enzymes [SHV-12: 1/77 (1%)]. The *bla*<sub>CTX-M-15</sub> gene was detected alone in 15 isolates (20%) or in association with other  $\beta$ -lactamase genes: *bla*<sub>OXA-1</sub> and *bla*<sub>TEM-1</sub> in 8 isolates (10.6%), *bla*<sub>TEM-1</sub> and *bla*<sub>SHV-1</sub> in 4 (5.3%), *bla*<sub>OXA-1</sub> and *bla*<sub>SHV-1</sub> in 8 (10.6%), *bla*<sub>TEM-1</sub> alone in 15 (20%), *bla*<sub>OXA-1</sub> alone in 23 (30.6%), and *bla*<sub>SHV-1</sub> alone in 2 (2.6%). The *bla*<sub>CTX-M-14</sub> gene was associated with the *bla*<sub>OXA-1</sub> gene while *bla*<sub>SHV-12</sub> was detected alone. The *bla*<sub>CTX-M-15</sub> gene was found in 59 *E. coli* strains, 15 *K. pneumoniae* isolates and the single *E. cloacae* strain. The *bla*<sub>CTX-M-14</sub> gene was detected in one *E. coli* strain (healthy volunteer) and *bla*<sub>SHV-12</sub> in one *K. pneumoniae* isolate (healthy volunteer) (Table 4).

#### *E. coli* phylogenetic groups and ST131 clone

The phylogenetic group analysis indicated that the 60 ESBL-positive *E. coli* isolates were distributed in six phylogenetic groups: D (18/60), A (14/60), B1 (12/60), C (8/60), B2 (4/60), F (3/60), and one unknown.

Group B1 *E. coli* isolates were only detected from hospitalized patients, while group D isolates were mostly from

TABLE 2. UNIVARIATE ANALYSIS OF ANTIBIOTIC USE AND PREVIOUS HOSPITALIZATION AMONG OUTPATIENTS AND INPATIENTS

Risk factors	Outpatients (n = 101)	Inpatients (113)	Odds ratio (95% CI)	P
Previous use of antibiotics				
Yes/no	22/45	58/37	0.31 (0.16–0.60)	<0.001
Previous hospitalization				
Yes/no	6/95	21/92	0.27 (0.10–0.71)	0.005

TABLE 3. ANALYSIS OF RISK FACTORS FOR ESBL-PE FECAL CARRIAGE IN THE STUDY POPULATION

Covariate	Outpatients (n = 101)				Inpatients (n = 113)			
	ESBL-positive (n = 22)	ESBL-negative (n = 79)	Odds ratio (95% CI)	P	ESBL-positive (n = 47)	ESBL-negative (n = 66)	Odds ratio (95% CI)	P
Age, years, mean (SD)	21.6 (14.5)	17.6 (11.8)		0.24	25.8 (15.4)	26 (21.7)		0.95
Male gender	7	40	0.46 (0.16–1.24)	0.12	16	29	0.62 (0.29–1.34)	0.28
Hospitalization duration, days, mean (SD)					12.1 (14.1)	8.9 (8.35)		0.09
Antibiotic use (last 3 months)								
Yes/no	15/4	7/41	20.47 (5.5–95.23)	<0.001	23/15	35/22	0.96 (0.41–2.23)	0.93
Anterior hospitalization (in last year)								
Yes/no	5/17	1/78	23 (2.51–209.17)	<0.001	9/38	12/54	1.01 (0.39–2.65)	0.89

healthy volunteers (12 out of 18). The ST131 clone was found only in healthy volunteers (Table 5).

## Discussion

In this study, we investigated the prevalence of healthy community volunteers and hospitalized patients with intestinal carriage of ESBL-PE in Burkina Faso. Overall, we found a prevalence of fecal ESBL-PE carriage of 32%, which is much higher than what is observed in the countries in the North, and particularly in Europe, where fecal carriage rates range from 0.6% to 11.6%.<sup>12–14</sup> However, our results are consistent with data from various countries of

Sub-Saharan Africa: 16–55% in Cameroon<sup>7,15</sup> and 31% in Niger.<sup>16</sup> This geographical difference is underlined by studies reporting that the antibiotic resistance level in low-income countries is dramatically increasing, mainly as a consequence of hygiene policy failure, poor drug quality, antibiotic misuse, and lack of surveillance programs.<sup>12,17,18</sup> In our study, the prevalence of fecal carriage was significantly higher in hospitalized patients compared with community volunteers (42% vs. 22%,  $p=0.02$ ), in agreement with previous work<sup>7,15</sup> showing that inpatients are more exposed to strong selective pressure of antibiotics or bacterial transmission. Nevertheless, the proportion of ESBL-PE fecal carriage in healthy volunteers was also high,

**FIG. 1.** Percentage of antimicrobial resistance of the ESBL-PE samples from healthy volunteers ( $n=22$ ) and hospitalized patients ( $n=47$ ). ESBL-PE, extended-spectrum  $\beta$ -lactamase-producing *Enterobacteriaceae*.

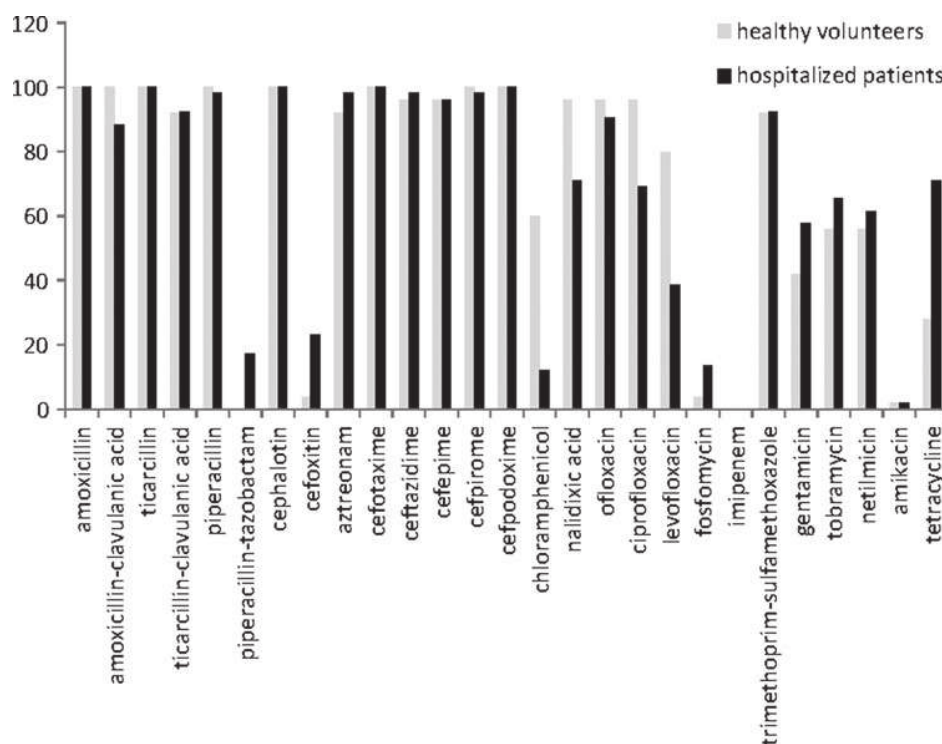




TABLE 4. CHARACTERIZATION OF THE ESBL-PE STRAINS ISOLATED FROM HEALTHY VOLUNTEERS AND HOSPITALIZED PATIENTS

	No. of ESBL producers (%)	All detected genes	ESBL genes	Other $\beta$ -lactamase genes
Healthy volunteers (n=22 ESBL-positive samples)				
<i>Escherichia coli</i>	21 (84)	CTX-M, TEM, OXA-1-like (2) <sup>a</sup> CTX-M, TEM (1) CTX-M, OXA-1-like (11) CTX-M-14 (1) CTX-M (6)	CTX-M-15 (2) <sup>a</sup> CTX-M-15 (1) CTX-M-15 (10) OXA-1 (1) CTX-M-15 (6)	TEM-1, OXA-1 (2) <sup>a</sup> TEM-1 (1) OXA-1 (10)
<i>Klebsiella pneumoniae</i>	4 (16)	CTX-M, SHV (1) CTX-M, SHV, OXA-1-like (1) CTX-M, TEM (1) CTX-M (1) SHV (1)	CTX-M-15 (1) CTX-M-15 (1) CTX-M-15 (1) CTX-M-15 (1) SHV-12 (1)	SHV-1 (1) SHV-1, OXA-1 (1) TEM-1 (1)
Hospitalized patients (n=47 ESBL-positive samples)				
<i>E. coli</i>	39 (75)	CTX-M, TEM, OXA-1-like (6) CTX-M, TEM (12) CTX-M, OXA-1-like (13) CTX-M, SHV, OXA-1-like (2) CTX-M (6)	CTX-M-15 (6) CTX-M-15 (12) CTX-M-15 (13) CTX-M-15 (2) CTX-M-15 (6)	TEM-1, OXA-1 (6) TEM-1 (12) OXA-1 (13) SHV-11, OXA-1 (2)
<i>K. pneumoniae</i>	12 (23)	CTX-M, SHV, OXA-1-like (5) CTX-M, SHV, TEM (3) CTX-M, SHV (1) CTX-M, TEM (1) CTX-M (2)	CTX-M-15 (5) CTX-M-15 (3) CTX-M-15 (1) CTX-M-15 (1) CTX-M-15 (2)	SHV-1, OXA-1 (5) TEM-1, SHV-1 (3) SHV-1 (1) TEM-1 (1)
<i>Enterobacter cloacae</i>	1 (2)	CTX-M, SHV, TEM (1)	CTX-M-15 (1)	SHV-1, TEM-1 (1)

<sup>a</sup>Number of samples.

suggesting that ESBL-producing isolates can spread in the community beyond the hospital environment (nosocomial infections), thus potentially increasing the seriousness of community-acquired infections.

Our risk factor analysis showed that in the community, previous hospital stays during the last year and antibiotic use

within the last 3 months are high risk factors of ESBL-PE fecal carriage, differently from what was reported by Rodriguez-Bano *et al.* in Spain and Wu *et al.* in Hong Kong.<sup>19,20</sup> This discrepancy could be explained by the different clinical practices in these three countries. Indeed in Burkina Faso, like in many less wealthy countries, the

TABLE 5. PHYLOGENETIC GROUP ASSIGNMENT OF THE 60 ESBL-PRODUCER *E. COLI* STRAINS CLASSIFIED ACCORDING TO THE  $\beta$ -LACTAMASE PRODUCTION

Detected genes	A	B1	B2	C	D	F	Unknown	ST131
Healthy volunteers (n=21 <i>E. coli</i> isolates)								
CTX-M, TEM, OXA-1-like (n=2 samples)	2	—	—	—	—	—	—	—
CTX-M, TEM (n=1 sample)	—	—	1	—	—	—	—	1
CTX-M, OXA-1-like (n=11 samples)	2	—	2	—	7	—	—	—
CTX-M (n=6 samples)	—	—	1	—	5	—	—	1
CTX-M, SHV (n=1 sample)	1	—	—	—	—	—	—	—
Hospitalized patients (n=39 <i>E. coli</i> isolates)								
CTX-M, TEM (n=4 samples)	1	1	—	—	1	1	—	—
CTX-M (n=2 samples)	1	1	—	—	—	—	—	—
CTX-M, OXA-1-like (n=4 samples)	1	—	—	—	3	—	—	—
CTX-M, TEM, OXA-1-like (n=2 samples)	—	2	—	—	—	—	—	—
CTX-M, TEM (n=6 samples)	2	1	—	2	—	1	—	—
CTX-M (n=2 samples)	—	2	—	—	—	—	—	—
CTX-M, OXA-1-like (n=3 samples)	2	1	—	—	—	—	—	—
CTX-M, TEM, OXA-1-like (n=2 samples)	—	—	—	2	—	—	—	—
CTX-M, TEM (n=2 samples)	—	—	—	2	—	—	—	—
CTX-M (n=2 samples)	—	2	—	—	—	—	—	—
CTX-M, OXA-1-like (n=6 samples)	2	1	—	—	2	1	—	—
CTX-M, TEM, OXA-1-like (n=2 samples)	—	—	—	2	—	—	—	—
CTX-M, SHV, OXA-1-like (n=2 samples)	—	1	—	—	—	—	1	—

number of physicians, microbiologists, and epidemiologists who can guide/supervise appropriate diagnostic testing, treatment, and control of infectious diseases is insufficient. Moreover, in Burkina Faso, antibiotics can be purchased over the counter without medical prescription. Patients may buy only a few tablets of an antibiotic because of limited availability of money. Moreover, patients may begin an antimicrobial regimen and stop it when they feel better, before the end of the treatment, to save the remaining tablets for another time. All these reasons can explain why prior hospitalization or previous antibiotic use can be serious risk factors of ESBL-PE fecal carriage in low- and middle-income countries, as previously highlighted by studies in Madagascar<sup>21</sup> and in rural Thai communities.<sup>22</sup> In the present study, ESBL producers were mainly *E. coli* and *K. pneumoniae* strains in hospitalized patients. Lonchel *et al.* in Cameroon and Zhang *et al.* in China reported a more important diversity of ESBL-PE species (*e.g.*, *Enterobacter* spp. and *Citrobacter* spp.).<sup>15,23</sup> Nevertheless, in all reports, *E. coli* was the most frequently identified species during colonization by ESBL-PE.<sup>15,23,24</sup> Importantly, all these bacteria that transit in the human intestine can become resistant through horizontal gene exchanges.<sup>25</sup>

In our study, *bla*<sub>CTX-M-15</sub> was the most frequently detected ESBL-encoding gene (98% of isolates), as previously reported in Cameroon (96% of isolates), Indonesia (94.5%), and Tunisia (91%).<sup>7,26,27</sup> *bla*<sub>SHV-12</sub>, which was previously reported in Cameroon,<sup>15,28</sup> was detected in a single *K. pneumoniae* isolate. The SHV-12 enzyme has evolved from SHV-1 and has been detected in different *Enterobacteriaceae* species in other African countries.<sup>29,30</sup> Nevertheless CTX-M-type ESBL, especially CTX-M-15, remain dominant worldwide, except in Asia where ESBL epidemiology shows specific characteristics, with the predominance of CTX-M-9 and 14.<sup>23,31</sup> ESBL-encoding genes are carried by plasmids, thus facilitating their transfer between different bacterial species both in hospitals and in the community.<sup>32,33</sup> Most of these plasmids typically carry resistance genes also to other drugs, such as aminoglycosides and fluoroquinolones.<sup>34</sup> Accordingly, in our study, CTX-M-15 producers also showed high-level resistance to non- $\beta$ -lactams, such as aminoglycosides and tetracycline in hospitalized patients and chloramphenicol and fluoroquinolones in the community volunteers. Most ESBL-PE samples from both populations were also resistant to sulfamethoxazole–trimethoprim. The high resistance level to chloramphenicol, sulfamethoxazole–trimethoprim, and fluoroquinolones was probably due to easy access and a large and uncontrolled use in the community. Concerning the level of resistance to tetracycline in hospitalized patients, except for an extensive use in hospitals, other risk factors have not been identified.

In Burkina Faso, like in many low-income countries, there are no restrictions on the use of antibiotics. Many of our patients and healthy volunteers had a history of broad-spectrum antibiotic consumption. Some antibiotics were prescribed by medical professionals, while others were not. Based on this irrational antibiotic use, we hypothesize that the high prevalence of fecal carriage of ESBL-PE that are resistant also to non- $\beta$ -lactam antibiotics is caused by the strong drug-selective pressure on bacterial populations that live in the human intestine, generally in good intelligence with their host,<sup>35</sup> and not to an epidemic-resistant clone. The

*E. coli* phylogenetic group assignment supports this hypothesis. Indeed, the 60 ESBL-producing *E. coli* isolates belonged to six of the eight different phylogenetic groups recently described by Clermont *et al.*<sup>10</sup> Moreover, most isolates were assigned to the commensal groups A and B1. This finding contrasts with previous studies that associated the dissemination of CTX-M-15-producing isolates with the spread of the epidemic ST131 *E. coli* strain belonging to group B2.<sup>36–38</sup> In the present study, only four isolates were assigned to group B2 and only two belonged to the epidemic ST131 clone, indicating that this clone is less represented in fecal ESBL-producing *E. coli*. Similar results were reported in Tunisia, Libya, and Spain.<sup>39–41</sup> Besides group A, B1, and B2, some ESBL-producing *E. coli* isolates belonged also to three other phylogenetic groups associated with CTX-M-15 dissemination: the virulent extraintestinal group D, previously found in a similar study in France,<sup>42</sup> and groups C and E, usually described in urinary tract infections.<sup>43</sup> This important genetic diversity among CTX-M producers in Burkina Faso suggests that horizontal plasmid transfer has played a more important role than clonal expansion in the community and hospital environments. Molecular epidemiology investigations, including multilocus sequence typing and variable number of tandem repeats analysis, two powerful tools for the genetic characterization of Enterobacteriaceae, are needed to complete this study.<sup>44,45</sup> This genetic characterization of Enterobacteriaceae (sensitive and resistant to antibiotics) population in Burkina Faso will enhance our understanding of epidemiology and the circulation of these bacteria within and between hospitalized patients and community populations in this country.

In summary, this first study on ESBL carriage among intestinal isolates in Burkina Faso shows that ESBL-PE intestinal carriage by asymptomatic humans in Burkina Faso is high. The characterization of  $\beta$ -lactamase-encoding genes highlights an important dissemination of the ESBL CTX-M-15 without clonal dissemination. History of hospitalization and previous antibiotic use are risk factors in the community. Public health efforts should focus on educating the population and healthcare professionals on the proper use of antibiotics.

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## Disclosure Statement

No competing financial interests exist.

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**Médecine et  
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Case report

## Intrafamilial transmission of pulmonary tuberculosis due to *Mycobacterium bovis*

*Transmission intrafamiliale de tuberculose pulmonaire due à Mycobacterium bovis*

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**Mots clés :** Tuberculose ; *Mycobacterium bovis* ; Transmission interhumaine

### 1. Introduction

*Mycobacterium bovis* (*M. bovis*) infections in humans have become quite rare in industrialized countries. Bovine tuberculosis (bTB) control programs have indeed reduced the rate of zoonotic transmission. The number of *M. bovis* tuberculosis (TB) case patients in France drastically reduced since the 1960s and amounted to only 0.5% of all French TB case patients in 1995 [1]. The person-to-person transmission of *M. bovis* TB, as confirmed by microbiological and epidemiological analyses, remains very rare [2–4]. We report the case of an intrafamilial transmission of *M. bovis* TB.

### 2. Observations

#### 2.1. First case patient

A 49-year-old male patient was admitted to the pulmonology department of the hospital of Bressuire (France) in March 2001 for a suspicion of pulmonary tuberculosis. The patient had been

working in a slaughterhouse as a butcher for several years. He presented with a persistent cough, asthenia, and weight loss for the past 4 months. The results of the chest X-ray revealed bilateral apical opacities, but no cavity. The tuberculin skin test result was negative (no induration). The HIV serology was negative. Microbiological analyses were performed on 3 sputum samples and no acid-fast bacilli were found at direct microscopic examination. However, the culture results highlighted the presence of mycobacteria in a specific media. Species identification was performed with the commercial kit GenoType MTBC (Hain Lifescience) and confirmed the *M. bovis* infection. A combination therapy with isoniazid (INH), rifampicin (RMP), pyrazinamide (PZA), and ethambutol (EMB) was administered for 3 months and later replaced by a two-drug combination therapy (INH + RMP) for 6 more months.

#### 2.2. Second case patient

The mother of the first case patient, a 72-year-old woman who had been living with her son for years, was admitted to the same pulmonology department in September 2003 for acute chest pain on the right lower part of her thorax. The patient was immunocompromised due to a long-term treatment with corticosteroids and methotrexate for rheumatoid arthritis. A family

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*Résultat du typage moléculaire spoligotype + MIRU-VNTR des deux souches humaines (fils et mère).*

Patient	Spoligotype profile (Sp1 to Sp43)	MIRU-VNTR profile							
		ETR-A	ETR-B	ETR-C	ETR-D	QUB11a	QUB11b	QUB26	VNTR3232
Son	1000000101111110111111111111111110111100000	7	5	3	2	11	4	7	7
Mother	1000000101111110111111111111111110111100000	7	5	3	2	11	4	7	7

cattle breeding activity, stopped for several years, was mentioned at anamnesis. The results of the chest X-ray revealed a right pleural effusion. The tuberculin skin test result was positive (15 mm). A direct microscopic examination was performed on sputum and pleural fluid samples. No acid-fast bacilli were found, but the culture results confirmed the presence of mycobacteria. Molecular species identification confirmed the *M. bovis* infection. A tuberculosis regimen (INH + RMP + EMB + PZA) was administered for 9 months. Both patients fully recovered after completing the treatment course.

The strains of *M. bovis* isolated from the mother and son were compared to try and establish a potential infectious epidemiological link between these 2 case patients. Two complementary and reference molecular epidemiological tests were used: spoligotyping and mycobacterial interspersed repetitive-unit-variable-number tandem-repeat (MIRU-VNTR) [2–9]. The results confirmed the genetic link between the isolates of the mother and the son. Identical DNA profiles were observed using the spoligotyping technique and the analysis of 8 loci with the MIRU-VNTR technique (ETR-A to ETR-D, QUB 11a, QUB 11b, QUB 26, VNTR 3232) (Table 1).

To confirm the hypothesis of a zoonotic transmission of *M. bovis* for those 2 case patients, their spoligotype profiles were compared with those of the national database on spoligotypes of *M. bovis* strains isolated from French animals between 1979 and 2014 (M.L. Boschirolì, personal communication, Anses). No similarity was found in the human and animal spoligotype profiles.

### 3. Discussion

The incidence of bTB in cattle herds and the risk of human transmission in industrialized countries significantly decreased with the systematic culling of cattle with a positive tuberculin skin test (performed since 1963 in France) and with the implementation of control measures for bovine tuberculosis [1,5]. Food transmission from cattle to humans is also no longer an issue because of the systematic pasteurization of the milk (since 1955 in France). In industrialized countries, *M. bovis* TB is therefore currently observed in patients born before the implementation of such measures or in individuals who come from a country with a high incidence of bTB and no control measures [1,2,4].

As *M. bovis* is innately resistant to PZA, the World Health Organization (WHO) recommends treating *M. bovis* pulmonary TB with a triple therapy (INH + RMP + EMB) for 3 months, followed by a two-drug combination therapy (INH + RMP) for

6 more months. These 2 patients did not receive the standard treatment due to the delayed identification of *M. bovis*. A conventional tuberculosis regimen was instead administered (INH + RMP + EMB + PZA). Treatment duration was however adjusted (9 months) once the results of the species identification were available. PZA should however have been discontinued when *M. bovis* was identified.

Genetic typing using the MIRU-VNTR and spoligotyping techniques is an efficient and discriminating test to confirm the epidemiological links between the mycobacteria isolates of infected patients. It is also useful to better understand the transmission of *M. tuberculosis* and *M. bovis* TB [2,4,6–9]. These molecular tests have been widely used to confirm epidemiological links in person-to-person transmission of TB or to compare human and cattle isolates in zoonotic transmission cases [2,4,6,7].

The present family under study used to own a cattle herd. One of the epidemiological hypotheses on the transmission of the infection could be that both mother and son were contaminated by the same animal and that several years later they presented with TB due to the reactivation of the same endogenous strain. However, the most likely hypothesis remains the occupational zoonotic contamination of the son by infected carcasses that had not been detected by the routine controls, followed by a person-to-person transmission from the son to the mother. The mother probably reactivated the tuberculosis 2 years after that initial contamination because of her immunocompromised status. Some authors suggested that patients presenting with tuberculosis and negative sputum results at direct examination, but positive culture results, played an important role in the person-to-person transmission of TB [10]. Those studies support the intrafamilial transmission hypothesis of our case patients despite the paucibacillary presentation of the son's TB. This hypothesis is even more relevant as person-to-person *M. bovis* transmission has already been reported in immunocompetent and immunocompromised patients [2,4]. The zoonotic origin of the infection could however not be clearly proven as no similarity on the genetic profile of those human strains were identified in the national database of cattle spoligotypes.

## 4. Conclusion

Human *M. bovis* tuberculosis remains rare in industrialized countries. These case patients point out to the discriminating aspect of molecular epidemiological techniques (spoligotyping and MIRU-VNTR) that helped confirm the suspicion of person-to-person transmission from the son to the mother. The zoonotic

origin of the infection could not be proven but these case patients highlight the need for a better prevention of occupational risks of animal to human *M. bovis* contamination for people working in the cattle industry (livestock farmers, butchers, and veterinaries).

### Authors' contribution

A.S.O., S.A. and N.B. performed the molecular typing of the strains and wrote the article.

A.B., A.K., H.J-P. and S.G. proofread the article.

### Disclosure of interest

The authors declare that they have no conflicts of interest concerning this article.

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