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Prevalence and molecular characterization of colistinresistant, ESBL-AmpC- and carbapenemase-producing Enterobacterales in humans, animals and in food chains in Lebanon

Hiba Al Mir

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Hiba Al Mir. Prevalence and molecular characterization of colistinresistant, ESBL-AmpC- and carbapenemase-producing Enterobacterales in humans, animals and in food chains in Lebanon. Microbiology and Parasitology. Université de Lyon; Université Libanaise, 2020. English. NNT : 2020LYSE1287 . tel-03489363

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N°d'ordre NNT : 2020LYSE1287

THESE de DOCTORAT DE L'UNIVERSITE DE LYON
opérée au sein de
L'Université Claude Bernard Lyon 1

Ecole Doctorale 205
Ecole Doctorale Interdisciplinaire Sciences Santé EDISS

Spécialité de doctorat : Medical and Food Microbiology

Soutenue publiquement le 07/12/2020, par :
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Prevalence and molecular characterization of colistin-resistant, ESBL/AmpC- and carbapenemase-producing Enterobacterales in humans, animals and in food chains in Lebanon

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Acknowledgments

First, I would like to express my sincere gratitude to my supervisor Dr. Marisa Haenni, for her guidance and continuous support through my PhD studies. You were always there, pushing me to learn, work and achieve my best. I have learned a lot from you, and for that, I am deeply grateful.

I would also like to thank Dr. Jean-Yves Madec for his enthusiasm, constant support, encouragement and patience. I am indeed blessed to be a part of your research group during my Master and PhD studies.

Additionally, my sincere thanks go to my mentor Pr. Monzer Hamze. Being in your team and one of your students was an honor. From you, I learned a lot in the research field. Thank you for your guidance, patience and the support that you gave me throughout this journey.

Many thanks are also owed to my co-supervisor Dr. Marwan Osman. Thanks for building this dream with me and for always being there no matter what the circumstances are.

I would also like to thank my thesis committee members Pr. Sima Tokajian, Pr. Mira El-Chaar, Pr. Didier Hocquet, Pr. Yves Millemann and Pr. Anne Vianney for their interest in my research work.

Furthermore, I would like to take this opportunity and dedicate my deepest love and gratitude to my best friend, sister, recent Dr. and future bride Fatima Halimeh, who was always with me through the ups and downs of this educational journey. I am so blessed to have the best caring, supportive and never changing friend like you.

I would also like to deeply thank my dear colleague Tony, who was a caring friend. Thanks for being so encouraging, optimistic and supportive all the time.

Similarly, many thanks go to Mona, Naima, Shaymaa, Nawrass, Reem, Ibaa, Iman, Hajar, Zena, Hadi, Ahmad, Sara, Sarah, Orsola, Soha, Zaher, Ali, Nawal, Abir, Mirna and Issam for being my support in France. When I was locked continents away from Lebanon during the tough quarantine, you made me feel like I have a home away from my family. The

same goes to Dr. Ayman El-Falou, Dr. Marwa El-Bouz, Hadil, Abdulgheni and Hadi. I am blessed for every single memory that we shared together.

Equally, I want to thank Raquel, Pierre, Estelle, Melody, Antoine, Henock, Rodolphe, Agnese, Isabelle, Rabah, Souad and all the Anses team for the precious moments and the beautiful memories of laughter that we had.

Dear Vero and Taha, no I didn't forget to mention you and I will never do. You were the persons who always motivated me, supported me, and, more importantly, lifted me up when I fell during my whole PhD journey. When things were not going well in the lab, you were always on my side giving me the strength to carry on. Thank you.

Last but not least, I would like to convey my deepest love, recognition and pride to the two people who aided me in growing up, to the eyes that looked after me days and nights, to the unconditional love, to my everything in this life "MOM and DAD".

Besides, my parents gave me three gifts in this life, my lovely sister and brothers Hana, Anas and Hamed. You are my joy, happiness, and life protectors.

Abbreviation list

AMR	Antimicrobial Resistance
ANSM	National Agency for Medicines and Health Products Safety
ANMV	National Agency for Veterinary Medicinal Products
ANSES	Agence Nationale de Sécurité Sanitaire de l'alimentation et du travail
<i>ampC</i>	ampicillin resistance genes (céphalosporinase)
AGISAR	WHO Advisory Group on Integrated Surveillance of Antimicrobial Resistance
<i>bla</i>	β -lactamase gene
CTX-M	Cefotaximase-Munich
CRE	Carbapenem-Resistant <i>Enterobacteriaceae</i>
CRA	Carbapenem-Resistant <i>Acinetobacter</i>
CRP	Carbapenem-Resistant <i>Pseudomonas</i>
CMY	plasmid-mediated class C beta-lactamases
CI	Chromosomal Integrons
COL-R	Colistin-Resistance
CP	Carbapenem Resistance
CMUL	Lebanese University bacterial collection
CA-SFM	Antibiogram Committee of the French Society for Microbiology
CGE	Center for Genomic Epidemiology
cgMLST	core genome Multi-Locus Sequence Type
DNA	Deoxyribonucleic Acid
DHA	plasmid-mediated AmpC β -lactamases
DNDi	Drugs for Neglected Diseases Initiative
<i>dfrA12</i>	genes encoding for resistance to trimethoprim
<i>E. coli</i>	<i>Escherichia coli</i>
ESC	Extended-Spectrum Cephalosporin
EUCAST	European Committee for Antimicrobial Susceptibility Testing
ESBL	Extended-Spectrum β -lactamases
EU	Europe
ECDC	European Centre for Disease Prevention and Control
EMA	European Medicines Agency

EFSA	European Food Safety Authority
FAO	Food and Agriculture Organization
<i>floR</i>	florfenicol/chloramphenicol resistance genes
FIND	Foundation for Innovative Diagnostics
GNB	Gram-Negative Bacteria
GC	Gene cassettes
GARDP	Global Antibiotic Research and Development Partnership
GAFFI	Global Action Fund for Fungal Infections
GALVmed	Global Alliance for Livestock Veterinary Medicines
GAMRIF	Global AMR Innovation Fund
GLASS	WHO Global Antimicrobial Surveillance System
HGT	Horizontal Gene Transfer
Inc	Incompatibility group
IS	Insertion Sequence
IACG	Interagency Coordination Group
JPIAMR	Joint Programming Initiative on AMR
ICARS	International Centre for Antimicrobial Resistance Solutions
IRT	Inhibitor Resistant TEM
InnoVet-AMR	Innovative Veterinary Solutions for Antimicrobial Resistance
<i>K. pneumoniae</i>	<i>Klebsiella pneumoniae</i>
KPC	<i>Klebsiella pneumoniae</i> carbapenemase
LVIF	Livestock Vaccine Innovation Fund
LPS	Lipopolysaccharide
LMSE	Laboratoire Microbiologie Santé et Environnement
LMICs	Low and Middle-Income Countries
MGE	Mobile Genetic Elements
<i>mcr</i>	<i>mobile colistin resistance gene</i>
MI	Mobile Integrations
MRI	Multidrug Resistance Integrations
MMV	Medicines for Malaria Venture
MDR	Multidrug-Resistant
MBL	metallo β -lactamases

MLST	Multilocus Sequence Typing
Mg ²⁺	Magnesium ions
MIC	Minimum Inhibitory Concentration
mg/L	milligrams/liter
MENA	Middle East and North Africa
MRSA	methicillin-resistant <i>Staphylococcus aureus</i>
MALDI-TOF	Matrix-assisted Laser Desorption/Ionisation Time-of-flight
NDM	New-Delhi Metallo-β-lactamase
ND4BB	Innovative Medicines Initiatives: New Drugs for Bad Bugs
NRC	National References Centers
NDARO	National Database of Antibiotic Resistant Organisms
OXA	Oxacillinase
OIE	World Organization for Animal Health
ONERBA	French national observatory for epidemiology of bacterial resistance to antibiotics
PBP	Penicillin-binding Proteins
Pb	Pairs Baise
PDR	Pandrug-resistant
pEtN	phosphoethanolamine transferase
PFGE	Pulsed-Field Gel Electrophoresis
PCR	Polymerase Chain Reaction
PBRT	PCR-Based Replicon Typing
RESAPATH	Surveillance Network for Antimicrobial Resistance in Pathogenic bacteria of Animal origin
RI	Resistant Integrons
SHV	Sulphydryle variable réactif
SPM	Sao Paulo metallo- β -lactamase
SME	Serratia marcescens enzymes
ST/pST	Sequence Type/plasmid Sequence Type
SNP	Single Nucleotide Polymorphism
<i>sul2</i>	Sulfonamide resistance genes
<i>tet(A)</i>	Tetracycline resistant genes
Tn	Transposons
TE	Transposable Elements

Tase	Transposases
TEM	Temoneira
TC	Transconjugant
UN	United Nations
UK	United Kingdom
UTI	Urinary Tract Infections
VIM	Verona integron-encoded metallo- β -lactamase
WHO	World Health Organization
WGS	Whole Genome Sequencing
XDR	Extensively drug-resistant

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Résumé

Globalement, il est désormais admis que le taux des bacilles à Gram-Négatif sécréteurs de β -lactamases, des carbapénèmases et/ou résistantes à la colistine ne cesse de croître pour atteindre des prévalences inquiétantes dans tous les continents. En parallèle, la quasi-totalité des classes d'antibiotiques utilisées chez l'Homme sont également utilisées chez les animaux destinés à l'alimentation. Enfin, les deux compartiments que sont l'Homme et le monde animal ne sont pas étanches, d'où la nécessité d'appréhender la résistance aux antibiotiques selon une approche « One Health ». A ce jour, les données existant au Liban sur la prévalence des gènes de résistance dans le secteur humain comme des filières animales et alimentaires sont préliminaires. De plus, les investigations dans la communauté et la dynamique de la transmission des bactéries multi résistantes restent inconnues. Ainsi, l'objectif du travail de thèse est de décrire l'épidémiologie des Enterobacterales productrices de BLSE / AmpC, carbapénèmases et résistantes à la colistine à l'interface de l'Homme et de l'animal au Liban, et d'élucider par la suite le dynamique de transmission de la résistance. Pour cela, nous avons établi une première étude portant sur la détection et la caractérisation des gènes de résistance à la colistine dans des souches cliniques humaines. En parallèle, une autre étude est menée chez les volailles pour décrire la prévalence des gènes de résistance, aussi bien que la population bactérienne véhiculant ces déterminants dans le secteur agroalimentaire au Liban. Une troisième étude faite vise à établir les états des lieux des déterminants génétiques des résistances et du dynamisme d'acquisition, et de persistance de ces dernières chez l'Homme dans la communauté. Le typage des bactéries par PFGE, par MLST, les Southern Blots ainsi que le séquençage du génome entier ont été utilisés dans les différentes études. Nos résultats indiquent que les élevages de poulets sont de puissants réservoirs de gènes de résistance BLSE et *mcr-1* au Liban. En parallèle, nous décrivons pour la première fois la dissémination des gènes *mcr-1* chez l'Homme dans le secteur clinique aussi bien que chez des porteurs sains dans la communauté Libanaise. Nos études soulignent une large dissémination des *Escherichia coli* multirésistantes, la présence de différents carbapénèmases ainsi qu'un fort dynamisme de perte et d'acquisition d'*E. coli* productrices des BLSE entre les secteurs, soulignant ainsi une urgence de recours à des stratégies efficaces de surveillance de l'antibiorésistance au niveau national.

Mots clés: Antibiorésistance, *mcr-1*, BLSE, carbapénèmases, poulets, communauté, One Health, Liban.

Abstract

Globally, it is relevant that the prevalence of Gram-negative bacilli producing β -lactamases, carbapenemases, and/or are colistin-resistant have been highly increasing all over the five continents threatening global public health. In parallel, the majority of antibiotics prescribed in human medicine have been also administrated in veterinary medicine and in food producing animal husbandry. However, all the sectors of the ecosystem, including Humans and animals are correlated, so that the antimicrobial resistance (AMR) issue should be addressed via a One Health approach. Nowadays, data on the prevalence of resistance genes and the occurrence of resistance determinants in humans as well as in animals and in the food sector in Lebanon are scarce. Moreover, the prevalence of AMR, the population structure of resistant isolates and the transmission of resistance determinants between sectors and in the Lebanese community remain unknown. The aim of this thesis was thus to further describe the epidemiology of ESBL/AmpC-, carbapenemase- producing and colistin resistant Enterobacteriales in the interface between humans and animals, and to fill up the gap in our knowledge on the transmission of resistances across sectors in Lebanon. Accordingly, we performed a first study on humans in order to detect and characterize colistin resistance genes in the clinical sector. In parallel, we conducted a study in the poultry sector in order to investigate the prevalence of resistance genes and the population structure of resistant isolates in food producing animals. Additionally, we conducted a third study on humans to explore the presence, the spread and the dynamic of acquisition, persistence and carriage of resistance determinants in the Lebanese community. Isolates have been characterized using PFGE, MLST, Southern blots and whole genome sequencing in all studies. Our data revealed that chickens are potent reservoirs of ESBL/AmpC and *mcr-1* genes in Lebanon. Hence, we described for the first time the presence of the *mcr-1* gene in humans from hospitals and the food sector. Results underlined also the huge diffusion of MDR *Escherichia coli*, the presence of different carbapenemases as well as the high genetic diversity and the important dynamic of acquisition and loss of ESBL-producing *E. coli* in Lebanon. These findings are alarming highlighting the urgent need to reevaluate effective antimicrobial stewardship in order to limit the emergence of AMR in Lebanon.

Keywords: AMR, *mcr-1*, ESBL, carbapenemases, poultry, community, One Health, Lebanon.

Chapter1 : General introduction

1. Antibiotics and antimicrobial resistance : From the 1928 serendipity to a potential world without antibiotics

1.1. Antibiotics : discovery, action and use

1.1.1. *Antibiotics discovery and timeline*

Microbes have survived on Earth for several billion years. Some of them, particularly many bacteria, led to serious and fatal infections worldwide, from tuberculosis to pneumonia to whooping cough. Nevertheless, it is back in 1928 that microbes have made the phenomenal contribution to the health and well-being of people [3]. By serendipity, Sir Alexander Fleming began the microbial drug era when he discovered that a compound produced by a mold was able to kill the bacteria [3]. The mold, identified as *Penicillium notatum*, produced an active agent, named penicillin, that killed staphylococcal colonies. The word “antibiotic” was first used in 1940 by Selman Waksman to describes any molecule made by a microbe that kill or inhibit the growth of other microorganisms [4, 5]. In 1941, this wonder drug was isolated and used as a potent antibacterial compound during World War II, and rapidly found practical application in combating infectious diseases, including endemic and almost incurable human infections such as syphilis, gastrointestinal infections and contagious childhood illnesses [6].

Soon after penicillin, naturally occurring antibiotics were isolated, such as chloramphenicol and streptomycin [7]. Later, different generations of new antibacterial agents have been produced by chemical modifications of existing antibiotics, either synthetically or semi-synthetically, with broad spectrum activity and improved efficacy (**Figure 1**) [7]. In the timeline of antibiotics, this period is called the “Golden age” of antimicrobial therapy [7]. It started with the discovery of penicillin in 1928 and lasted for several decades, until all antibiotics classes known to date were discovered, such as β -lactams, sulfonamides, aminoglycosides, tetracyclines, chloramphenicol, macrolides, glycopeptides, ansamycins, quinolones, streptogramins, oxazolidinones and lipopeptides. Almost all bacterial infections were treatable with these drugs and become no longer fatal, opening a new era in medicine where men felt invulnerable against bacterial diseases (**Figure 1**) [7].

This invulnerability was unfortunately only an illusion. Back in 1945, Alexander Fleming, warned in an interview with The New York Times, that misuse of the drug could result in selection for resistant bacteria: “the greatest possibility of evil in self-medication [with

penicillin] is the use of too-small doses, so that, instead of clearing up the infection, the microbes are educated to resist penicillin and a host of penicillin-fast organisms is bred out which can be passed on to other individuals and perhaps from there to others until they reach someone who gets a septicemia or a pneumonia which penicillin cannot save” [8]. Fleming had foreseen that antibiotic treatments have two consequences: in the short-term, antibiotics combat bacterial growth and cure bacterial infections; but in the long-term, the use of antibiotics accelerates the evolution of resistance. Eighty years later, bacteria that are resistant to one, two or even to all available antibiotics, are reported worldwide, in particular, in Enterobacterales, *Acinetobacter baumannii*, and *Pseudomonas aeruginosa* [9].

During the golden age of antibiotics, the solution to the problem has depended primarily on the development of novel antimicrobial agents. Conversely, the number of new classes of antimicrobial agents being developed has decreased dramatically in recent years, and the nightmare of an “antibiotics apocalypse” remains a reality (**Figure 1**) [10].

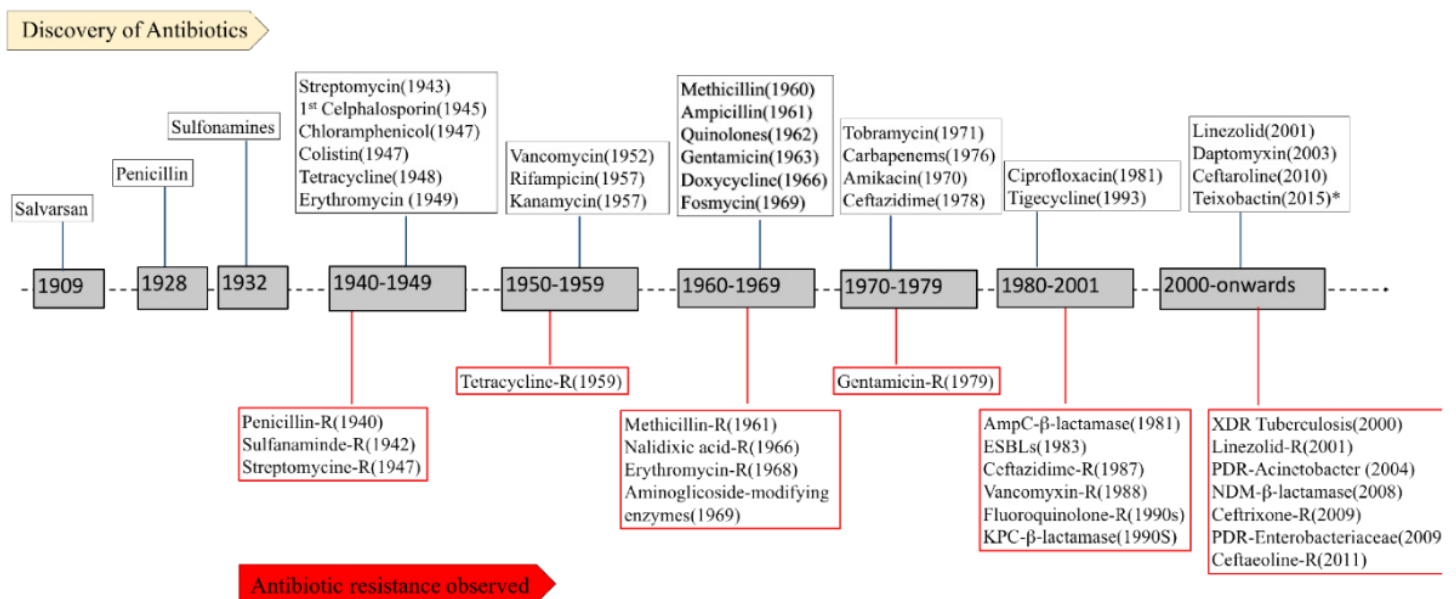


Figure 1: Key dates in the history of antibiotic discovery and subsequent resistance development. -R: resistance; XDR: extended-drug resistance; PDR: Pan-drug resistance. *Teixobactin is not in clinical use yet. (Updated from Bbosa *et al.* 2014 and, Ventola *et al.* 2015).

1.1.2. Antibiotics targets and modes of action

Antibiotics have various modes of action on the bacterial cell, by which they antagonize the multiplication and growth (bacteriostatic activity) and contribute to the destruction of bacteria (bactericidal activity) [11]. Antibiotic targets must be specific to prokaryotic cells, so that the use of these drugs will not be toxic for the human body. Bacterial cells present several potential targets, such as ribosomes, cell membranes and cell wall, bacterial cellular metabolism, nucleic acids and bacterial cellular enzymes (**Figure 2**) [12].

Consequently, antibiotics inhibit and kill the bacteria through different mechanisms, including i) inhibition of nucleic acid synthesis, both the DNA synthesis and RNA synthesis, ii) inhibition of cell wall synthesis, iii) inhibition of protein synthesis, both 50S and 30S, iv) disruption of cell-membrane function, and v) action as antimetabolites (**Figure 2**) [13].

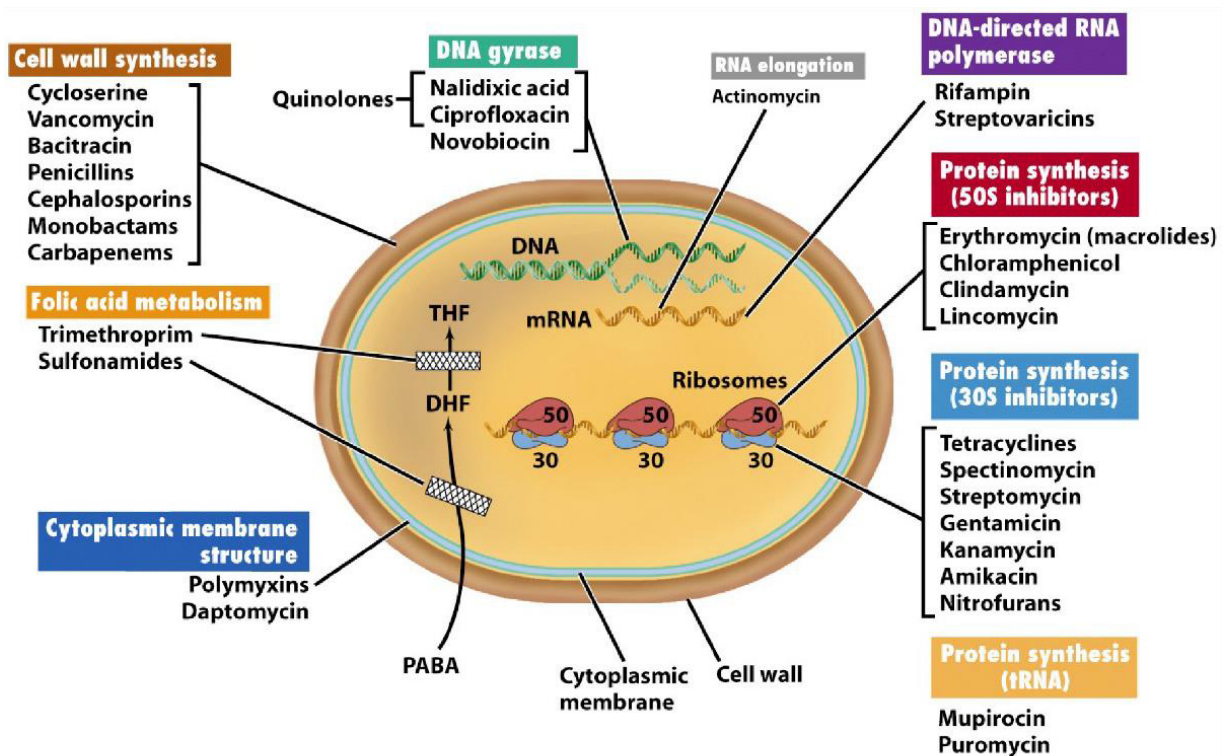


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Figure 2: Classes of antibiotics and their mode of actions (Adapted from Bbosa *et al.* 2014).

1.1.3. *Antibiotics use*

Antibiotics have been used for decades to prevent infectious disease in humans. Alongside, they have been used across other sectors such as animal husbandry and agriculture [14]. Those which are used in veterinary and agriculture are often used for several purposes: therapeutical (for therapy), metaphylactical (for disease, prophylaxis and therapy), prophylactical (for disease prevention) and subtherapeutical (for growth promotion, feed efficiency and disease prophylaxis) [14].

In 1950s the use of antibiotics in livestock, as growth promoters for example, has been increasing by 10- to 20-fold, and since then, it has become common practice worldwide [15]. Hence, to improve feed efficiencies and growth in farm animals, antibiotics are administrated in water, food or parenterally to animals which intend to increase harmful drug residues in meat, milk, and eggs products [14]. At subtherapeutic levels, antibiotics affect host animals immune system [16]. Antibiotics used as growth promoters also shift the balance among gut microbial species and modulate the metabolic activity of bacteria leading to weight-gain benefits. They suppress certain sensitive bacteria that compete with host animals for nutrients and inhibit the effects of subclinical disease on growth [17]. At metaphylactic levels, antimicrobial drugs are administrated in mass-medication procedures, especially in food animal production, such as in poultry, where, treating entire groups of animals is more efficient than individual medication [14].

However, the use of antibiotics as growth promoters was banned in EU since 1999 [18]. Consequently, animal welfare has suffered. In poultry for example, it is reported in both France and Denmark that clostridial necrotic enteritis has emerged and require therapy after withdrawn of bacitracin. Hence, the ban has tend to decrease the weight gain, increase the mortality and infections rate in relation to salmonellae, campylobacters and zoonotic strains of *Escherichia coli* in husbandry. In order to encounter those effects, the use of therapeutic antibiotics has increased in veterinary and agriculture [18].

Antibiotic molecules used in the clinical sector or in agriculture are mostly identical, but they are used in different proportions [2]. According to a comparative study conducted by Moulin *et al.* in 2008, tetracyclines, sulphonamides, trimethoprim, β -lactams and aminoglycosides presented more than 80% of the antimicrobials sold in veterinary medicine in

France between 1999 and 2005 [2]. Conversely, during the same period, β -lactams constituted more than 50% of the total consumption in human medicine [2]. Thus, as shown in **Figure 3**, some antibiotics were used almost exclusively either in animals such as apramycin (aminoglycosides), chloramphenicols, polymyxins and tetracyclines, or in humans such as nitrofurans derivatives, carbapenems and other antimicrobials [2].

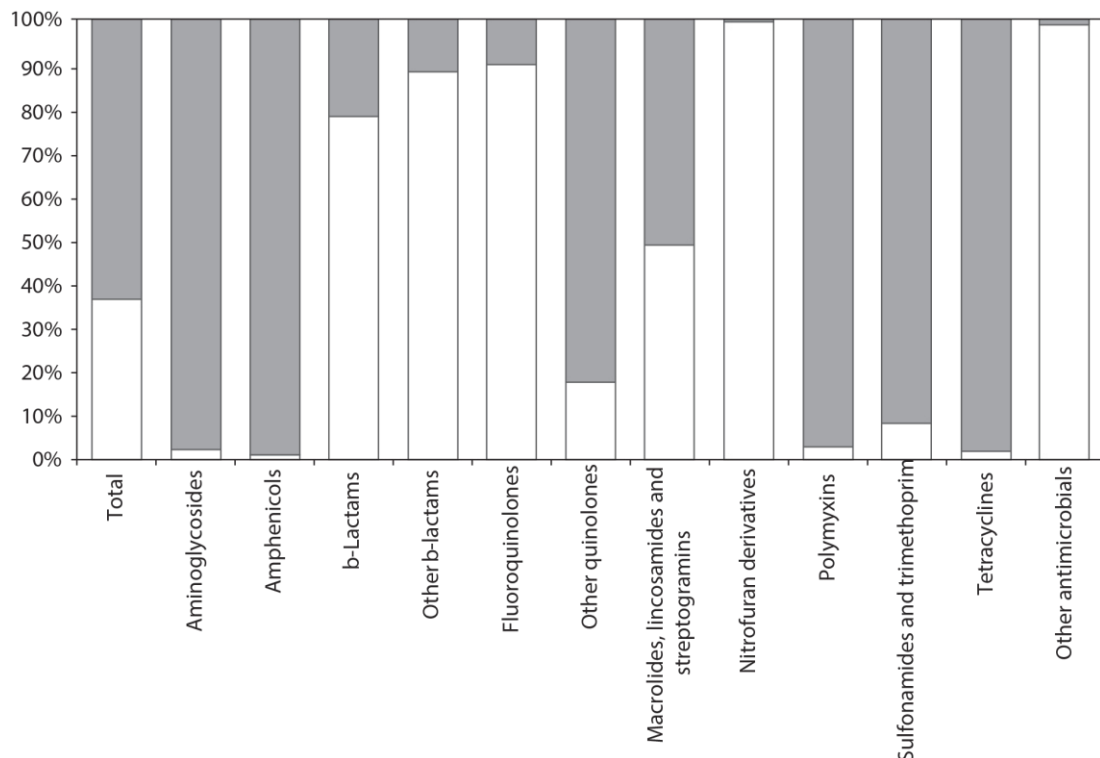


Figure 3: A comparison between total sales recorded in France in 2005 of antimicrobials approved for human (in white) and animal (in grey) usage (Adapted from Moulin *et al.*) [2].

However, the fact that antibiotics are heavily demanded across many sectors for decades, especially in developing countries, has allowed for less expensive and off-label uses of drugs. Thus, almost all the antibiotics can be bought in the developing world without medical prescription, due to their availability over the counter [19].

1.2. Antimicrobial resistance: from phenotype to genotype

1.2.1. *Definition of resistance*

Antibiotics have been named miracle drugs for decades. But sixty years of use and misuse of these compounds in medical and veterinary practice, communities, farmers, agriculture, food industries and self-medication, has increased frequencies of resistance for most combinations of antibiotics and bacteria [20]. By definition, antibiotic resistance occurs when a drug loses its cytogenic and cytotoxic activity on bacteria, meaning that bacteria want, survive and multiply in the presence of the drug whose activity is lost [19].

Resistance can be either intrinsic or acquired. Intrinsic (or natural) resistance is independent of antibiotic exposure, shared universally within a bacterial species or genus, and not related to horizontal gene transfer [21, 22]. One common example of intrinsic resistance is the reduction of the outer membrane permeability (most specifically the lipopolysaccharide, LPS, in gram negative bacteria- GNB) and the natural activity of efflux pumps [23, 24]. Acquired resistance is conferred either by modification of the existing bacterial genetic material or by acquisition of exogenous genetic material [23, 24]. Acquired resistance determinants can be further transferred between individual bacteria of the same species (from *Escherichia coli* (*E.coli*) to *E. coli*) or even between different species (from *E. coli* to *Klebsiella pneumoniae*) [23, 24].

Two definitions of “resistance” coexist. Epidemiological resistance encompass all bacteria that have derived from the wild-type fully susceptible population, whatever the level of resistance conferred. On the contrary, clinical resistance comprise bacteria that have a high probability to survive to the appropriate antibiotic treatment. This clinical definition of resistance is the one that will be used in the experimental part of this work.

Numerous studies have shown that emergence of resistance appears shortly after the introduction of a new antibiotic, and resistance to virtually all available antimicrobial molecules have been developed over the past [25]. In the 1970s, the major problem was the multidrug resistance of GNB, but later in the 1980s the Gram-positive bacteria became important, including methicillin- resistant staphylococci, penicillin-resistant pneumococci and vancomycin-resistant enterococci. Today, the emergence of multidrug- and pandrug-resistant

pathogens makes certain bacterial infections almost untreatable with antibiotics: we are now in the post-golden age of antibiotics and the “safe heaven” is over [26].

1.2.2. Phenotypical and molecular aspects of resistance

Several bacterial mechanisms for antibiotic resistance have evolved as shown in (Figure 4) and they implicate i) inactivation of the drug, ii) limiting drug uptake from the cell, or iii) modifying the target site [23, 24]. These mechanisms can be native to the microorganisms, or acquired from other microorganisms. [23, 24].

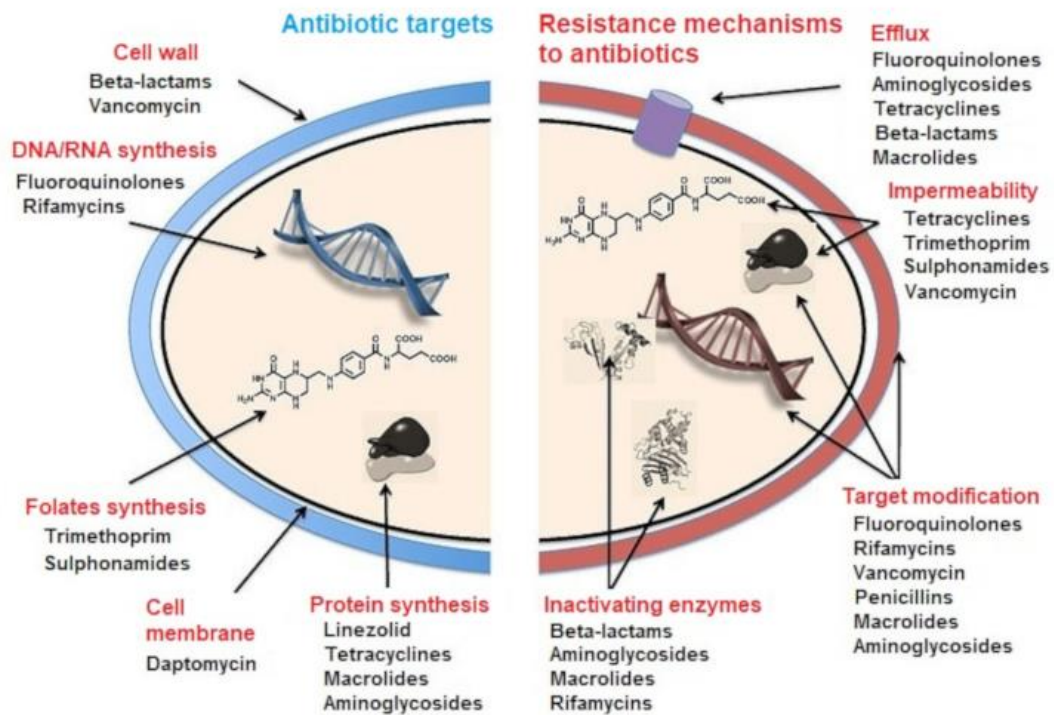


Figure 4: An overview on bacterial targets of antibiotics and primary resistance mechanisms (Adapted from <https://www.encyclopedie environnement.org/en/health/antibiotics-antibiotic-resistance-and-environment/>).

i) Drug inactivation

Bacteria can inactivate drugs by degradation of the drug, or by transfer of a chemical group to the drug. The best-known drug hydrolyzing enzymes are β -lactamases (for details, see chapter 2.2.1). Tetracyclines can be also inactivated by hydrolyzation via the *tetX* gene [27, 28].

Other enzymes, the transferases, mediate drug inactivation by transfer of a chemical group to the drug. There are a large number of transferases that have been studied. Acetylation is known to be used against the aminoglycosides, streptogramins, chloramphenicol and fluoroquinolones, and are the most diversely used mechanism. Phosphorylation and adenylation are known to be used against the aminoglycosides (**Figure 4**) [28-31].

ii) Limitation of drug uptake

Modification of the drug uptake includes both impermeability and efflux phenomena. Hydrophilic molecules can access to GNB via the porin channels [29, 32]. Alteration of porin channels, either by a decrease in their number or by mutations modifying their selectivity, limits drug uptake and thus leads to impermeability [33]. Porin modification is a common carbapenem-resistance mechanism especially in *Enterobacter spp.* Alongside, high-level resistance can be caused by mutation that induced or overexpressed the transport channel of bacterial efflux pumps. Efflux pump promote the rid of toxic substances from bacterial cell and are implicated in the transport of numerous compounds. Modifications in GNB efflux pumps enhance drug uptake and can confers resistance to penicillins, macrolides, chloramphenicol, fluoroquinolones and tetracycline in GNB [29, 34].

iii) Modification of the target site

As shown in **Figure 4**, bacterial cell presents different antibiotic targets that may be modified by the bacteria to enable resistance to these drugs. One emblematic example of target modification is resistance to fluoroquinolones, which is mediated by point mutations in the DNA gyrase and topoisomerase genes (*gyrA* and *parC*). These mutations decrease or eliminate the ability of fluoroquinolones to bind to the target component [35]. β -lactam resistance can also be achieved via target modification. In the case of these molecules, resistance is due to alterations in the structure and/or number of penicillin-binding proteins (PBPs). PBPs are transpeptidases involved in the last step of peptidoglycan biosynthesis, which allow the correct construction of the bacterial cell wall. A change in the number of PBPs affects the amount of drug that can bind to that target. A change in structure (e.g. PBP2a in *S. aureus* by acquisition of the *mecA* gene) decreases the ability of the drug to bind, or totally inhibit drug binding [36]. Resistance to several other molecules involves target modification, including resistance to glycopeptides and lipoptides (modification of the cell wall) or aminoglycosides (inhibition of the 30S ribosomal subunit).

1.3. Resistance transmission pathways

Acquired resistance can be caused by chromosomal mutations or genes acquired on mobile genetic elements (MGE). Resistance mediated by chromosomal mutations can only be transferred from the mother cell to its daughter cells in a vertical manner. On the contrary, MGE can promote horizontal gene transfer (HGT), which can involve intra- or inter-species transmission. The spread of antibiotic resistance among bacteria is mediated by (i) plasmids, (ii) transposons or (iii) integrons carrying resistance genes of resistance through transduction, conjugation or transformation (**Table 1**).

Table 1: Characteristics of different elements involved in resistance gene spread [12, 21, 22]

Element	Characteristic	Role in spread of resistance genes
Self-transmissible plasmid	Circular, autonomously replicating element; carries genes needed for conjugal DNA transfer	Transfer of resistance genes; mobilization of other elements that carry resistance genes
Conjugative transposon	Integrated elements that can excise to form a non-replicating circular transfer intermediate; carries genes needed for conjugal DNA transfer	Same as self-transmissible plasmid
Mobilizable plasmid	Circular, autonomously replicating element; carries gene that allows it to use conjugal apparatus provided by a self-transmissible plasmid	Transfer of resistance genes
Transposon	Can move from one DNA segment to another within the same cell	Can carry resistance genes from chromosome to plasmid or vice versa
Gene cassette	Circular, nonreplicating DNA segments containing only open reading frames; integrates into integrons	Carry resistance genes
Integron	Integrated DNA segment that contains an integrase, a promoter, and an integration site for gene cassettes	Forms clusters of resistance genes, all under the control of the integron promoter

1.3.1. *Plasmids*

Plasmid-mediated transmission of resistance genes is the most common route for acquisition of exogenous genetic material [23, 24]. As described in **(Table 1)**, plasmids are extrachromosomal circular DNA molecules capable of autonomous replication, and can mediate resistance to several classes of antimicrobials, including β -lactams, tetracyclines, aminoglycosides, sulfonamides, chloramphenicol, trimethoprim, colistin, macrolides and quinolones [37, 38]. Plasmids mobilize resistance genes and promote the horizontal transfer of resistance determinants among bacteria of different species, genera and kingdoms, depending on their narrow or broad host range, conjugative properties and efficiency of conjugation [39].

Plasmids can be classified in a groups called Incompatibility groups (Inc) [40]. Efficient conjugative plasmids (i.e. IncI1 and IncX) are often associated with the spread of epidemic resistances such as extended-spectrum cephalosporin (ESC), carbapenem and colistin-resistances mediated by *bla*_{CTX-M-1}, *bla*_{CTX-M-15}, *bla*_{OXA-48}, *bla*_{NDM-1} or *mcr-1* genes [37]. The prevalence of many resistant determinants located on other plasmids (i.e. IncB/O, IncK, IncN, IncT) can increase on the local level due to selective pressures when corresponding antibiotics are used [37]. Moreover, a same plasmid can confer resistance to different classes of antimicrobial drugs, when carrying multiple physically linked genetic determinants, such as ESBL- and *mcr-1*- encoding genes [41]. This increases the chances of these resistance genes to be maintained and transmitted in case of treatment with one or the other antibiotic. This also favors the emergence of highly multi-drug resistant isolates.

1.3.2. *Transposons*

Transposons (Tns) or transposable elements (TEs) are a group of mobile genetic elements that can jump into different places of the genome. The existence of the enzyme in Tns, named transposase (Tase) causes their transposition from the DNA molecule to other places on the same DNA or other DNA molecules [42]. It is by this transposition phenomenon that Tns usually stay in the genome for a long time and cause mutations. Tns, which contain antibiotic resistance genes, cause the resistance transmission among bacteria. TEs can be divided into different categories including insertion sequence (IS), composite Tns and non-composite Tns (Tn3 family) [43].

Composite Tns are flanked by IS [44]. Drug resistance genes are often carried by composite Tns and their transmission among bacteria is a serious challenge in the treatment of infectious diseases [45]. The most important composite Tns in bacterial antibiotic resistance, especially *E. coli*, are Tn903 (carrying resistances to kanamycin and neomycin), Tn9 (carrying resistances to chloramphenicol), Tn10 (carrying resistances to tetracycline) and Tn5 (carrying resistances to neomycin/kanamycin, bleomycin, and streptomycin) [46].

IS are the smallest genetic elements (length less than 2500 bp) and the simplest TEs that cause mutation and create different phenotypes by transmitting to the bacterial genome. For example, IS256 that exists in the composite Tns of Tn4001 is responsible for resistance to aminoglycosides [47].

1.3.3. *Integrans*

As shown in (Table 1), Integrans are integrated DNA segments containing an integrase, a promoter, and an integration site for gene cassettes (GCs). They are bacterial genetic elements able to promote expression and acquisition of genes embedded within GCs [48]. GCs are circular, non-replicating DNA segments containing only open reading frames and that can integrate into integrans. [49]. Studies describes two major groups of integrans: chromosomal integrans (CIs), and mobile integrans (MIs).

CIs are located on the chromosome of bacterial species, while MIs are not self-transposable elements but are located on mobile genetic elements such as transposons and plasmids. MIs contain a limited number of GCs encoding antibiotic-resistance determinants [50]. MIs are also called resistant integrans (RIs) or multidrug resistance integrans (MRIs). Most MIs have been described in an important range of GNB [49].

Five classes of MIs have been described, among which classes 1 and 2 are the most commonly studied ones [51]. Class 1 MIs present a broad distribution among GNB of clinical interest and are the most reported ones in animals and humans. They are mainly associated with functional and non-functional transposons derived from Tn402. The non-functional type is described in clinical isolates [52]. These structures are frequently embedded in plasmids or larger transposons, such as those of the Tn3 family (Tn21, Tn1696) allowing their rapid dissemination [53]. Class 2 MIs are mainly composed of the GC *sat2* (involved in the resistance to streptothricin), *dfrA1* (involved in the resistance to trimethoprim), and *aadA1* (involved in the resistance to streptomycin and spectinomycin) [54].

1.4. The “One Health” concept applied to AMR

The One Health concept is defined as “ a collaborative, multi-sectoral, and trans-disciplinary approach, working at local, regional, national, and global levels, to achieve optimal health and well-being outcomes recognizing the interconnections between people, animals, plants and their shared environment” (https://www.onehealthcommission.org/en/why_one_health/what_is_one_health/). AMR fits particularly well in this concept (**Figure 5**).

Resistance genes emerged on Earth far before the antibiotic era by providing a selective advantage to resistant bacteria in their conquest of ecological niches [55]. However, in the last century, antibiotic resistance exploded and disseminated worldwide as a consequence of the partially uncontrolled use of antibiotics in both human and veterinary medicine. AMR has now percolated in all sectors, including in the most pristine environments, so that scientific and institutional actions to limit the burden of resistance have now to be thought in a One Health context.

All the sectors of the ecosystem are connected and antibiotics enter the environment in a complex vicious cycle (**Figure 5**) [56, 57]. Antimicrobials misuse and abuse in multiple sectors are one of the major drivers of AMR leading to the creation of an important reservoir of resistant bacteria and resistance genes that are going back to the environment through fecal contamination, wastewater treatment plants being renowned hotspots (**Figure 5**). Thus, inadequate treatment of residential, industrial and farm waste further expand the resistome in the environment [58]. Contamination of water, soil, wildlife, and many other ecological niches arose through resistance dissemination from farm and domestic animals as well as from aquaculture sites, due to pollution by sewage, and manure runoff from farms and direct dispersion in water (**Figure 5**) [9, 56]. The occurrence of bacterial human pathogens in the environment increases the risk of emergence of new pathogens consecutive to the transfer of new resistance genes coming from environmental bacteria. This already occurred in the past, with CTX-M enzymes originating from *Kluyvera* spp. and the *mcr-1* gene most probably originating from *Moraxella* spp. [59, 60].

AMR remains one of the biggest ecological threats to public health that involve complex interactions of microbial populations affecting the health of animals, humans and the environment (**Figure 5**). An urgent and heightened awareness has emerged of the need to

address health issues through health management at the interfaces of all sectors to attain optimal health for humans, wildlife, domestic animals, plants and environment through multisector and coordinated approaches [61, 62].

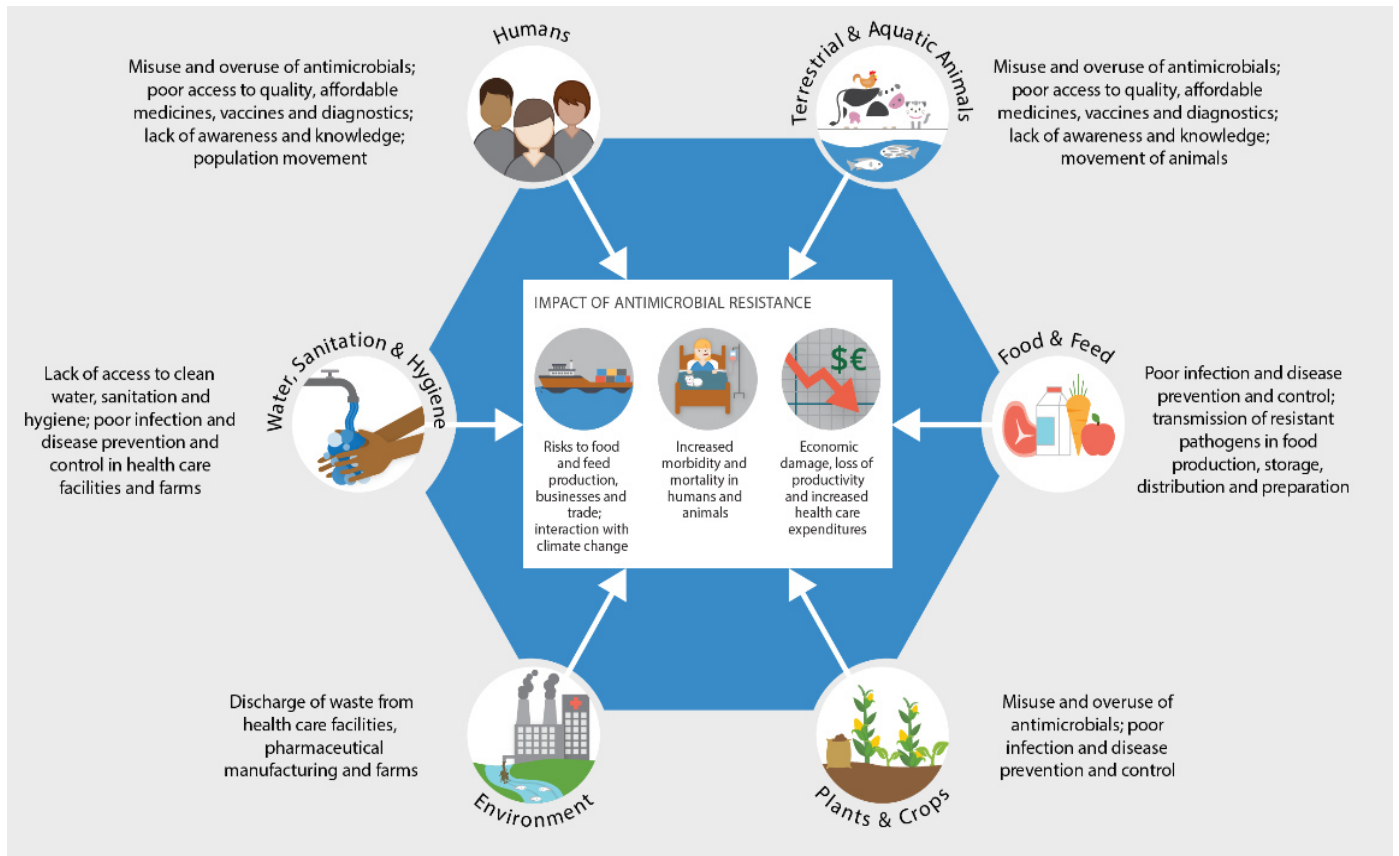


Figure 5: A One Health response to address the drivers and impact of antimicrobial resistance (adapted from the IACG 2019 final summary; https://www.who.int/antimicrobial-resistance/interagency-coordination-group/IACG_final_summary_EN.pdf?ua=1)

1.5. Institutional actions to limit the burden of antibiotic resistance

1.5.1. At an international level

Global, multinational and national frameworks for a One Health approach focuses on comprehensive action plans to address AMR crisis [61, 63-70]. The leading global plan embracing the One Health approach to address AMR is the WHO Global Action Plan. It encounter different major axes regarding the various dimensions of the One Health approach and the different drivers of AMR (**Figure 5**), and calls on member countries to do the same

when developing their own action plans [61, 71]. In this contest, a manual has been developed by WHO, in collaboration with the Food and Agriculture Organization of the United Nations (FAO) and the World Organization for Animal Health (OIE) to assist countries in preparing or refining their national action plans (<https://www.who.int/antimicrobial-resistance/national-action-plans/en/>).

Furthermore, the United Nations High-Level Meeting on AMR in 2016 has highlighted the urgent need to combat AMR at a global level. Consequently, the Interagency Coordination Group (IACG) on AMR directed by the Secretary-General of the United Nations and the WHO has been created where the WHO provided the secretariat for the IACG with contributions from the FAO and OIE. Similarly, the EU collaborate various associations and organizations working in different sectors including the European Centre for Disease Prevention and Control (ECDC), the European Medicines Agency (EMA) and European Food Safety Authority (EFSA), to address AMR at the interfaces between animals and humans [72-74].

However, plans are collecting data and require an integrated analysis and interpretation, involving teams of sectoral experts who also possess intersectional knowledge and understanding and the collaborative skills to work with those from other sectors, including social scientists and behavior change experts [70]. **Table 2** summarized several international initiatives and partnerships addressing AMR to date [75].

At the most basic level, awareness should be improved on principles of basic hygiene in all sectors. It is essential for everyone to understand the need to follow the antibiotics and antimicrobials prescriber's instructions for treatment, and to have a basic appreciation of the risks to themselves and others, which is improved through effective communication, education, and training. Otherwise, the use of antimicrobials in human and veterinary medicines can be optimized through interventions taken to restrict, ban and monitoring antimicrobials utilization and mechanisms. While drug classification plays a judicious tool to tackle AMR in all sectors, WHO, OIE and other individual national organizations, has developed schemes to classify antimicrobials used in humans into categories such as critically important, highly important, and important, to guide risk management strategies to prevent and control AMR especially from food animal production.

Although, AMR reduces the effectiveness of antimicrobial therapies and increase the severity, incidence and cost of infections. Consequently, there is an urgent need to address the economic case for sustainable investment for all countries and increase investment in new medicines, diagnostic tools, vaccines, and other non-antimicrobial disease control strategies.

Table 2 : Examples of international initiatives and partnerships addressing AMR with their focus areas (adopted from Mattar *et al.* 2020) [75].

Name	Data and Surveillance	Policy & Advocacy	Partnership Development
The Joint Programming Initiative on AMR (JPIAMR)	x		
Innovative Medicines Initiatives: New Drugs for Bad Bugs (ND4BB)			x
The Global Antibiotic Research and Development Partnership (GARDP)	x	x	
Livestock Vaccine Innovation Fund (LVIF)			x
Innovative Veterinary Solutions for Antimicrobial Resistance (InnoVet-AMR)		x	x
Bill & Melinda Gates Foundation			x
Wellcome Trust	x	x	x
International Centre for Antimicrobial Resistance Solutions (ICARS)	x		x
The Fleming Fund	x		
Global Action Fund for Fungal Infections (GAFFI)	x	x	
TB Alliance		x	x
Drugs for Neglected Diseases Initiative (DNDi)	x		x
Foundation for Innovative Diagnostics (FIND)			x
Medicines for Malaria Venture (MMV)		x	x
Global Alliance for Livestock Veterinary Medicines (GALVmed)		x	x
Global AMR Innovation Fund (GAMRIF)	x		x
CGIAR AMR Hub		x	x
Antibiotic Research UK		x	
PEW Trust		x	
Innovative Medicines Initiatives: AMR Accelerator			x

1.5.2. Surveillance systems

Surveillance and research identify AMR problems and how to prevent them. Strengthen the knowledge and evidence base through targeted research is essential to demonstrate how

resistance develops and spreads within and between species of bacteria and ecological niches, including environmental bacteria and enteric commensals as well as pathogens. Isolates collected in these studies include appropriate bacteria from specimens collected in humans, animals, environmental settings such as hospitals, health care and community settings, veterinary clinics, farms, wildlife, soil and water. All countries have a vital interest in elaborating critical capacities, ensuring that data is used to orient responses, and supporting global-level surveillance within initiatives such as the WHO Advisory Group on Integrated Surveillance of Antimicrobial Resistance (AGISAR) and the WHO GLASS [76, 77].

Various institutional actions highlight the importance of a multisectoral One Health response to limit the burden of antibiotic resistance (**Table 3**), such as WHO Global Action Plan on AMR (WHO 2015), United States National Action Plan for combating antibiotic-resistant bacteria (White House 2015), Declaration from the 2016 high-level meeting on AMR at the United Nations General Assembly (OPGA/ WHO/FAO/OIE 2016), and the FAO/OIE/WHO Tripartite Collaboration (FAO/OIE/WHO 2017) [61, 63-70].

Table 3: Antimicrobial resistance surveillance systems (Adapted from Queenan *et al.* 2016, WHO 2014).

Surveillance system, country	Surveillance of resistant bacteria from:					Bacterial species included				
	Healthy animals	Diseased animals	Food	Healthy humans	Diseased humans	Salmonella	Campylobacter	Escherichia coli	Enterococci	Animal pathogens
CIPARS, Canada	X	X			X	X	X	X	X	X
DANMAP, Denmark	X	X	X	X	X	X	X	X	X	X
FINRES-Vet, Finland	X	X	X		X	X	X	X	X	X
GermVet, Germany		X				X		X	X	X
ITAVARM, Italy	X	X	X		X	X		X	X	X
JVARM, Japan			X			X	X	X	X	
NARMS, USA	X		X		X	X	X	X	X	X
NethMap/MARAN, The Netherlands	X	X			X	X	X	X	X	X
NORM/NORM-VET, Norway	X	X	X	X	X	X	X	X	X	X
ONERBA, France	X	X	X		X	X	X	X	X	X
SWEDRES/SVARM, Sweden	X	X	X	X	X	X	X	X	X	X

CIPARS, Canadian Integrated Program for Antimicrobial Resistance Surveillance; DANMAP, Danish Integrated Antimicrobial Resistance Monitoring; FINRES-Vet, Finnish Veterinary Antimicrobial Resistance Monitoring and Consumption of Antimicrobial Agents; GermVet, German National Antibiotic Resistance Monitoring System; ITAVARM, Italian Veterinary Antimicrobial Resistance Monitoring; JVARM, Japanese Veterinary Antimicrobial Resistance Monitoring System; MARAN, Monitoring of Antimicrobial Resistance and Antibiotic Usage in Animals in The Netherlands; ONERBA, Observatoire de l'Épidémiologie de la Résistance Bactérienne aux Antibiotiques; SVARM, Swedish Veterinary Antimicrobial Resistance Monitoring Programme.

In France for example, several action plans monitored by Ministry of Public Health and Ministry of Agriculture, address AMR in both human and veterinary medicines. [78]. One of these plans, the EcoAntibio1 2011-2016, aimed to thus reduce the exposure to antibiotics and improve the implementation of several measures to control the use of critical antibiotics and

lower its negative impacts across sectors. Due to positive outcomes established, a second plan EcoAntibio2 2017-2021 have been enrolled [79].

Therefore, exposure to antibiotics in animals and humans is estimated through the consumption of antibiotics in humans and animals by the French National Agency for Medicines and Health Products Safety (ANSM) and the French Agency for Veterinary Medicinal Products (ANMV) in the he French Agency for Food, Environmental and Occupational Health & Safety (ANSES), respectively. Data of AMR surveillance in the human sector are provided by hospitals and national references centers (NRCs) within the French national observatory for epidemiology of bacterial resistance to antibiotics (ONERBA) [80]. While in veterinary medicine, data are provided by the ANSES through NRCs and the French surveillance network for antimicrobial resistance in pathogenic bacteria of animal origin (RESAPATH) which is also a member of ONERBA [81].

In addition, infection control programs play major role in reducing the incidence of infection in health care settings, veterinary clinics and in most food animal industries, particularly for intensive production in the poultry and swine sectors. It is also important to implement measures to promote sanitation, hygiene and microbiological quality through appropriate treatments of environmental sources to reduce exposure to bacteria and human-human transmission of enteric susceptible and resistant bacteria.

1.5.3. At a national level: example of an AMR awareness campaign (Adopted from Al Omari et al.)

In Lebanon, numerous studies have investigated the epidemiology of certain types of resistance which seem to grow rapidly and uncontrollably [82, 83]. Behaviors at individual level play a major role in the spread of resistant pathogens. Therefore, an initiative was planned in the form of an awareness campaign of multiple events in diverse regions and settings, mainly to target vulnerable populations. The Lebanese Association for Development initiated the first Lebanese Antibiotic Awareness Week campaign, synchronized with the WHO's Antibiotic Awareness Week (13th -19th November 2017), to highlight the issue of AMR and the misuses that lead to its incredible spread in the community.

The campaign was delivered via interactive awareness lectures in various locations in Lebanon, a documentary featuring interviews from the streets of Tripoli to highlight the wide spread of misconceptions among the public, research questionnaires which aimed to measure the knowledge, practices and attitudes of the population regarding antibiotics, and posters and a Facebook page in order to raise global awareness.

In parallel, a quantitative, cross-sectional epidemiological study was conducted in the same period, between 13th and 19th November 2017. In total, 1187 surveys were completed by people from seven governorates: Akkar, Baalbek-Hermel, Bekaa, Beirut, Mount Lebanon, Nabatieh and North Lebanon (**Figure 6**). The investigation was conducted in accordance with the Declaration of Helsinki and national and institutional standards. (Detailed study is shown in **Annex**).

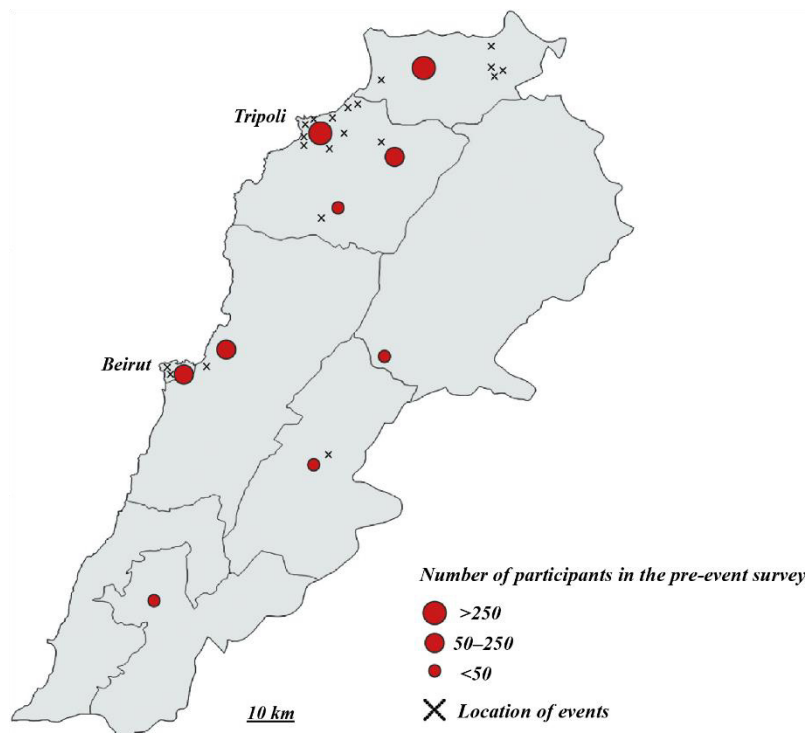


Figure 6: Geographic locations targeted in this study in Lebanon.

This study highlighted a poor understanding of the prudent and adequate use of antibiotics except in educated people. This confusion is probably attributed to the lack of proper communication by physicians prescribing antibiotics, and lack of awareness by schools and governmental agencies. Younger participants seem to be more aware of the issue, and this might be attributed to the fact that they are more exposed to social media platforms where information about antibiotics can be shared. Results of this study are consistent with other studies in Lebanon [84] and in nearby Middle Eastern countries such as Kuwait [85], Jordan [86] and Saudi Arabia [87], which also reported a significant lack of awareness about this global health issue.

This study has shown high public support regarding application of the recommendations and the behavioral changes needed. However, the main limitations remain those related to the healthcare system and socio-economic factors. In Lebanon, most people are of low socio economic status, with only 45.9% of the population covered by the governmental health insurance. In addition, there is a lack of primary healthcare facilities. Moreover, due to the ongoing Middle Eastern conflicts, huge numbers of immigrant populations, including Syrian and Palestinian refugees displaced in Lebanon, face dire levels of poverty and vulnerability, and represent an ideal environment for the development and dissemination of infectious diseases. Thus, a remarkable percentage of the community does not see a doctor to save money, but goes to the pharmacy where they receive antibiotics without prescription.

In conclusion, further actions need to be taken at a policy-based level. These actions include (i) extended awareness campaigns targeting the public with various means, (ii) a pressing movement to ensure more surveillance of pharmacists and doctors by the Lebanese Government, and (iii) the coordination between healthcare facilities and the Ministry of Public Health to further control the spread of resistance in hospital settings. In parallel, an urgent intervention is needed in the animal sector, which serves as a significant reservoir of resistant bacteria.

2. Antibiotic resistance in Enterobacterales: an old story in constant evolution

2.1. Enterobacterales: what to know about this family?

The order Enterobacterales includes 7 families: *Erwiniaceae*, *Pectobacteriaceae*, *Yersiniaceae*, *Hafniaceae*, *Morganellaceae*, *Budviciaceae* and *Enterobacteriaceae* [88]. Members of this order involve a diverse and large Gram-negative, non-spore-forming, facultative anaerobic, rod-shaped bacteria [89]. Enterbacterales have been found in different ecological niches. They inhabit water, soil, and can be found in association with living organisms such as insects, plants, animals and humans [90].

The *Enterobacteriaceae* family is considered one of the most taxonomically diverse bacterial family [88]. Many members of the order *Enterobacteriaceae* are normal inhabitants of the intestinal flora while others can be pathogens in animals and humans. This family includes *Escherichia* spp., *Klebsiella* spp., *Enterobacter* spp. or *Salmonella enterica*, but excludes *Proteus* spp., *Morganella* spp. and *Serratia* spp. which belongs respectively to the *Morganellaceae* and *Yersiniaceae* families [90]. *Enterobacteriaceae* are important causes of cystitis to pyelonephritis, peritonitis, meningitis, bloodstream infections, urinary tract infections (UTIs), hospital- and healthcare- associated pneumoniae, and various intra-abdominal infections. UTIs are frequently caused by *E. coli*. Additionally, pneumonia is caused by *Klebsiella* spp and *Enterobacter* spp and all GNB of the *Enterobacteriaceae* family are related to bloodstream infections, peritonitis, cholangitis, and other intra-abdominal infections [91]. On the other hand, gastroenteritis and, in some patients, invasive infection, are caused by *Salmonella* spp.

Members of the *Enterobacteriaceae* family are usually highly susceptible to all available antibiotics. Unfortunately, resistance to β -lactams, carbapenems and colistin sequentially emerged and disseminated worldwide among *Enterobacteriaceae*, causing a global threat on conventional antibiotic treatments. Today, multidrug-resistant (MDR) have spread in humans, animals and the environment, and extensively drug-resistant (XDR) and pandrug-resistant (PDR) bacteria are already emerging in humans in certain region of the world [9] (**Figure 7**).

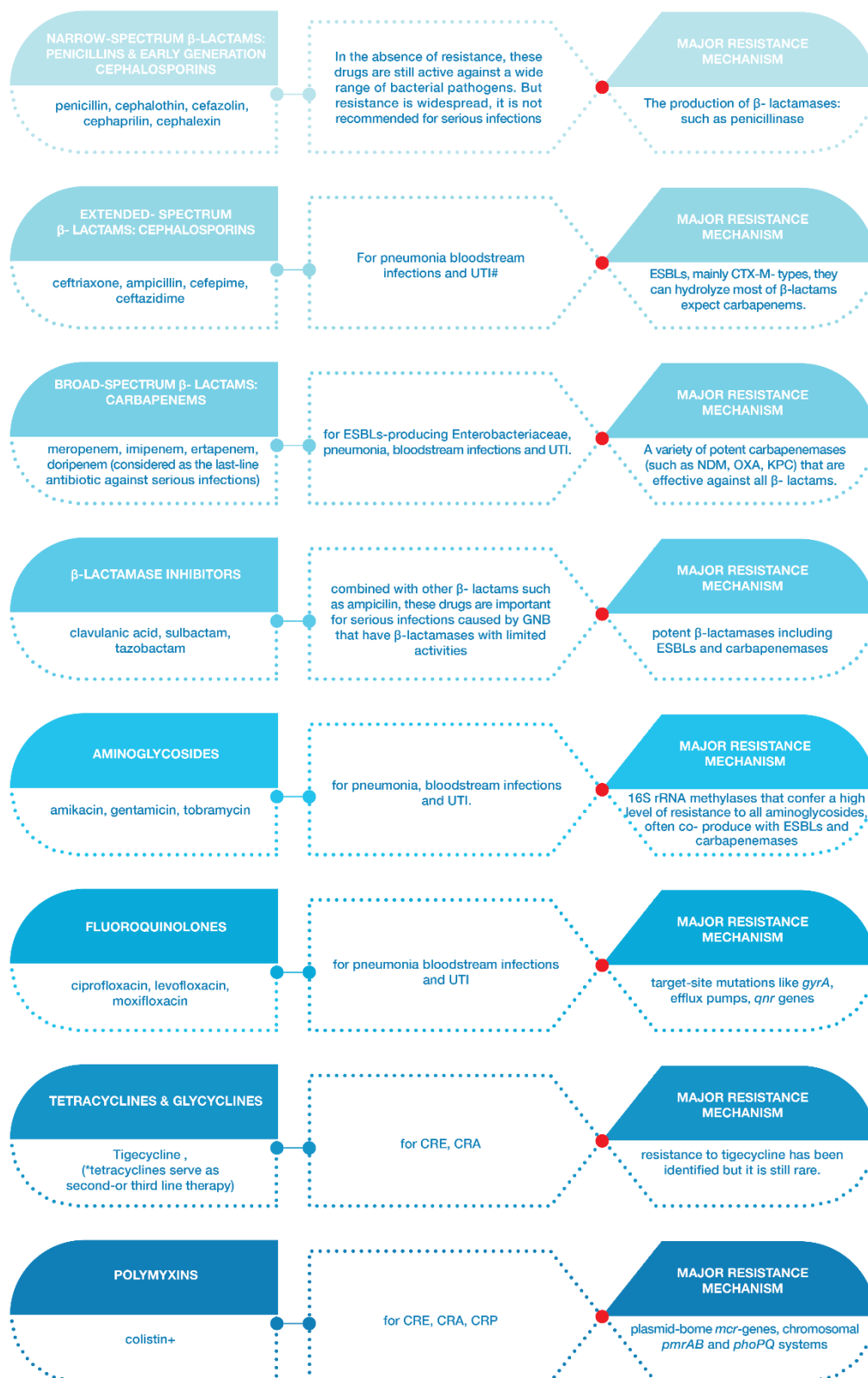


Figure 7: Currently available antibiotics for the treatment of GNB infections.

Footnotes: * tetracyclines are not first-line treatment options, but increasing resistance to other classes, it is considered a therapeutic option). + tigecycline and colistin are last-line antibiotics for the treatment of serious infections caused by CRE (Carbapenem-Resistant *Enterobacteriaceae*), CRA (Carbapenem-Resistant *A. baumannii*) and CRP (Carbapenem-Resistant *P. aeruginosa*). # UTI: urinary tract infection. (Adapted from Yang *et al.* 2018).

2.2. Resistance to broad-spectrum cephalosporins in Enterobacterales

2.2.1. Classification of β -lactamases

The largest family of antibiotic molecules prescribed to treat infections is called β -lactams. β -lactam family members share the presence of a β -lactam ring in their structure and belong to four classes: penicillins, cephalosporins, carbapenems, and monobactams, which all inhibit peptidoglycan biosynthesis. Resistance to these antibiotics is globally ubiquitous. It occurs through three mechanisms, namely i) drug hydrolysis by β -lactamase enzymes, ii) modification of the PBP target, usually by modifying the ability of the drug to bind to the PBP by alterations in existing PBPs or acquisition of other PBPs; iii) the presence of efflux pumps that can eject β -lactam drugs [92].

Production of β -lactamases is the most important resistance mechanism against penicillin and cephalosporin drugs, and the most common resistance mechanism used by GNB against β -lactam drugs. Originally called cephalosporinases and penicillinases, β -lactamases inactivate β -lactam drugs by hydrolyzing a specific site in the β -lactam ring structure, blocking the drug to bind to their target [93]. Structurally, β -lactamases are classified into four main categories (A, B, C, or D) (**Table 3**). On a functional level, they are classified in three groupings based on the substrate specificity: the cephalosporinases, the serine β -lactamases, and the metallo (zinc-dependent) β -lactamases [93, 94]. The β -lactamases genes confer different levels of resistance, ranging from amoxicillin (*bla*_{TEM}, *bla*_{SHV}) to last generation cephalosporins (*bla*_{ESBL}) and carbapenems (*bla*_{OXA-like}, *bla*_{NDM}). (**Table 3**) [94].

The first described β -lactamase was from *E. coli* and is chromosomally encoded by the *ampC* gene (named for ampicillin resistance). This gene is constitutively repressed in *E. coli* but mutations in its promoter may result in its overexpression, resulting in a resistance to ESC. On the other hand, this *ampC* gene is constitutively expressed in other species, such as *Enterobacter* spp., where promoter mutations lead to its hyper-expression. Specific AmpC-type genes are plasmid-encoded and can spread among *Enterobacteriaceae*. The most prevalent and widely disseminated ones are the CMY-2-like enzymes in *E. coli* and the inducible DHA-like β -lactamases in *K. pneumoniae* [95].

Table 4: Main classes of β -lactamases (Adapted from Bush *et al.* 2020) [2].ESBL, extended-spectrum β -lactamase; IRT, inhibitor-resistant TEM; MBL, metallo- β -lactamase.

Molecular class/subclass	Functional group/subgroup	Common name	Clinically relevant enzyme(s) or enzyme family(ies)
A	2a	Penicillinase	PC1/ <i>blaZ</i>
A	2b	Penicillinase	TEM-1, SHV-1
A	2be	ESBL	TEM-10, SHV-2, CTX-M-15
A	2br	IRT	TEM-30 (IRT-2)
A	2e	ESBL cephalosporinase	CepA
A	2f	Carbapenemase	KPC
A	2f	Carbapenemase	SME
B1, B3	3a	MBL, carbapenemase	IMP, NDM, VIM, SPM
B2	3b	MBL, carbapenemase	L1, CphA
C	1	Cephalosporinase	AmpC
D	2d	Oxacillinase	OXA-1
D	2df	Carbapenemase	OXA-23, OXA-48, OXA-181, OXA-232

2.2.2. CTX-M-type β -lactamases

Until 1991, all detected extended-spectrum β -lactamases (ESBLs) were derived from plasmid mediated SHV- or TEM- type enzymes [96, 97]. Soon after, CTX-Ms disseminated worldwide thanks to their epidemic success [98]. CTX-Ms were first identified in France, Germany and South America [99-101] in the human sector in the 1980s, and emerged twenty years later in animals and the environment. Transmission of ESBL-producing *Enterobacteriaceae* and ESBL genes between humans, animals and the environment has been exemplified, and proportions of ESBLs in these different sectors are now used as an indicator of the resistance burden [102].

The occurrence of *bla*_{CTX-M} genotypes is particularly well-characterized in *E. coli* [103, 104], where *bla*_{CTX-M-15} and *bla*_{CTX-M-14} are the predominant genotypes [98]. *bla*_{CTX-M-14} genes are dominant in South Korea, China, South-East Asia, Japan and Spain, while *bla*_{CTX-M-2} was dominant in South America (Figure 8). In parallel, *bla*_{CTX-M-1} is usually strongly associated with an animal or environmental origin and has maintained a significant dissemination in various European countries (Figure 8).

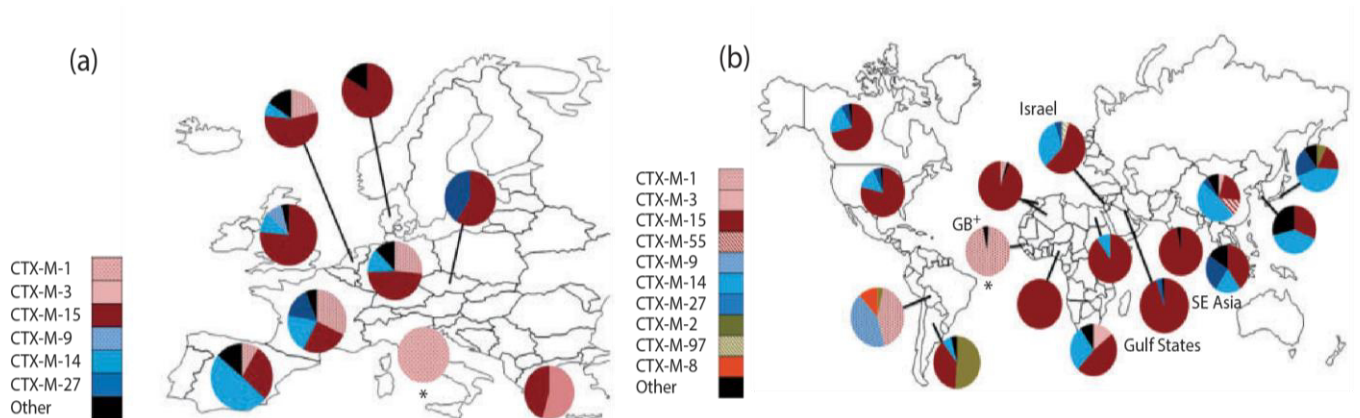


Figure 8: CTX-M trends between 2009 and 2017 in the world (a) and for Europe (b). Data include both hospital and community isolates. An asterisk indicates that, for these areas, only CTX-M grouping was done and these were not subdivided into genotypes (Adapted from Bevan *et al.* 2017) [1].

Even though chromosome-encoded CTX-Ms are more and more often reported, these enzymes are still largely carried by plasmids. Plasmids of the IncF family have been shown to be the main carriers of *bla*_{CTX-M-15} and *bla*_{CTX-M-14} [105-108], whereas, *bla*_{CTX-M-1} is often carried by IncII plasmids, among which the plasmid sequence type 3 (pST3) is predominant in the animal sector in Europe [109]. Although, the dissemination of CTX-Ms is enhanced by a wide diversity of genetic platforms via transposons, ISs and integrons [59, 110]. The vast majority of the *bla*_{CTX-M} genotypes have been associated with the insertion element ISEcp1 [110]. The dissemination of group 1 and group 9 *bla*_{CTX-M} variants have also been associated with the transposition element IS26 [110]. Alongside, *bla*_{CTX-M}-bearing plasmids can capture additional resistance determinants, including carbapenemase- and colistin-resistance genes [41, 111-114].

2.2.3. Importance of ESBLs

The emergence of ESBL-producing isolates is becoming a critical issue globally. The prevalence of β -lactamases increased among patients infected with bacterial pathogens treated heavily with β -lactams such as extra-intestinal infections caused by *Enterobacteriaceae*. Consequently, resistant *Enterobacteriaceae* have been selected and transmitted between patients and health care workers [115]. In addition, ESBL-producing isolates were not limited to hospital settings, but expanded at a One Health scale into nursing homes, extra-hospital settings and ultimately to the community [116-118]. Unlike methicillin-resistant *Staphylococcus aureus*, whose proportion is on a constant downward trend since the hygiene campaign and the use of hydro-alcoholic solutions in hospitals, the proportion of ESBL is on the upward trends because of its dynamic in the community that cannot be stopped by simple hygiene measures.

Studies reporting Multilocus Sequence Typing (MLST) showed that epidemics and outbreaks were due to resistant pathogens highly associated with various widely disseminated sequence types (STs). Extra-intestinal infections caused by *E. coli* have been related to the ST131 clone. Although, the dissemination of CTX-M-15 has been associated with the spread of the same clone *E. coli* ST131 that has been identified as a predominant lineage causing infections in both the hospital and community settings [119]. Studies indicated that 40 to 80% of ESBL-producing isolates were associated to this particular lineage [120].

Otherwise, ST117 and ST354 were commonly identified from ESBL-producing *E. coli* isolated from chicken meat as well as from clinical dominant clonal groups of CTX-M-producing non-ST131 isolates in France. Interestingly, these two STs were also identified as clonal ESBL-producing *E. coli* strains isolated from healthy people in the Paris area [121-125]. Other circulating clones were reported in healthcare settings in which ST131, ST69, ST405, ST410 and ST38 are dominating [126-129]. Another circulating clone reported in several sectors remain the ST1011 identified from ESBL-producing *E. coli* [130, 131].

With the dissemination of ESBLs, the choice of empirical antimicrobial therapy was affected, and carbapenems were one of the few remaining β -lactam antibiotics left for the treatment of Gram-negative infections, promoting, unfortunately, the global spread of carbapenem resistant isolates [132].

2.3. Resistance to carbapenems in Enterobacterales

2.3.1. Main classes of carbapenemases

Carbapenems are the last generation of antibiotics belonging to the β -lactamase family. They are considered as last-resort antibiotics mostly reserved to human treatment, and their use notably increased with the emergence of ESBL-positive pathogens [133]. Resistance to this class of molecules emerged soon after its first use in humans. Resistance can occur through limitation of the drug uptake through porin channel changes or/and by the production of hydrolyzing specific enzymes, but the main mechanism involves carbapenemases. These enzymes are found primarily in *Enterobacteriaceae*, but also in *Pseudomonas* spp. and *Acinetobacter* spp. [134].

Carbapenemases are β -lactamases with the ability of hydrolyzing carbapenems at a rate that is clinically relevant. They belong to structural class A, B, or D, or to the functional groups 2df, 2f or 3 (**Table 4**). Carbapenemase genes belonging to class A (KPC), class B (IMP, VIM, NDM) or class D (OXA-48, OXA-181) are mostly plasmid-located (**Table 4**).

i) Ambler class A carbapenemases

Class A carbapenemases includes the chromosomal Nmc-A/SME, and the plasmidic IMI/KPC and GES enzymes [135]. KPC enzymes remain the most clinically significant enzymes among class A β -lactamases. KPC-2 was firstly isolated from the clone ST258 of *K. pneumoniae* and has been extensively identified worldwide [136].

ii) Ambler class B metallo β -lactamases (MBL)

The most common types of MBLs in *Enterobacteriaceae* include NDM and VIM groups [137]. VIM-2 genes are among the most often reported MBLs in *P. aeruginosa*. NDM genes are more frequently found in *Enterobacteriaceae*, often in association with other resistance genes. For example, the *bla*_{NDM-1} gene has disseminated worldwide in *E. coli* clones co-producing the *bla*_{CTX-M-15} gene [138], while the *bla*_{NDM-5} gene harbored by an IncX3 plasmid represents another epidemic MBLs frequently associated with the *mcr-1* gene and identified in humans worldwide as well as in food products and in livestock [139-141].

iii) The Ambler class D carbapenemases

Class D includes OXA family of β -lactamases, some of which have a carbapenemase activity. The most prevalent OXA-type carbapenemase is OXA-48, with its related variants, OXA-162, OXA-181, OXA-244 or OXA-232 [142]. OXA-48-producing *Enterobacteriaceae* represent the most common described pathogens among nosocomial outbreaks. Hospital outbreaks and sporadic infections related to OXA-48-producing *E. coli*, *K. pneumoniae* or *Enterobacter cloacae* are worsened by the high capacity of this gene to spread thanks to its location on a small and highly transmissible IncL plasmid. The detection of OXA-48-like enzymes can be difficult unless those isolates possess associated mechanisms of resistance including ESBL production and/or permeability defects. Although, reports show that OXA-181 is extensively associated with the geographical parameter, enterobacterial isolates from India or from patients with a link to the Indian subcontinent have been found associated to the spread of these variants [143-145]. OXA-181 have also been identified on an IncX3 plasmid in pigs in Italy isolated from an *E. coli* carrying the *mcr-1* gene [146]. In recent years, an increase in OXA-244-producing isolates has been seen worldwide [147]. This OXA-48 variant was identified from clinical strains, community well as from river water and is silently disseminating thanks to relatively low carbapenem minimum inhibitory concentrations (MICs) that are very close to the breakpoints [148-155].

2.3.2. Emergence of carbapenem-resistant *Enterobacteriaceae*

Carbapenem-resistant *Enterobacteriaceae* (CRE) are considered as a cause of human severe infections worldwide. In Europe, the prevalence of carbapenem-resistant *K. pneumoniae* causing invasive infections increase extensively, strongly related to healthcare settings [156]. The most frequently described risk factors associated with the spread of CRE include geographical location, population density, hygiene, intensive care therapy and hospital admission in the preceding 6 months [157]. A study conducted by Shen *et al.* highlights the prevalence of CRE in healthy people from China [139]. The NDM-5 gene was the predominant determinant in these CRE and was carried by an IncX3 plasmid [139].

Furthermore, OXA-48, OXA-181, OXA-232, OXA-204, OXA-162, and OXA-244 represent – in that order, the major common OXA-48-like carbapenemases. Reports highlight the association between the endemic OXA-48 and different Tn1999 variants located on IncL plasmids isolated in North Africa and the Middle East, which then invaded Southern Europe.

OXA-181 and OXA-232 are endemic in Bangladesh, Sri Lanka, Pakistan, and certain African countries. These specific carbapenemases are correlated to ISEcp1 and Tn2013 on ColE2, and IncX3 plasmids. Studies reported a correlation between the global dispersion of OXA-48, OXA-181, OXA-232, and OXA-204 and clonal dissemination of either *K. pneumoniae* ST147, ST307, ST15, and ST14 or *E. coli* ST38 and ST410 [158].

Last-resort antibiotics are mostly reserved to human use and are not approved for livestock based on the Animal Medicinal Drug Use Clarification Act of 1994 in the USA and similar regulations in other countries [159]. The detection of CRE in animals and other extra-hospital reservoirs alarmed public health authorities and clinicians (**Figure 9**) [160, 161]. Alongside, ESBL-producing *E. coli* has been controversially suspected to promote the transfer of carbapenem-resistant genes and variants via food products [112, 162-164].

In Europe, while VIM-1 and OXA-181 genes have been identified from livestock and seafood in Germany and Italy respectively (**Figure 9**) [134], the presence of OXA-48 in either *K. pneumoniae* or *E. coli* has been sporadically reported in pets [134, 165, 166]. In China, Wang *et al.* performed a study on humans with contact to the animals [167] in which all the samples were collected from one commercial chicken farm. Results showed that 50% of the tested samples isolated from farmers and chickens were positive for *bla*_{NDM-5}. Conversely to the study of Shen *et al.*, *bla*_{NDM} genes were harbored by different plasmids including HI2, Y, FrepB, A/C, FIB, FIC, I1 and HI2 [167]. In addition, analysis underlined that isolates from two different farmers shared ST10 and ST746 *E. coli* with isolates from local dogs, flies, and chickens and clustered closely together, suggesting transmission on the farms [167]. Although, clonality was detected between *bla*_{NDM}-positive *E. coli* strains from chicken farms, slaughterhouses, supermarkets, and humans, belonging to ST10 and ST156 clones [167].

Therefore, prevention of CRE occurrence and spread in all human-animal-environment sectors should be a major public health priority to protect both persons with direct and occupational exposure and consumers on a One Health scale [160].

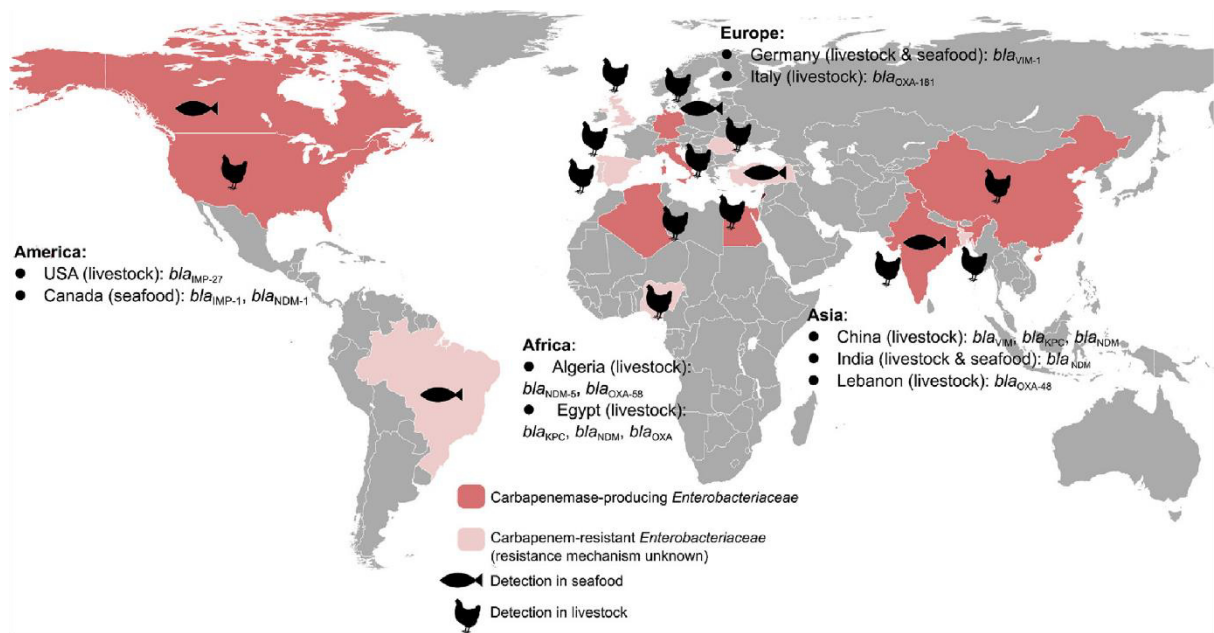


Figure 9: Global distribution of carbapenem-resistant and carbapenemase-producing *Enterobacteriaceae* in livestock and seafood (Adapted from Kock *et al.* 2018) [6].

2.4. Resistance to colistin in Enterobacterales

2.4.1. *The specific case of colistin*

Carbapenem-resistance is often accompanied by numerous other associated resistances, so that treatment options are regularly limited to fosfomycin, tigecycline, and colistin. Consequently, colistin regained interest to combat CRE [168]. Colistin is a cationic polypeptide consisting of a cyclic heptapeptide, a linear tripeptide and a long hydrophobic tail which are integrated into the bacterial membrane to produce its damage like a detergent [168]. Polymyxin A, C and D, are highly toxic. Only polymyxin B and E (also referred to as colistin) have been used in clinical practice, but renal and neurological toxicity was also reported in association with the intravenous use of colistin and polymyxin B leading to its ban in the early 1980s [169]. Colistin was reintroduced as a last-line drug for treating severe bacterial infections due to outbreaks of MDR superbugs, such as NDM-1-producing bacteria [168, 170]. Thus, studies showed that a therapy including the combination of colistin with tigecycline, and meropenem was associated with a lower mortality risk caused by *K. pneumoniae* KPC-positive infection in patients [171].

Colistin sulphate is toxic for humans but not for animals, so that this molecule has been heavily used for decades in veterinary medicine for the treatment of intestinal infections in pigs, cattle and poultry, which were caused mainly by *E. coli* and *Salmonella* spp. [38]. In the Netherlands, colistin is one of the most used antimicrobials in addition to tetracyclines, trimethoprim/sulfonamides, macrolides, and lincosamides [172]. In France, colistin is administered under different forms in swine, bovine, and chicken husbandry. The ANMV surveillance reports highlight a diminution of colistin exposure in these animals between 2014 and 2018. Recently, colistin consumption strongly decreased in France, from 40 tons in 2014 to 13 tons in 2018 (<https://www.anses.fr/fr/content/suivi-des-ventes-dantibiotiques-v%C3%A9t%C3%A9rinaires>).

Colistin-resistance went unnoticed for years since susceptibility testing for colistin is challenging due to its cationic nature, its ability to binds to plastic materials because of its amphipathic nature and its poor diffusion in agar [173-175]. These methodological difficulties explain why only whole-genome sequencing allowed scientist to discover the existence of the plasmid-borne *mcr* genes. Nowadays, colistin-resistant *Enterobacteriaceae* have been tracked down and found in numerous isolates from livestock and companion animals, from various food sources, from vegetables as well as from the environment (including rivers and lakes water), infected patients, and asymptomatic human carriers [38, 139, 167].

GNB acquire resistance to colistin as a result of intrinsic or transferable mechanisms. In both cases, LPS modifications alter the negative charge of the outer-membrane and reduce the affinity between colistin and their target in the bacterial cell resistance [176].

2.4.2. Chromosomal resistance

It has been proved that chromosomal mutations in the two-component systems *pmrA/pmrB* and *phoP/phoQ* in *E. coli*, in addition to insertional inactivation of *mgrB*-encoding regulator in *K. pneumoniae*, generates colistin-resistance [177]. These mutations affect the drug target of the bacterial cell, i.e. the LPS by modifying the surface charge of the cell wall and thus weaken the binding of polymyxins (**Figure 10**) [177]. Moreover, colistin-resistance of clinical isolates can be a result from a combination of porin mutations and overexpression of efflux pump systems [177].

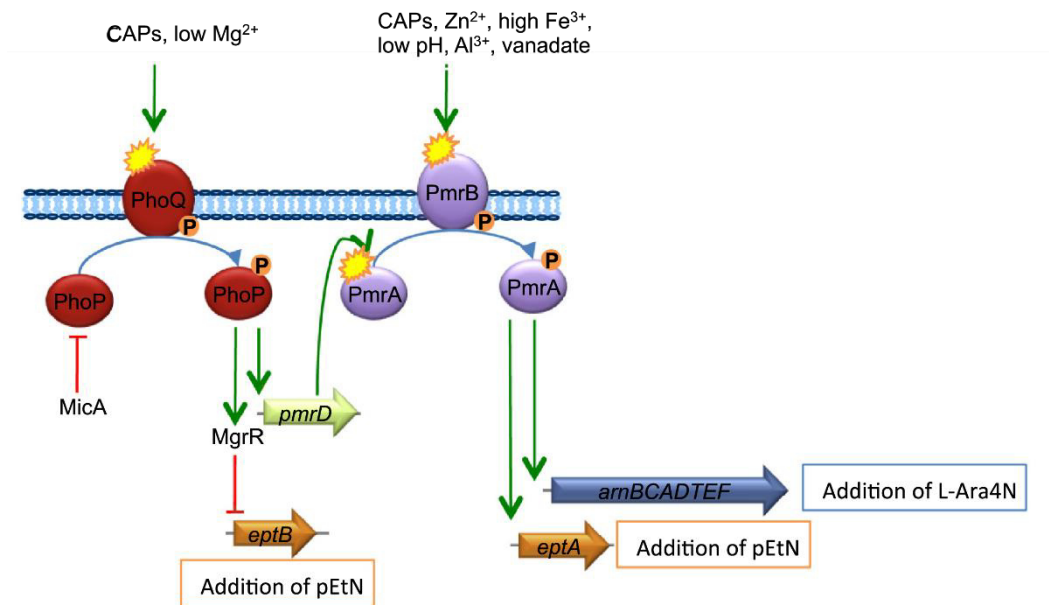


Figure 10: Diagram of the regulations putative two-component systems, *phoPQ*, and *pmrAB* responsible for polymyxin resistance in *E. coli* (Adapted from Jeannot *et al.*) [4, 5].

As shown in **Figure 10**, high concentrations of polymyxins and low Mg^{2+} constitute an environmental stress, which affect the phosphorylation of the sensor PmrB and PhoQ, respectively. Consequently, PmrA is activated and induce several lipopolysaccharide-modifying genes including *arn* operon and *pmrABC* operon. Once activated, it induces the phosphorylation of *pmrA*. The activation of the *phoQ-phoP* system affects *arn* operon directly or indirectly via the activation of PmrD, which in turn activates *pmrA*. Besides, the activated PhoP affects other genes such as *pagL*, *pagP*, and *lpxO*, which modify(ies) lipid A [178]. In *K. pneumoniae*, the *mgrB* can exerts negative feedback on the *phoQ-phoP* system. Hence, mutations in *mgrB* lead to constitutive induction of the *phoQ-phoP* system.

2.4.3. Plasmidic resistance

Liu et al. published the first description of a novel conjugable plasmid-mediated colistin-resistance gene, *mcr-1*, conferring colistin resistance in animals and humans in China [38]. The *mcr-1* gene encodes for a phosphoethanolamine transferase (pEtN transferase), which modifies the LPS by the addition of pEtN to lipid A [179]. Epidemiological studies performed

following this discovery proved that *mcr-1*-mediated colistin-resistance emerged as early as 2010 and spread worldwide [180]. Until October 2020, there have been various reports published about newly discovered plasmid-mediated colistin resistance genes *mcr-2* up to *mcr-10* (**Figure 11**) [181-189].

mcr genes are thought to have an environmental origin, with *mcr-1* and *mcr-2* potentially originating from *Moraxella* spp and *mcr-3* and *mcr-7* potentially originating from *Aeromonas* spp. [184, 186, 190]. The distribution of *mcr* genes differs depending on the bacterial species, with *mcr-8* mainly detected in *K. pneumonia* and *mcr-9* mostly prevalent in *S. enterica* [187, 188]. The National Database of Antibiotic Resistant Organisms (NDARO) reported that most *mcr*-positive isolates are restricted *Enterobacteriaceae* species, particularly *E. coli*, *S. enterica*, *K. pneumoniae*, and *Enterobacter* spp..

The *mcr-1* gene was firstly detected on IncI2 plasmid, This gene was then predominantly associated to IncX4 and IncHI2 plasmids, but was also identified on 12 other plasmid backbones, including IncFII, IncX3, IncX1-X2, IncHI1 and IncP plasmids [191-193].

Generally, *E. coli* strains with the *mcr-1* gene are characterized by the low-level colistin resistance with a MIC in the range of 2–8 mg/l. Zhang *et al.* have shown that the expression of the *mcr-1* gene in *E. coli* led to a higher mutation rate in the chromosomal polymyxin resistance cascade genes and produced higher MIC values (≥ 64 mg/l) [194].

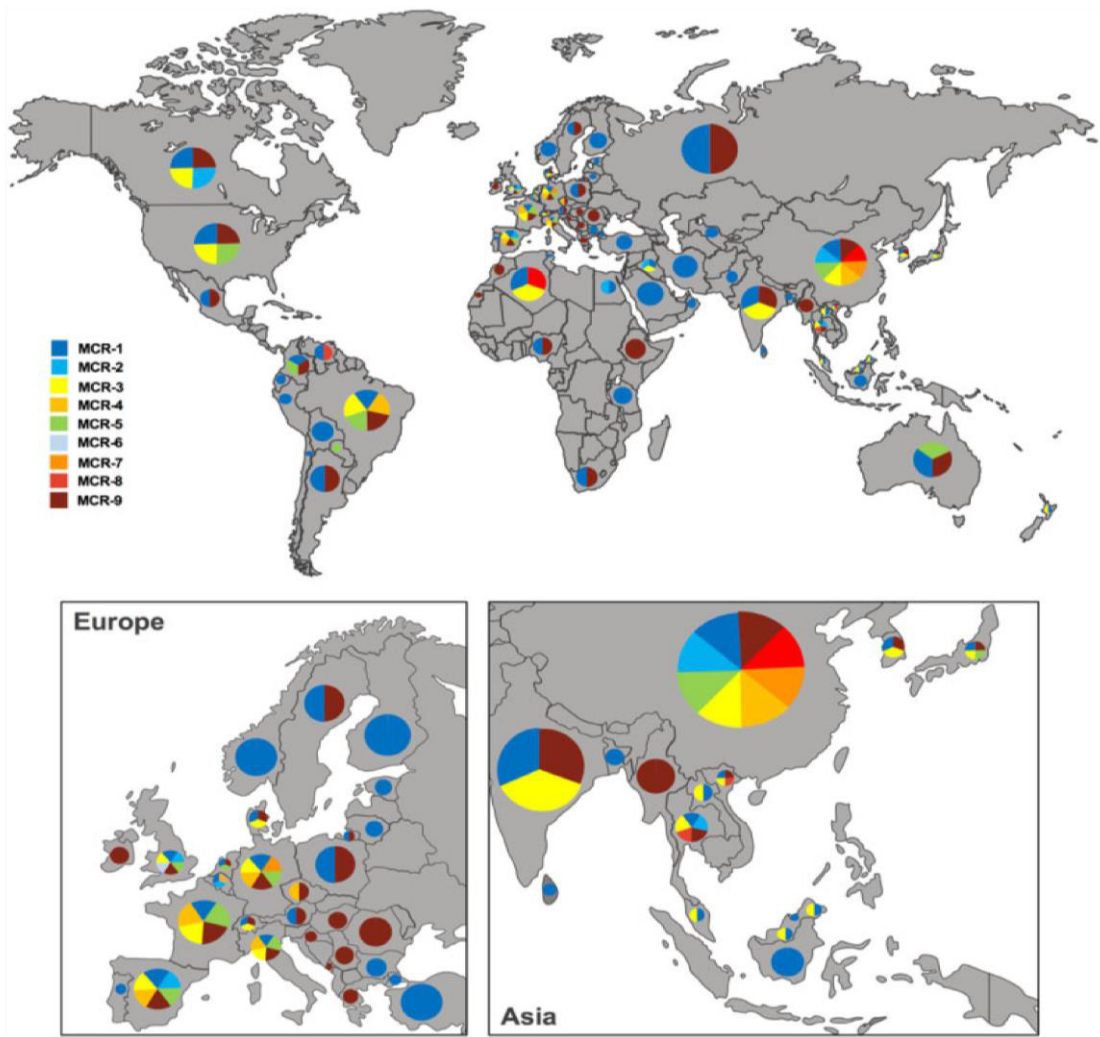


Figure 11: MCR-family gene diversity in different countries (Adapted from Ling *et al.* 2020, where data was collected until July 2020) [3].

3. Epidemiology of antibiotic resistance in Lebanon: where are we?

3.1. Global context of antibiotic use and antibiotic resistance in Lebanon (adapted from Osman *et al.*)

Recently, a high profile report warned that 700 000 annual deaths globally attributed to AMR in 2015 could climb to 10 million deaths in 2050 if nothing is done to reverse the trend [195]. Besides these serious issues, AMR has a negative economic impact on communities, leading to huge annual costs almost similar to those of the global financial crisis [196]. A recent study estimated the GDP loss due to AMR in the Middle East and North Africa (MENA) region, including Lebanon, to range between US\$2 billion and US\$159 billion per year over 40 years [196]. Unfortunately, Lebanon, like the majority of developing countries, still suffers from high levels of AMR.

Despite the implementation of consistent strategies and measures to prevent and control the emergence and spread of AMR in developed countries, this issue continues to grow in a large number of these countries [197]. However, the AMR issue is more complicated in developing countries, such as Lebanon, since numerous factors leading to the emergence and spread of MDR strains remain uncontrollable.

The increasing availability of antibiotics in Lebanon is generally a good thing, however the misuse of these drugs has created and promoted the transmission of antimicrobial-resistant strains of deadly bacteria, particularly in poorer communities such as Syrian and Palestinian refugees and the vulnerable Lebanese population [198]. Besides the availability of antibiotics without prescription in pharmacies, various factors are key drivers of AMR in Lebanon, including (i) poor antibiotic awareness both among prescribers and the general public (see the subsection 1.5.3), (ii) extensive antibiotic use in humans, animals and agriculture, (iii) excessive use of antibiotics as growth-promoting factors in livestock, (iv) non-compliance with antibiotic therapy, (v) promotion of antibiotic self-medication, (vi) poor antibiotic stewardship and (vii) inadequate infection control. Moreover, the availability of antibiotics for purchase on websites increases the circulation of counterfeit drugs and decreases the quality of care in the country [199].

Therefore, despite the outstanding progress to develop a national plan of action to combat the spread of AMR in Lebanon, AMR rates are at the higher end of levels reported globally [200]. Several cross-sectional studies showed a high prevalence of inappropriate antibiotic prescription in Lebanon, regardless of doctors' specialty [201, 202]. Similarly, large amounts of antibiotics are used in the veterinary, livestock and agriculture fields without surveillance and control, causing a rapid increase in the prevalence of AMR and the use of last-resort antibiotic compounds both in clinical and veterinary fields in Lebanon. On the other hand, a cross-sectional nationwide study conducted in 2017, in synchronization with the first Lebanese antibiotic awareness campaign, revealed very insufficient knowledge, poor attitudes and inadequate practices towards antibiotics among the resident populations in Lebanon [203].

Unfortunately, AMR bacteria and genes are now widely disseminated among humans, animals and the environment, and their presence in these sectors have also been regularly reported in Lebanon [112, 204-208].

3.2. Resistance to broad-spectrum cephalosporins, carbapenems and colistin in humans

Fortunately, surveillance of AMR in Lebanon has improved during the last years. Moreover, Lebanon has been enrolled in the WHO's GLASS since April 2017. In this context, numerous epidemiological studies were conducted in the last decade in Lebanon that highlighted the worrying situation of AMR in the country and described high rates of methicillin-resistant *Staphylococcus aureus* (MRSA) and differentially resistant GNB [82, 83, 200].

3.2.1. Resistance to broad-spectrum cephalosporins

One nationwide study performed at 16 Lebanese tertiary care centers between 2011–2013 reported a rise in the prevalence of common MDR pathogens including ESBL-producing *E. coli* [200]. Another study performed at El Youssef Hospital Center in North Lebanon between 2015–2017 reported that 35.7% of uropathogenic *E. coli* were ESBL-producers [209]. Other clinical epidemiological studies highlight the predominance of CTX-M-15 and SHV-15a in *K. pneumoniae* and *E. coli* [210, 211]. Hence, clonal diversity have been reported within ESBL producing *K. pneumoniae* and *E. coli* isolates [211, 212]. A study conducted by Tokajian *et al.* characterized clones circulating in healthcare settings, where five lineages

(ST131, ST648, ST405, ST73 and ST38) predominated [212]. In the same study, resistant *E. coli* harbored *bla*_{OXA-1}, *bla*_{TEM-1B}, *bla*_{TEM-1C} on Col156, IncFIA, IncFIB, IncFII, and IncX1 plasmids [212].

Conversely, data on the ESBL-producing *Enterobacteriaceae* fecal carriage in the community in Lebanon are very scarce. Two studies have investigated MDR Enterobacterales among elderly residents of nursing homes in Lebanon [213, 214]. Dandachi *et al.* reported a high prevalence of ESBL-producers strains (91.5%) and the occurrence of CTX-M-15 among resistant isolates [213]. Another study conducted by Hijazi *et al.* in healthy infants underlined the prevalence of CTX-M-9 followed by CTX-M-15 and CTX-M-2 in the community [215]. Indeed, unprofessional prescription of antibiotics, environmental decontamination, waste disposal, and hygiene practices in these whole country promote the easy spread of ESBL-producing GNB.

3.2.2. Resistance to carbapenem

A study carried out in North Lebanon at Nini Hospital reported that 24.4% of cancer patients were colonized in the gut with carbapenem-resistant GNB [216]. Thus, cancer patients undergoing chemotherapy have been reported as highly carrier of carbapenem resistant strains including OXA-48 and CTX-M *E. coli* [216].

Overall, the most common reported carbapenemases detected among *Enterobacteriaceae* in clinical settings were OXA-48, OXA-232 *E. coli*, ST14 NDM-1 *K. pneumoniae*, and OXA-48, OXA-162, OXA-232 *K. pneumoniae* [217-220]. Moreover, *bla*_{OXA-181} gene has been identified recently on IncX3 plasmid in a ST940 carbapenem-resistant *E. coli* recovered from a Lebanese ICU patient [221]. Furthermore, the same strain harbored OXA-1, CMY-42, and TEM-1 genes [221]. In two studies, the OXA-48 gene was located on the same plasmid IncL/M (in both *E. coli* and *K. pneumoniae*) suggesting the spread of resistant determinants between species [219, 220]. Although, Moussa *et al.* showed that OXA-48 can be carried on IncFII plasmid within Tn6237 [222]. Conversely, clonality was not observed among the same species [223]. On the other hand, the *bla*_{NDM-1} gene has been identified in ST14 and ST15 carbapenem-resistant *K. pneumoniae* [220, 224]. Interestingly, NDM-5 has been also reported in a ST383 carbapenem-resistant *K. pneumoniae* [225].

3.2.3. Resistance to colistin

Resistance to last-resort antibiotics, including carbapenems, is frequent in healthcare settings in Lebanon, whereas colistin resistance has only rarely been identified [116]. Conversely, to the best of our knowledge, *mcr* genes have never been reported from human isolates in Lebanon until 2019 [226]. Clinical colistin-resistant *Enterobacteriaceae* have been described in two previous studies from Lebanese hospitals, but always associated to chromosomal mutations [227, 228]. In addition to *mcr-1* detected isolates, a very recent study conducted by Salloum *et al.* reported a positive *mcr-8* ST-15 *K. pneumoniae* isolate located on an IncFII-type conjugative plasmid [229].

3.3. Resistance to broad-spectrum cephalosporins, carbapenems and colistin in non-human settings (adapted from Osman *et al.*)

Animals, food and the environment are generally considered reservoirs of MDR and XDR bacteria, particularly *Enterobacteriaceae* [208, 230]. Nevertheless, bacterial strains carried by animals can be easily transferred to humans through direct contact or by handling or consumption of animal products [231].

During the last decade, very few investigations have targeted the AMR issue in Lebanese in non-clinical settings. However, the prevalence of ESBL-, and carbapenemase-producing GNB in addition to colistin resistant GNB, has become widely reported in animals, food and the environment in Lebanon. Interestingly, the majority of investigations were conducted either in overcrowded urban regions or in rural regions with high agricultural production.

3.3.1. Resistance to broad-spectrum cephalosporins

In recent years, Diab *et al.* reported the first ESBL-producing *E. coli* in animals in the country [208]. The same paper also described massive colonization of the Lebanese bovine sector with ESBL-producing *E. coli*, with a prevalence of 23% among healthy adult cattle and 84% among randomly selected cattle farms [208].

In contrast to worldwide findings, the enzymes responsible for the ESBL phenotype found in Lebanon were mostly CTX-M-15, followed by other ESBL types such as SHV-12, CTX-M-9, CTX-M-14, CTX-M-27 and CTX-M-55 [112, 207, 208, 230, 232]. Globally, CTX-M-1 is the most widely identified enzyme responsible for the ESBL phenotype in livestock, followed by CTX-M-14 [233-235]. However, recent data highlighted the presence of the *bla*_{CTX-M-15} gene in non-clinical contexts [207, 209].

Interestingly, MLST and pulsed-field gel electrophoresis (PFGE) results revealed high genetic diversity of CTX-M-15- producing strains circulating in Lebanon. The isolated *E. coli* strains belonged to ST10, ST58, ST69, ST155, ST617 and ST1303, which have been observed both in humans and animals, and to ST162, ST196, ST218, ST457, ST515, ST540, ST746, ST1140, ST1294, ST1303, ST2325, ST2450, ST4252, ST4623, ST5204, ST5442 and ST5728, which have no common history [208]. However, none of the isolated strains belonged to ST131, ST405 or ST648 clones, which are the most responsible for the spread of CTX-M-15 in humans.

Similarly, water contamination by ESBLs is potentially a major risk to public health in Lebanon. A recent nationwide study reported a high diversity of *E. coli* STs in sewage water in Lebanon, including ST38, ST90, ST617, ST4144, ST4608, ST6222, ST6450, ST6470, ST6480 and ST6894 [232]. Another study conducted in North and South Lebanon also described very similar findings associated with high ESBL levels (59%) in water sampled from estuaries [207]. As described above, CTX-M-15 was the predominant ESBL identified in water sources including estuaries, wells and springs. This study described for the first time the presence of *E. coli* isolates belonging to ST131 and ST405 clones that are commonly reported in clinical settings [212]. Worryingly, wells and spring water in Lebanon, particularly in rural areas, is directly intended for human and animal consumption without any further treatment. In addition, estuary water is commonly used to water animals and/or irrigate crops.

In the poultry-farm sector, a cross-sectional study conducted at a national level revealed the presence of STs common to both humans and animals, particularly ST155 [206]. More recently, Dandachi *et al.* reported the presence of ST101, ST746, ST1196, ST359, ST1140, ST2220, ST5687 and ST2481 in chicken, farmers and surrounding environment in Lebanon [236]. Surprisingly, none of the aforementioned studies reported the occurrence of *bla*_{CTX-M-1}, which is mainly associated with animal sources.

All these findings are coherent with the hypothesis that livestock, poultry and water in Lebanon are inhabited by genetically diverse *E. coli* carrying and disseminating *bla*_{CTX-M-15} plasmids in different genetic backgrounds and imply an association with clinical isolates [237].

3.3.2. Resistance to carbapenem

Regarding carbapenemases, Al Bayssari *et al.* published the first description of *bla*_{VIM-2}-carrying *P. aeruginosa* and *bla*_{OXA-23}-carrying *A. baumannii* strains isolated from Lebanese livestock and poultry [238]. Moreover, they also detected the co-occurrence of *bla*_{OXA-23} and *bla*_{OXA-58} in the same strain of *A. baumannii* isolated from fowl. Similarly, Rafei *et al.* identified a *bla*_{OXA-143}-harbouring *A. baumannii* and a *bla*_{OXA-24}-harbouring *Acinetobacter pittii*, both from horses [239]. In addition, two *Acinetobacter calcoaceticus* strains carrying the *bla*_{OXA-72} carbapenemase gene have also been isolated from vegetables [240]. Furthermore, a case report showed the presence of an OXA-48-producing ST38 *E. coli* clone in a Lebanese fowl (*Gallus domesticus*). This latter represents an emerging human clone in the Mediterranean basin region but has also been identified as an OXA-244-producer in estuaries water in Lebanon [207, 230, 232]. Furthermore, an OXA-48-producing *K. pneumoniae* ST530 was recently detected in raw bovine milk [112]. In addition, *E. coli* and *K. pneumoniae* strains harboring *bla*_{OXA-48} were also reported in estuary water, confirming the diversity of bacterial species producing OXA-48 and their probable endemic status in Lebanon [112, 204, 230, 241]. Likewise, a recent study reported for the first time NDM-1-producing *E. cloacae* in Lebanon [242]. The NDM-1 enzyme was previously detected in Lebanon among *A. baumannii* and *A. pittii* isolated from clinical samples, but not in *Enterobacteriaceae* [243, 244]. Besides, GNB producing ESBLs and carbapenemases are co-resistant to other veterinary and clinically licensed antibiotics, further worsening the AMR situation.

3.3.3. Resistance to colistin

The extent of colistin usage in farms and veterinary medicine remains unclear. However, unpublished data have revealed the heavy use of this compound both in humans and animals in Lebanon. As a consequence, colistin-resistance has been reported in animals and the environment.

A case report published in January 2018 showed the emergence of an *mcr-1* plasmid-mediated colistin-resistant *E. coli* strain isolated from Lebanese poultry [245]. The isolated strain belonged to ST515, which was lately observed in Lebanese livestock [208]. In the same year, the same research group reported 23 colistin-resistant *E. coli* strains harboring the *mcr-1* gene isolated from pigs [246]. Of the 23 colistin-resistant strains, only 4 were ESBL-producers whilst the remaining strains were susceptible to the majority of β -lactams [246]. A nationwide study carried out between September 2017 and March 2018 in three main poultry farms in Lebanon revealed widespread resistance to colistin, with an alarming percentage of *E. coli* isolates harboring the *mcr-1* gene (98%) [247]. More recently, Dandachi *et al.* reported the presence of ST746, ST101 and ST3941, and ST1011 colistin-resistant *E. coli* isolated from chicken farm, litter, feed samples and workers in South Lebanon [236].

Similarly, water contamination by colistin resistant *E. coli* have been reported in several water sources in Lebanon [248, 249]. ST93 and ST602 *E. coli* have been reported from seawater drinking-, well-, and camp-generated sewer waters where colistin-resistant strains harbored the *mcr-1* gene on IncI2, IncX4, and IncF plasmids [248, 249].

Table 5: Epidemiology of antimicrobial resistance in non-clinical settings in Lebanon.

Investigation site	Targeted population	Type of sample	Year(s) of survey	No. of isolates ESBL-producing	No. of isolates Carbapenem-resistant	Presence of Colistin resistance	Antibiotic resistance (%)								β-lactam resistance genes (n)	Reference
							among ESBL-producing and/or carbapenem-resistant strains									
							TET	NAL	ENR/CIP	TMP	SXT	GEN	STR	CHL		
North and South Lebanon (Search for MDR bacteria)	Healthy adult cattle	Stool	October 2012 - February 2013	<i>Escherichia coli</i> (n=40)	0	No	83	30	18	75	- ^a	23	78	30	<i>bla</i> _{CTX-M-15} (24) <i>bla</i> _{SHV-12} (10) <i>bla</i> _{CTX-M-14} (2) <i>bla</i> _{CTX-M-15} + <i>bla</i> _{SHV-12} (3) <i>bla</i> _{CTX-M-14} + <i>bla</i> _{SHV-12} (1)	[112]
South Lebanon (Search for MDR bacteria)	Swine	Stool	May 2017	<i>E. coli</i> (n=105) <i>K. pneumoniae</i> (n=2) <i>E. fergusonii</i> (n=4)	0	<i>mcr-1</i> (n=23)	-	-	78	-	89	42	-	-	[<i>bla</i> _{CTX-M} (83) <i>bla</i> _{TEM} (57) <i>bla</i> _{SHV} (9) <i>bla</i> _{CMY} (13) alone or in combination]	[246]
North Lebanon	Animals (pigs, fowl and cattle)	Stool	2013	-	<i>A baumannii</i> (n=5) <i>P. aeruginosa</i> (n=4)	No	-	100	100	-	-	100	-	-	<i>bla</i> _{OXA-23} (4) <i>bla</i> _{VIM-2} (4) <i>bla</i> _{OXA-23} + <i>bla</i> _{OXA-58} (1)	[238]
Case report	Poultry (fowl)	Stool	December 2013	-	<i>E. coli</i> (n=1)	No	-	-	-	100	-	100	-	-	<i>bla</i> _{OXA-48} + <i>bla</i> _{CTX-M-14} + <i>bla</i> _{TEM-1} (1)	[230]
Case report (Sidon)	Poultry	Rectal swab	14 August 2015	<i>E. coli</i> (n=1)	0	<i>mcr-1</i> (n=1)	-	-	100	100	100	100	-	-	<i>bla</i> _{TEM-135-like} (1)	[245]

Nationwide	Poultry	Rectal swabs	August - December 2015	<i>E. coli</i> (n=103) <i>K. pneumoniae</i> (n=5) <i>E. cloacae</i> (n=3) <i>E. fergusonii</i> (n=1)	0	No	-	-	62	-	68	71	-	-	<i>bla</i> _{CTX-M} (2) <i>bla</i> _{TEM} (36) <i>bla</i> _{SHV} (17) <i>bla</i> _{CTX-M} + <i>bla</i> _{TEM} (52) <i>bla</i> _{CTX-M} + <i>bla</i> _{TEM} + <i>bla</i> _{SHV} (5) [<i>bla</i> _{CMY} (152): alone or in combination with other genes]	[206]
Nationwide	Poultry	Fecal samples	September 2017 – March 2018	-	-	<i>mcr-1</i> (n=88)	89	-	91	-	84	61	80	95	<i>bla</i> _{TEM-1} (84) <i>bla</i> _{CTX-M} (31)	[247]
South Lebanon	Poultry, environment	Fecal samples, soil, feed samples, litter samples	May 2017	<i>E.coli</i> (n=110) <i>K.pneumoniae</i> (n=1) <i>A.baumannii</i> (n=4)	0	<i>mcr-1</i> (n=278)	-	-	-	-	-	-	-	-	<i>bla</i> _{CTX-M} (192) <i>bla</i> _{TEM} (300) <i>bla</i> _{SHV} (55)	[236]
Beqaa Valley	Agri-food environment	Vegetables; Knife & board swabs; Fresh produce; Water	July - August 2013 and July 2014	- (100% of isolates were resistant to cefalotin)	-	No	-	-	-	-	-	-	-	-	-	[250]
North Lebanon	Food	Akkawi cheese	May - June 2011	<i>E. coli</i> (n=3) <i>K. pneumoniae</i> (n=2) <i>Rhanella aquatilis</i> (n=1)	0	No	17	17	17	-	33	50	-	-	-	[204]

North Lebanon	Food	Raw bovine milk	September – November 2015	<i>E. coli</i> (n=7) <i>K. pneumoniae</i> (n=36)	<i>K. pneumoniae</i> (n=1)	No	-	-	-	-	-	-	-	-	-	<i>bla</i> CTX-M-15 (38); <i>bla</i> CTX-M-15 + <i>bla</i> SHV-12 (4) <i>bla</i> CTX-M-15 + <i>bla</i> OXA-48 (1)	[112]
North Lebanon (Tripoli)	Food	Raw meat	April – May 2010	<i>E. coli</i> (n=2)	0	No	100	-	50	-	-	0	-	-	-	-	[251]
Nationwide	Water	seawater	2019-2020	-	0	<i>E. coli mcr-1</i> (n=16)	81	-	63	69	-	-	-	63	-	-	[248]
Nationwide	Water	Sewage water	Spring 2016	<i>E. coli</i> (n=21)	0	No	71	-	81	-	81	43	-	-	-	<i>bla</i> CTX-M-15 (2); <i>bla</i> CTX-M-9 (1); <i>bla</i> CTX-M-15 + <i>bla</i> OXA-1 (3) <i>bla</i> CTX-M-15 + <i>bla</i> OXA-1 + <i>bla</i> SHV-12 (3) <i>bla</i> CTX-M-15 + <i>bla</i> OXA-1 + <i>bla</i> TEM-1 (1) <i>bla</i> CTX-M-15 + <i>bla</i> TEM-1 (2) <i>bla</i> CTX-M-15 + <i>bla</i> TEM-1 + <i>bla</i> SHV-12 (1) <i>bla</i> SHV-12 + <i>bla</i> OXA-1 (1) <i>bla</i> CTX-M-27 + <i>bla</i> TEM-1 (1) <i>bla</i> CTX-M-14 + <i>bla</i> OXA-1 (1)	[232]
North and South Lebanon (Search for MDR bacteria)	Water	Dug wells; Spring water; Estuaries	March 2014 - January 2015	<i>E. coli</i> (n=23) <i>K. pneumoniae</i> (n=4) <i>Citrobacter freundii</i> (n=1) <i>Citrobacter braakii</i> (n=1)	<i>E. coli</i> (n=3) <i>K. pneumoniae</i> (n=1)	No	67	58	48	58	-	33	42	15	-	<i>bla</i> CTX-M-15 (24); <i>bla</i> CTX-M-14 (1); <i>bla</i> CTX-M-55 (1); <i>bla</i> SHV-12 (1) <i>bla</i> OXA-48 (1) <i>bla</i> OXA-244 (1) <i>bla</i> OXA-48 + <i>bla</i> CTX-M-14 (1) <i>bla</i> OXA-244 + <i>bla</i> CTX-M-14 + <i>bla</i> SHV-12 (1) <i>bla</i> CTX-M-15 + <i>bla</i> CMY-42 (2)	[207]
Beirut and North Lebanon	Water	Hospital wastewater	August 2011 - June 2012	<i>E. coli</i> (n=45) <i>K. pneumoniae</i> (n=6)	<i>E. cloacae</i> (n=2)	No	-	-	-	-	53	41	-	-	-	[<i>bla</i> NDM-1 (2) <i>bla</i> CTX-M (45) <i>bla</i> TEM (19) <i>bla</i> SHV (23)	[242]

Syrian refugee camps in Lebanon	Water	drinking -, well-, and camp-generated sewer waters	February 2019	-	0	<i>mcr-1</i> (n=36)	-	-	-	-	-	-	-	-	-	<i>bla</i> _{OXA} (29) alone or in combination] <i>bla</i> _{CTX-M} (30) <i>bla</i> _{TEM} (31) <i>bla</i> _{SHV} (5) <i>bla</i> _{OXA-48} (1)	[249]
Akkar district	Water	Dug wells	April – May 2010	<i>E. coli</i> (n=3) <i>C. freundii</i> (n=1)	n=0	No	75	75	50	-	-	67	-	-	-	-	[252]
Nationwide (Search for <i>Acinetobacter</i> spp.)	Agri-food environment	Soil ; Water; Vegetables; Seawater fishes; Cow's milk; Animals	September 2014 – Feb 2015	n=0	<i>Acinetobacter calcoaceticus</i> (n=2)	No	-	-	0	-	-	-	-	-	-	<i>bla</i> _{OXA-72} (2)	[240, 243]
Nationwide (Search for <i>Acinetobacter</i> spp.)	Agri-food environment	Soil; Animals; Food	February 2012 - October 2013	n=0	<i>A baumannii</i> (n=1) <i>Acinetobacter pittii</i> (n=1)	No	-	-	-	-	-	-	-	-	-	<i>bla</i> _{OXA-143} (1) <i>bla</i> _{OXA-24} (1)	[239]

ESBL, extended-spectrum β -lactamase; TET,tetracycline;NAL,nalidixic acid;ENR,enrofloxacin;CIP,ciprofloxacin;TMP,trimethoprim;SXT,trimethoprim/sulfamethoxazole;GEN,gentamicin;STR,streptomycin;CHL, chloramphenicol;MDR,multidrug-resistant.

^a Indicates not available .

Chapter 2: Experimental studies

1. Objectives of the experimental work

AMR is a complex multifaceted problem that threatens global public health and causes global economic loss. The AMR crisis is not any more confined to clinical settings but has disseminated in the community and all sectors, so that it has now to be tackled in a One Health manner. Animals as well as foodstuffs and the environment are significant reservoirs of AMR in countries all over the globe, including Lebanon. Although few epidemiological studies have been conducted, and the available data indicates that MDR and XDR bacteria are ubiquitous in Lebanon. Diab *et al.* described a high prevalence of ESBL-producing *E. coli* among healthy cattle in Lebanese farms. Additionally, carbapenemases have been highly emerged in livestock and different water sources in this country. Other studies have been underlined the wide dissemination of CTX-M-15 and OXA-48 producing *Enterobacteriaceae*, both in clinical and non-clinical samples. Lately, the presence of *mcr-1*-positive *E. coli* has been described in poultry, swine, and the environment but have never been studied in humans neither in hospitals nor in community settings. Unfortunately, tackling the Lebanese AMR burden remains complicated due to the poor and mostly unknown information on the population structure as well as the plasmids carrying the resistance genes.

Our research purpose was to gain new insights into ESC, carbapenem, and colistin resistant Enterobacteriales on epidemiological and molecular levels in different linked sectors, in order to fill up the gaps in Lebanese data, and to complete what our colleagues starts.

The lack of reports on *mcr* genes in the Lebanese clinical settings despite its rising trend worldwide in the same sector prompted us to work on human clinical isolates. In parallel, the global increasing rates of *mcr* resistant determinants in healthy animals and retail meat probably enhances the contamination either by human or animal activities and represents a prominent public health issue in Lebanon. In this context, we performed a study in the Lebanese poultry sector, in which we collect feces and meat samples from chicken, to further describe the prevalence and the population structure of *E. coli* isolates harbored the *mcr-1* gene and to point the possibility of cross-contamination within humans and food animal's production. Otherwise, data in the Lebanese community are scarce and the dynamic of MDR persistence and carriage has never been studied. Accordingly, we performed a longitudinal study on healthy adults working in the food sector in North Lebanon.

In a long term, our results aim at enhancing Lebanese authorities in order to develop comprehensive and integrative measures and effective preventive and control strategies to tackle AMR burden in the country.

2. Emergence of clinical *mcr-1*-positive *Escherichia coli* in Lebanon.

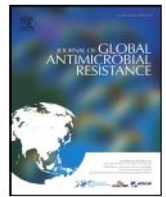
The emergence of carbapenem-resistance in infections caused by already multi-drug resistant gram-negative pathogens such as *P. aeruginosa*, *A. baumannii*, *K. pneumoniae* and *E. coli* raised the specter of pan-resistant clinical pathogens. In numerous cases, the only antibiotics that are still active against these bacteria are tigecycline, fosfomycin and colistin. Colistin, which is an older class of antimicrobial drugs that has widely been used to treat livestock, had only limited use in humans because of its nephrotoxicity and neurotoxicity in people [253]. The emergence of carbapenem-resistant pathogens thus shed a new light on colistin, which gained importance as a last-resort drug for parenteral use in the treatment, mainly in intensive care units. Since the identification of the first plasmid-mediated colistin-resistance gene *mcr-1* in 2015, *mcr* genes have been increasingly reported worldwide in animals, but also to a lesser extent in humans [254]. In Lebanon, resistance to last-resort antibiotics is frequent in healthcare settings, but only chromosomal colistin resistance mechanisms have been identified yet [116, 228]. Conversely, *mcr-1*-carrying *E. coli* have been reported in poultry, swine and the environment [206, 236, 245-249]. The goal of this study was to retrospectively look for colistin resistance by plating all clinical Enterobacterales isolates stored between 2006 and 2019 in the Lebanese University bacterial collection (CMUL) on MacConkey supplemented with colistin (3.5 mg/L). Among 998 isolates, 35 were colistin-resistant, among which six were *mcr-1*-positive *E. coli*. Isolates were further characterized for their sequence type, antimicrobial susceptibility, and the plasmid carrying the *mcr-1* gene was identified. It is important to note that colistin-resistant *E. coli* had been wrongly reported to physicians as susceptible to this drug, so that our work should help reevaluating the procedures to properly detect colistin-resistance in hospital settings in Lebanon.

Article 1: Accepted and published in Journal of Global Antimicrobial Resistance



Contents lists available at ScienceDirect

Journal of Global Antimicrobial Resistance

journal homepage: www.elsevier.com/locate/jgar

Letter to the Editor

Emergence of clinical *mcr-1*-positive *Escherichia coli* in Lebanon

Sir,

Antimicrobial resistance has become a global health crisis, and Lebanon does not escape this situation. A study in Lebanese hospitals reported a critical increase in antimicrobial resistance rates among clinical isolates, mainly Gram-negative bacteria. Moreover, resistance to last-resort antibiotics, including carbapenems, is frequent in healthcare settings in Lebanon, whereas colistin resistance is only rarely identified [1]. Surprisingly, the prevalence of colistin resistance in *Escherichia coli* from Lebanese poultry was found to be among the highest worldwide, and the plasmid-mediated *mcr-1* gene has been detected in Lebanese poultry and swine [2,3]. A recent study also found *mcr-1*-positive *E. coli* isolates in water sources in this country [4]. Conversely, to the best of our knowledge, *mcr* genes have never been reported from human isolates in Lebanon.

In this study, a total of 988 non-duplicate Lebanese clinical Enterobacteriaceae isolates stored in the Lebanese University bacterial bank (CMUL) between 2006–2019 were screened on MacConkey agar (Bio-Rad, Hercules, CA, USA) supplemented with 3.5 mg/L colistin (Sigma-Aldrich, St Louis, MO, USA). Regrettably, colistin use in the patients had not been recorded. All isolates grown on selective agar were purified and were identified by matrix-assisted laser desorption/ionisation time-of-flight (MALDI-TOF). Of the 988 isolates screened, 36 (3.6%) were resistant to colistin, with minimum inhibitory concentrations (MICs) ranging from 4 mg/L to 64 mg/L as determined by broth microdilution according to European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines. Among the colistin-resistant isolates, *E. coli* was predominant (26/36; 72.2%), however *Klebsiella pneumoniae* (7/36; 19.4%), *Salmonella enterica* (2/36; 5.6%) and *Enterobacter cloacae* (1/36; 2.8%) were also detected.

By PCR for *mcr-1* to *mcr-5* genes and sequencing, *mcr-1* was detected in six *E. coli* isolates recovered in 2011 ($n=1$), 2013 ($n=1$), 2017 ($n=2$), 2018 ($n=1$) and 2019 ($n=1$), whereas no other *mcr* genes were identified [5]. Chromosomal mechanisms conferring colistin resistance, such as mutations in the *mgrB* gene in colistin-resistant *K. pneumoniae*, were not further investigated. Also, since not all variants of the *mcr* gene were searched in this study, we cannot exclude possible additional *mcr*-positive isolates in this collection. As assessed by disk diffusion according to EUCAST guidelines, five out of the six *mcr-1*-positive isolates were resistant to all classes of β -lactams except carbapenems. These isolates also presented associated resistance to sulfonamides (6/6), tetracyclines (5/6), trimethoprim (5/6), streptomycin (4/6), chloramphenicol

(4/6), nalidixic acid (4/6), enrofloxacin (4/6), kanamycin (3/6) and gentamicin (2/6) but remained susceptible to amikacin (Table 1). Two *mcr-1*-positive *E. coli* additionally harboured an extended-spectrum β -lactamase (ESBL) (CTX-M-15) and three harboured an AmpC phenotype (CMY-2), as shown by synergy test or ceftoxitin resistance, respectively (Table 1).

Multilocus sequence typing (MLST), *E. coli* phylogrouping, and pulsed-field gel electrophoresis (PFGE) using *Xba*I restriction revealed fully different genetic backgrounds for the six *mcr-1*-positive *E. coli* isolates (Table 1; Supplementary Fig. S1), which also differed from the only *mcr-1*-positive animal isolate, a ST515 *E. coli* collected from poultry, for which MLST data are available in Lebanon [6]. This genetic diversity demonstrates horizontal transfer of the *mcr-1* gene rather than clonal spread of a single clone. Using the PCR-based replicon typing scheme (PBRT Kit; Diatheva, Fano, Italy) and Southern blotting on S1-PFGE gels, the *mcr-1* gene was shown to be located on an IncX4 plasmid in all six isolates (Table 1). IncX4 plasmids are self-transferable at high frequency and have been widely reported to disseminate *mcr-1* in Enterobacteriaceae both from human and animal origins [7]. Conjugation experiments using *E. coli* BM21 as recipient were successful for all isolates, showing the capacity of all plasmids to easily disseminate the *mcr-1* gene horizontally. None of the transconjugants harboured an ESBL/AmpC gene as assessed by PCR, demonstrating that *mcr-1* and ESBL/AmpC genes were not located on the same plasmid. Moreover, all transconjugants were fully susceptible to non- β -lactam antibiotics, verifying that the IncX4 plasmid carried only the colistin resistance determinant.

In Lebanon, the frequency of colistin prescription in the clinic remains unclear even though it has most likely increased in parallel to the growing prevalence of carbapenem-resistant bacteria in hospitals. In addition, the amount of colistin imported for human use has increased between 2010 and 2017, and drugs containing colistin are widely and legally available without prescription for veterinary purposes. Of note, all six colistin-resistant *E. coli* had been wrongly reported to physicians as susceptible to this drug. The majority of Lebanese diagnostic laboratories still use the Kirby–Bauer disk diffusion method, which cannot clearly discriminate isolates as susceptible or resistant to colistin, thus predisposing patients to treatment failures.

To the best of our knowledge, this is the first report of *mcr-1*-positive Enterobacteriaceae in humans in Lebanon. Coupled with the low awareness of the antimicrobial resistance threat in the Lebanese community, these findings highlight the need for better infection control and antimicrobial stewardship programmes in this country, including the implementation of reliable tests to be used in clinics before treatment with colistin. A One Health approach should be urgently considered by Lebanese authorities to promote the responsible use of antibiotics and to address risk factors associated with antimicrobial resistance in all sectors.

Table 1
Characteristics of six clinical *mcr-1*-positive *Escherichia coli* of human origin in Lebanon.

Isolate	Source	Patient age (years)	Hospital (region)	Isolation date	Phylogenetic group	MLST	β -Lactam enzyme	Colistin MIC (mg/L)	Plasmid content ^a	Additional resistance
CMUL371	Semen	18	Nini Hospital (Tripoli)	2011	B1	ST1431	CTX-M-15	8	11/FIB/Y/ <u>FII/X4</u>	GEN, STR, TET, SUL, TET, NAL, ENR
CMUL1076	Urine	1	Nini Hospital (Tripoli)	2013	B2	ST1011	CMY-2	4	<u>FIB/X4</u>	KAN, STR, CHL, TET, SUL, NAL, ENR
CMUL834	Stool	77	Hôtel-Dieu de France (Beirut)	2017	A	NT	CMY-2	4	FIA/FIB/W/T/ <u>X1/Y/FII/X4</u>	STR, CHL, TET, SUL, TET
CMUL832	Stool	84	Hôtel-Dieu de France (Beirut)	2017	A	ST2705	CMY-2	8	11/X1/FII/ <u>FIB/X4</u>	TET, SUL, TET
CMUL1075	Urine	65	Hamidi Medical Center (Tripoli)	2018	D	NT	–	8	<u>FII/Y/X4</u>	KAN, CHL, SUL, TET, NAL, ENR
CMUL1030	Stool	50	El Youssef Hospital Center (Halba)	2019	A	NT	CTX-M-15	6	11/ <u>X4</u>	GEN, KAN, STR, CHL, TET, SUL, TET, NAL, ENR

MLST, multilocus sequence typing; NT, not typeable (combination of alleles not available in the Achtman database); MIC, minimum inhibitory concentration; GEN, gentamicin; STR, streptomycin; TET, tetracycline; SUL, sulfonamides; TMP, trimethoprim; NAL, nalidixic acid; ENR, enrofloxacin; KAN, kanamycin; CHL, chloramphenicol.

^a *mcr-1*-carrying plasmids are underlined. Incompatibility groups were determined using a PBRT Kit (Diatheva, Fano, Italy).

Funding

This work was supported by internal funding of the French Agency for Food, Environmental and Occupational Health & Safety (ANSES), and by a grant from the Hamidi Medical Center (Tripoli, Lebanon). HA-M was supported by a fellowship from AZM & Saadeh Association, Lebanese Association for Scientific Research (LASER) and Université de Lyon.

Competing interests

None declared.

Ethical approval

This study was approved by the Azm Center/Lebanese University Ethical Committee [document CE-EDST-3-2018], authorised by the Lebanese Ministry of Public Health.

Acknowledgments

The authors thank Taha Abdou, Asmaa Alloush, Mariam Yehya, Imane Darwish, Mariane Ecco, Houssein Khouja and Nancy Mostafa for helpful assistance.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.jgar.2019.08.019>.

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Received 19 June 2019

Available online 4 September 2019

3. Resistance to colistin in Lebanese poultry at slaughterhouse

Colistin is a last-resort antibiotic, which have been used widely in veterinary medicine for over 50 years, but neglected in humans due to its nephron- and neurotoxicity [255]. Consequently, colistin quantities consumed for food animals vastly exceed those used in humans, and resistant isolates first emerged in the veterinary field [256]. Liu *et al.* identified the first plasmid-mediated colistin-resistant gene from chicken isolates in 2015 [38]. This discovery was soon after followed by an important number of studies worldwide which revealed *mcr-1* to be widespread in isolates from poultry, swine and environmental sources. The plasmid location of *mcr-1*, mostly on the highly transferable IncX4 and IncI2 plasmids, also triggered worldwide concern about the prospect of horizontal transfer of this gene from animal to humans, in which colistin is an important last-resort molecule [254]. Firstly detected by Dandachi *et al.* in 2015, *mcr-1* gene has also been identified in poultry, swine and water sources in Lebanon [206, 236, 245-249]. Nevertheless, these studies gave very little information on the population structure of *E. coli* carrying this resistance and on their genetic support. This prompted us to look for colistin-resistance in poultry samples originating from 32 farms throughout the country but all slaughtered in one place in North Lebanon. All non-duplicate colistin-resistant isolates were kept for further characterization, independently of the presence or absence of a *mcr* gene. Isolates were fully sequenced using the Illumina technology, which allowed fine analyses of resistance genes as well as the population structure of colistin-resistant *E. coli*. Our data revealed a high prevalence of *mcr-1*-mediated colistin-resistance in poultry farms, urging authorities to implement antibiotic stewardship programs in Lebanon. Interestingly, a direct link was established with our previous study on *mcr-1*-positive *E. coli* in clinical isolates. Indeed, one clinical isolate collected in 2013 belonged to ST1011, an ST typically associated to poultry in the present study, and more generally in animals, thus strongly suggesting a transfer from animals to humans.

Article 2: to submit in the research topic “Zoonotic microorganisms and spreads of acquired polymyxin resistance determinants” of Frontiers in Microbiology journal.

**High prevalence of *mcr-1* mediated colistin-resistance in poultry in Lebanon due to IncX4-
and IncI2-carrying plasmids**

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Running title: *mcr-1* in Lebanese poultry

Keywords: *mcr-1*, poultry, Lebanon, IncX4, IncI2

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Abstract

Poultry and poultry meat are important contributors to the global antimicrobial burden. This sector has been regularly pointed out because of the high prevalence of resistance to Extended-Spectrum Cephalosporins (ESC), but the *mcr-1* has also been detected since its discovery in 2015. In Lebanon, the first *mcr-1*-positive *E. coli* found in the poultry sector dates back to 2015. Since then, a few Lebanese reports of increasing proportions of *mcr-1*-positive *E. coli* have been reported in poultry, swine, humans and the environment, but the population structure of *E. coli* as well as the plasmids carrying the *mcr-1* gene mostly remained unknown. This study aimed at determining the prevalence of *mcr*-positive *E. coli* in poultry originating from numerous farms across the country, but slaughtered in the same place. We report here that 27/32 (84.4%) presented at least one *mcr-1* positive sample. A total of 84 non-duplicate *E. coli* were collected, of which 62 presented the *mcr-1* gene. Numerous associated resistances were identified, including to ESC through the presence of *bla*_{CTX-M} genes, mostly *bla*_{CTX-M-3} and *bla*_{CTX-M-65}, which differ from the *bla*_{CTX-M-15} usually found in humans. The *mcr-1* gene was mostly carried by IncX4 (n=36) and Inc12 (n=24) plasmids, which are both known for their efficient transfer capacities. A high genetic diversity was detected, arguing for the lack of contamination during the slaughter process. ST744 and ST1011 were the most widely identified clones, which have been both regularly associated to *mcr-1* carrying *E. coli* and to the poultry sector. The wide dissemination of colistin resistance, coupled to resistance to ESC and numerous other molecules, should urge authorities to implement efficient guidelines for the use of antibiotics in the poultry sector in Lebanon.

1. Introduction

Since the discovery of the plasmid-mediated colistin-resistance gene *mcr-1* in 2015 [38], this gene has been extensively described in the poultry sector worldwide [257]. In line with the reports of colistin-resistance in healthy animals, the presence of this resistance has also been detected in retail meat, suggesting a possible transfer to humans through under-cooked meat or cross-contamination [258, 259]. Moreover, most of the studies showed the presence of associated resistances, including high proportions of resistances to Extended-Spectrum Cephalosporins (ESC) [260, 261]. In the poultry sector, *mcr-1* gene has mostly been found located on IncX4 and IncI2 plasmids, and to a lesser extent on IncHI2 plasmids [262]. The usually high transfer capacity of both IncX4 and IncI2 most probably explain their wide geographical dissemination and their occurrence in a large variety of hosts, both human and animals. Colistin-resistance has often been studied under the prism of plasmid-mediated resistance, so that only a few studies reported the characterization of *mcr*-negative isolates and the role of PmrAB and PhoPQ mutations [263].

In Lebanon, the first case of *mcr-1*-mediated colistin resistance was reported in 2015 in poultry [245]. This *mcr-1*-positive *E. coli* was recovered from one rectal swab over 982 samples (0.1%) performed in 49 farms for surveillance purposes. In 2017, 23 over 114 fecal samples from swine were resistant to colistin due to the presence of the *mcr-1* gene, and four of these isolates co-harbored resistance to extended-spectrum cephalosporins (ESC) [246]. Between 2017 and 2018, 88/93 (94.6%) fecal samples collected from the three major poultry farms in Lebanon presented the *mcr-1* gene, among which 35.5% co-harbored a *bla_{CTX-M}* gene [247]. The presence of the *mcr-1* gene was also detected in water samples, from either irrigation water or the Mediterranean sea [248, 264], suggesting environmental contamination. Finally, *mcr-1* has also been reported in human clinical isolates, among which one had been collected in 2011 [226]. The plasmidic location of the *mcr-1* gene has rarely been investigated: it was found located on an IncX4 plasmid in human isolates, and on IncX4 and IncI2 in water isolates [226, 248]. Likewise, data on the population structure hosting *mcr-1*-mediated colistin resistance remains largely unknown.

The goal of this study was thus to look for the presence of colistin-resistance in samples collected from 32 farms located in three governorates: Akkar, North Lebanon, and Mount Lebanon. Based on both phenotypic and molecular analyses (including next-generation sequencing), we characterized the population structure of colistin-resistant *E. coli*, the genetic support of colistin-

resistance (*mcr*-dependent of *mcr*-independent resistance) and the plasmids carrying the *mcr* genes in order to lay the foundations for a better understanding of colistin-resistance spread in poultry, but also in humans and the environment.

2. Material and Methods

2.1. Ethics.

This investigation was approved by the Azm Center/Lebanese University ethical committee (document CE-EDST-3-2018), authorized by the Lebanese Ministry of Public Health.

2.2. Bacterial isolation and identification.

Between May and August 2018, poultry samples were collected in one big slaughterhouse in North Lebanon. Animals came from 32 individual farms located in seven districts from three governorates: Akkar, North Lebanon, and Mount Lebanon (**Figure 12**). From each farm, five different samples were collected: three ceaca and two necks (carcasses) were randomly taken with all precautions needed to avoid inter-sample contamination. All samples were put in plastic bags, conserved at 4°C and rapidly transported to the laboratory. Resistant Enterobacterales were isolated on MacConkey agar (Bio-Rad, Hercules, CA, USA) supplemented with colistin (3.5 mg/L) (Sigma-Aldrich, St Louis, MO, USA). Selective plates were incubated at 37°C for 24 h. One presumptive *E. coli* colony was arbitrarily selected from each selective plate. Identification of isolates was performed using matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI TOF MS) (VITEK MS Version 3.0, bioMérieux, Marcy L'Etoile, France).

2.3. Antibiotic susceptibility testing and phenotypic characterization.

Susceptibility testing was performed by the disc diffusion method on Mueller–Hinton agar according to the guidelines of the Antibiogram Committee of the French Society for Microbiology (CA-SFM) (www.sfm-microbiologie.fr). The *E. coli* ATCC 7624 strain was used as the quality control. A total of 16 beta-lactam (amoxicillin, piperacillin, ticarcillin, amoxicillin-clavulanic acid, piperacillin-tazobactam, ticarcillin-clavulanic acid, cefalotine, cefuroxime, cefotaxime, ceftiofur, ceftazidime, ceftiofur, cefepime, cefquinome, aztreonam, and ertapenem) and 14 non-beta-lactam (tetracycline, kanamycin, tobramycin, gentamicin, amikacin, apramycin, netilmicin, streptomycin,

florfenicol, chloramphenicol, sulfonamides, trimethoprim, nalidixic acid, and enrofloxacin) antibiotics of both veterinary and human interest was tested. Minimum inhibitory concentrations (MICs) was determined by broth microdilution for colistin, according to European Committee for Antimicrobial Susceptibility Testing (EUCAST). The presence of the colistin resistance genes *mcr-1* to *mcr-5* was systematically screened by multiplex PCR.



Figure 12: Map of the districts where sampled were performed.

2.4. Molecular typing of the isolates.

The detection of the major *E. coli* phylogenetic groups (A, B1, B2, or D) was performed as recently described by Doumith *et al.* [265]. PFGE was performed on all isolates using the restriction enzyme *Xba*I. Electrophoresis was conducted in a CHEF Mapper XP system using 6V/cm at 14°C

for 24 h, with pulse times ranging from 10 to 60 sec using an angle of 120°C. PFGE results were interpreted according to international recommendations [266].

2.5. Whole-genome sequencing.

DNA was extracted using the NucleoSpin Microbial DNA extraction kit (Macherey-Nagel, Hoerd, France) according to the manufacturer's instructions. Library preparation was performed using the Nextera XT technology and sequencing was performed on a NovaSeq instrument (Illumina, San Diego, USA). After sequencing, reads were quality trimmed and *de novo* assembled using Shovill 1.0.0 and the quality of assemblies was assessed using QUAST v4.5. STs, resistance genes and plasmid content were determined using the CGE online tools MLSTFinder 2.0.4, ResFinder 3.2 and PlasmidFinder 2.0.1 (<http://www.genomicepidemiology.org/>). The PmrA, PmrB, PhoP and PhoQ amino acid sequences were extracted from the assemblies of all isolates and compare using Clustal Omega with the *E. coli* K12 reference strain (NP_418537.1 for PmrA, NP_418536.1 for PmrB, NP_415648.1 for PhoP, and NP_415647.1 for PhoQ).

2.6.Characterization of the *mcr-1*-carrying plasmids.

The plasmidic content was determined from the NGS data using PlasmidFinder 2.0.1 (<http://www.genomicepidemiology.org/>). Plasmids carrying the ESBL/pAmpC genes were detected using PFGE-S1 gels (6V/cm for 20 hours with an angle of 120° at 14°C with pulse times ranging from 1 to 30 seconds) followed by Southern blot using adequate probes as previously described [266]. Plasmid co-localization was assessed by comparison between the bands corresponding to the resistance gene and those corresponding to the Inc type of the plasmid.

When Southern blots did not lead to interpretable results and for all isolates that could not be typed by PFGE (smearing profile), the plasmid of interest was transferred by conjugation in an *E. coli* J53 rifampicin-resistant recipient strain. Conjugation was performed in liquid medium using rifampicin and cefotaxime (5 mg/L) or colistin (2 mg/L) to select transconjugants (TC). Only TC presenting a unique plasmid, as assessed by S1-PFGE, were further characterized by PBRT and Southern blots as described above.

2.7. Phylogenetic analysis.

The cgMLST (core genome multi-locus sequence type) was extracted from the whole genome sequence (WGS) data. The pan-genome was determined and core gene alignments were generated, for each collection, with Roary v. 3.11.0 [267] using a Protein BLAST identity of 80% and a core

definition of 90%. In the first step all assemblies were annotated *de novo* with Prokka using default settings [268]. The Prokka annotations were provided to Roary as input; in turn, Roary produced a gene presence/absence matrix, a multi-FASTA alignment of core genes using PRANK v.0.140603 and a tree based on the presence and absence of accessory genes using FastTree v.2.1.8 [269, 270]. Subsequently, recombination was removed with gubbins v2.3.4 and a maximum likelihood tree was constructed from the core gene alignment produced by Roary using RAxML v.8.2.8 with 100 bootstrap repeats. Pairwise single nucleotide polymorphism (SNP) distances were calculated from core genome alignments generated by Roary using snp-dists (<https://github.com/tseemann/snp-dists>). The SNP distance matrix is provided as Supplemental Table 1. The resulting tree for both analysis/collections were visualized using iTol v.5.5.1 (<http://itol.embl.de/itol.cgi>) [271].

2.8. Accession number(s).

The whole genome shotgun project will be deposited in DDBJ/EMBL/GenBank and a BioProject accession number will be obtained.

3. Results

3.1. Detection of colistin-resistance.

All presumptive colistin-resistant *E. coli* isolated on selective plates were characterized according to their resistance phenotype, *mcr* resistance gene(s), phylogroup and PFGE, and only non-duplicate isolates were kept for further studies. Among the 32 farms tested, 27 presented at least one colistin-resistant isolate (27/32, 84.4%), with MICs ranging from 4 to 36 mg/L. Since several different isolates were retrieved from one farm (up to 12), and also from one animal (up to three), a total of 84 colistin-resistant isolates were collected (supplemental Table S1).

3.2. Plasmid-mediated colistin-resistance.

The *mcr-1* gene was detected in 62 *E. coli* isolates (62/84, 73.8%) coming from 21 different farms, while no other *mcr* genes were detected. Two copies of the gene were found in one isolate (CMUL??), located on the same contig. The plasmidic location of the *mcr-1* gene was proved in 61/62 of the isolates. The *mcr-1* gene was mostly carried by an IncX4 plasmid (n=36), followed by an IncI2 (n=24) and IncHI2 (n=1) plasmid.

3.3. Chromosome-mediated colistin-resistance.

Amino-acid variations in the PhoP, PhoQ, PmrA and PmrB were extracted from the WGS data of all *mcr-1*-positive and *mcr-1*-negative isolates (**Table S1, Table 6**). Modifications were found at one site in PhoP, 11 sites in PhoQ, 2 sites in PmrA and 6 sites in PmrB. Two modifications in PhoQ (3 isolates with the V228I modification, and one isolate with the T348I modification) were exclusively found in *mcr-1*-negative isolates. On the contrary, 11 modifications (one in PmrA and 10 in PhoQ) were found only in *mcr-1*-positive isolates (**Table 6**). Four modifications (H2R and D283G in PmrB, I44L in PhoP and A482T in PhoQ) were found in *mcr-1*-positive and *mcr-1*-negative isolates.

3.4. Associated resistance genes.

Among the 62 *mcr-1*-positive isolates, 30 presented an associated ESBL gene, including *bla*_{CTX-M-65} (n=15), *bla*_{CTX-M-3} (n=9), *bla*_{CTX-M-14} (n=2), *bla*_{CTX-M-15} (n=2), *bla*_{CTX-M-64} (n=1), and *bla*_{SHV-12}

(n=1) (**Figure 13**). Plasmidic AmpC genes were also detected in 17 isolates, with *bla*_{CMY-2} in 16 of them. All but one CTX-M-3-positive isolate also displayed CMY-2. On the contrary, only one CTX-M-65 and nine CMY-2 were identified among *mcr-1*-negative colistin-resistant isolates.

Independently of the genes responsible for colistin-resistance, isolates mostly presented resistance genes to aminoglycosides (75/84, 89.3%), tetracyclines (71/84, 84.5%), sulfonamides (66/84, 78.6%), trimethoprim (62/84, 73.8%) and phenicols (53/84, 63.1%). The corresponding genes were mainly *tet(A)*, *sul2*, *dfrA12* and *floR*, while aminoglycoside resistance was largely multifactorial. None of the isolates was resistant neither to ertapenem nor to amikacin (**Table 7**).

3.5. Population structure of the *mcr-1*-positive and *mcr-1*-negative *E. coli* isolates.

The 84 isolates belonged to 38 different STs (**Figure 13 and Table S1**). Two STs were predominant (ST93 (n=14) and ST1011 (n=11, including one SLV), while two were each found in five different isolates (ST744 and ST1140). The remaining isolates were identified in three or less isolates. Inside one ST, colistin-resistance was usually mediated by the same mechanism (either *mcr-1* or chromosomal mutations). However, six STs (ST10, ST48, ST155, ST744, ST1140 and ST1635) encompassed isolates that were resistant to colistin either through plasmid- or chromosome-mediated mechanisms.

Table 6: Amino-acid modifications in *mcr-1*-positive and *mcr-1*-negative isolates.

	PmrA		PmrB					PhoP	PhoQ												
	G29S	V129L	H2R	A242V	D283G	V351F	Y358N	A360V	I44L	I175F	V228I	A341T	N346K	T348I	T348N	V373I	V386L	A390T	E464D	L467M	A482T
<i>mcr-1</i> -positive isolates	1	2	61	15	27	15	11	2	21	2	0	1	1	0	2	1	1	1	19	2	30
<i>mcr-1</i> -negative isolates	1	0	21	1	17	2	7	2	4	0	3	0	0	1	0	0	0	0	0	0	0
Total	2	2	82	16	44	17	18	4	25	2	3	1	1	1	2	1	1	1	19	2	30

Table 7: Associated resistance phenotypes.

	<i>mcr-1</i> -positive isolates (n=62)		<i>mcr-1</i> -negative isolates (n=22)		Total (n=84)	
	No.	Percentage	No.	Percentage	No.	Percentage
Streptomycin	31	50.0	17	77.3	48.0	57.1
Kanamycin	44	71.0	15	68.2	59.0	70.2
Amikacin	0	0.0	0	0.0	0.0	0.0
Apramycin	1	1.6	0	0.0	1.0	1.2
Gentamicin	26	41.9	9	40.9	35.0	41.7
Tobramycin	24	38.7	9	40.9	33.0	39.3
Netilmicin	11	17.7	6	27.3	17.0	20.2
Chloramphenicol	42	67.7	11	50.0	53.0	63.1
Florfenicol	35	56.5	9	40.9	44.0	52.4
Tetracycline	51	82.3	17	77.3	68.0	81.0
Sulfonamides	46	74.2	18	81.8	64.0	76.2
Trimethoprim	47	75.8	17	77.3	64.0	76.2
Nalidixic acid	53	85.5	17	77.3	70.0	83.3
Enrofloxacin	47	75.8	9	40.9	56.0	66.7

Among the STs presenting five representatives or more, only ST93 formed a homogeneous group with isolates differing by 14 to 72 SNPs. On the other side, ST744, ST1011 and ST1140 were split in two groups, each differing by >900, >2700 SNPs and >600 SNPs, respectively (Table S2). Number of SNPs were also detailed for all isolates sharing the same ST. ST359 presented two highly similar isolates (25 SNPs) coming from two different animals from the same farm, while ST616 and ST1196 each comprised two isolates from different farms that only differed by 3 SNPs. On the contrary, ST48 and ST6856 each included two isolates from different farms, respectively differing by >5000 and 391 SNPs. ST4162 comprised three isolates from different farms, of which two (farms F1 and F32) were fully identical, while the third one (F8) differed by 27 SNPs. ST155 encompassed three isolates from three different farms: isolates from farm F16 and F29 displayed the *mcr-1* gene and differed by 34 SNPs, while the isolate from F18 was *mcr-1*-negative and differed from the two others by >5000 SNPs. ST1638 comprised three isolates: two were from the same farm F9 and were highly similar (5 SNPs) while the third one from farm F22 clearly diverged (>5000 SNPs). Finally, ST10 included three isolates from three different farms that were not similar, isolates from farm F2 and F20 differing by >200 SNPs, and further differing by >6000 SNPs from the isolate from farm F26.

Tree scale: 100000

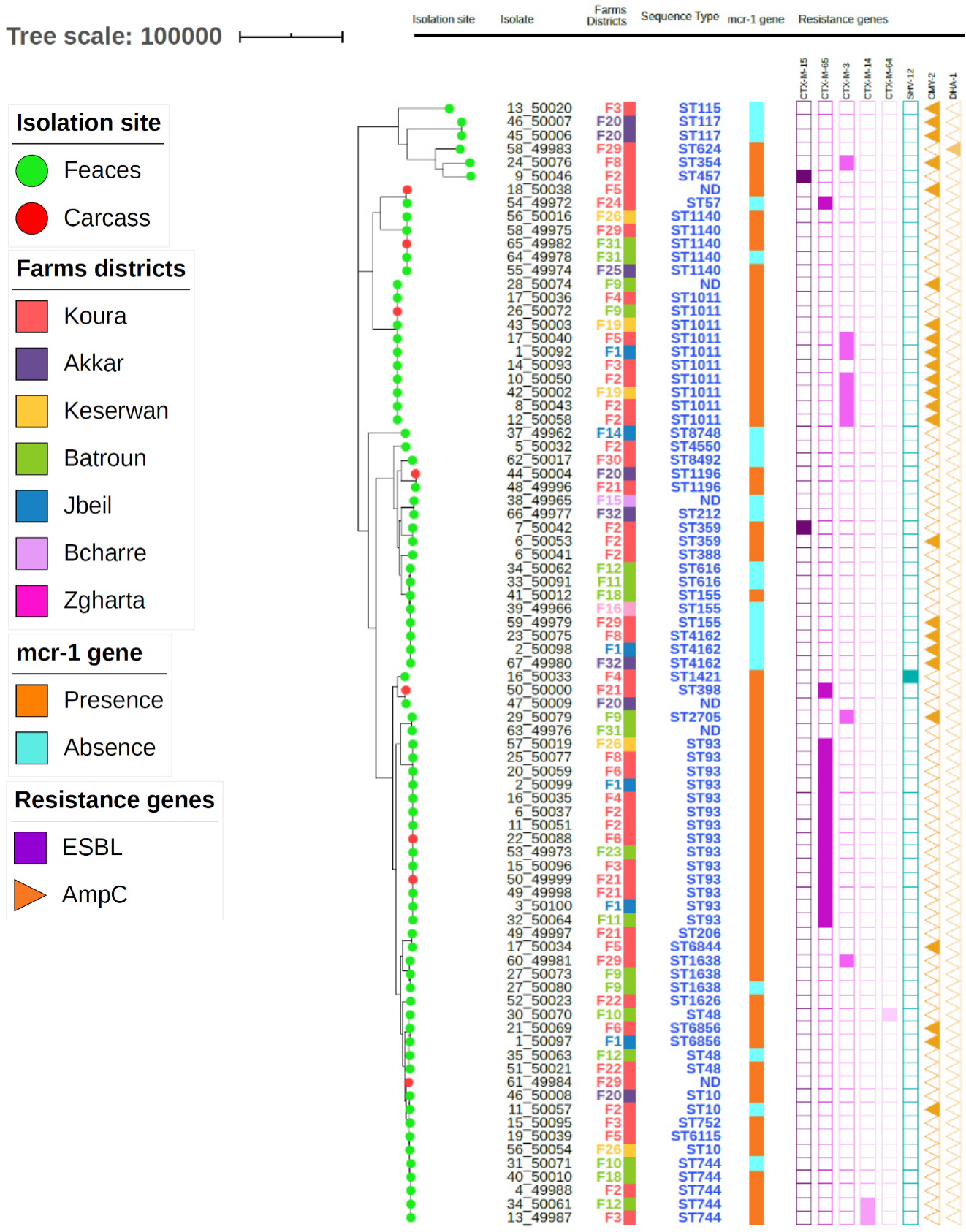


Figure 13: Maximum-likelihood phylogeny of *E. coli* isolates from poultry. The phylogenetic tree was constructed based on nucleotide sequence alignments of the core genes. Metadata columns include farm district, STs, presence/absence of the *mcr-1* gene and resistance genes.

Discussion

This study reveals a high prevalence of colistin-resistance in poultry farms in Lebanon (27/32, 84.4%), with the wide dissemination of the *mcr-1* gene (21/27 positive farms). This high prevalence can most probably be explained by the use of colistin in this country in the poultry sector, without implementation of antibiotic stewardship programs. Moreover, 49/84 *E. coli* isolates additionally carried a pAmpC/ESBL gene, while 28 of the 35 remaining ones could be considered as multi-resistant (resistance to three or more antibiotic families). This suggests that colistin-resistance would be co-selected in the poultry gut by the use of most of the veterinary-licensed antibiotics. The *bla*_{ESBL} genes found in these isolates (*bla*_{CTX-M-3}, *bla*_{CTX-M-65}) do not betray a human origin, where the *bla*_{CTX-M-15} gene, which was only detected in two poultry isolates, is clearly dominating.

The mechanism of colistin-resistance in *mcr*-negative isolates was not elucidated here. Numerous mutations were observed compared to the K12 reference strain, most of which were detected in both *mcr*-positive and *mcr*-negative isolates, if not only in *mcr*-positive ones. The rare mutations that have been associated with resistance (R81S in PmrA; T156K, A159V, G161V in PhoP; E375K in PhoQ) were not detected here [272]. Likewise, the N346K modification highlighted by Luo *et al.* as a possible colistin-resistance related modification [273] was found here associated to *mcr-1*-positive isolates. We might hypothesize that resistance-related in PmrAB and PhoPQ may also arise in *mcr-1*-positive isolates, in order to increase MICs, but the highest MICs (≥ 16 mg/L) did not correlate with a specific modification pattern. Moreover, several modifications detected here (S29G in PmrA, H2R in PmrB, D283G in PhoQ) have also been described in susceptible isolates [273]. Consequently, further studies are needed to describe other genes that may be associated to colistin-resistance.

The population structure described here is very diverse, with 38 different STs detected. Massive contamination of the animals inside the slaughterhouse – all animals were sampled in the same slaughterhouse, even though poultry came from 32 different farms – is thus very unlikely. A one-source contamination cannot however be excluded in the cases of ST616 and ST1196 (two isolates each, coming from different farms) and ST93 (recovered in 14 isolates collected from 10 different farms), which only differed by a few SNPs. Clonal spread of *mcr-1*-carrying ST93 has been described in companion animals in China [274], suggesting that this clone may

survive in the environment (a clinic or a slaughterhouse) before further dissemination. Interestingly, the *mcr-1* gene has also been detected in ST93 *E. coli* coming from a human patient in Uruguay and in Finland [275, 276]

Despite the high clonal diversity, two main STs (ST744 and ST1011) were detected. These STs have already been both concomitantly reported in *mcr-1*-positive isolates from poultry origin in Czech Republic and in colistin-susceptible poultry isolates from Algeria [277, 278]. ST1011 has also been reported in *mcr-1*-positive environmental samples of swine farms in Germany, in pigs in China and Belgium, in poultry in Egypt and in companion animals in China, as well as in colistin-susceptible poultry meat isolates in Egypt [274, 279-283]. Consequently, the *mcr-1*-positive ST1011 *E. coli* isolate that was identified in 2013 in a Lebanese patient may well have a poultry (or at least an animal) origin [226]. ST744 has also been reported in *mcr-1*-positive isolates from poultry in Romania and from swine in China, from *mcr-3*-producing *E. coli* in veal calves in France [261, 279, 284], but also from *mcr-1*-positive clinical isolates in Portugal [285], suggesting that this clone may be particularly prone to harbor colistin-resistance.

All *mcr-1* genes were detected on plasmids, mostly on IncX4 (n=36) but also on IncI2 (n=24). IncX4 is the main plasmid worldwide [191]. IncX is a family of small narrow-range plasmids, and experiments proved that it has a very weak fitness cost and high transfer frequencies at 30°C, allowing its wide spread in environmental settings [286]. IncI2 plasmids are also spreading efficiently, and a recent study proved *in situ* in a mouse model that this plasmid family has a particularly high capacity to transfer DNA in the gut [287]. This high transfer capacity of both IncX4 and IncI2 plasmids may explain the occurrence of *mcr-1* in such a high number of genetic background, and the relative absence of clonal transmission on farm, since animals from the same farm mostly carried different *E. coli* clones.

Conclusion

We report a high prevalence of *mcr-1*-positive *E. coli* in poultry farms in Lebanon. The colistin-resistance gene *mcr-1* has widely disseminated in the poultry sector in diverse genetic backgrounds on IncX4 and IncI2 plasmids. On the contrary, no other *mcr* gene disseminated

until now in *E. coli* from poultry. The STs detected, as well as the *bla*_{ESBL} genes, suggest an animal epidemiology. These STs could further spread to humans, as suggested by the occurrence of ST1011 both in poultry, where it is a major clone, and in a human patient in Tripoli. The wide dissemination of colistin resistance, coupled to resistance to ESC and numerous other molecules, should urge authorities to implement efficient guidelines for the use of antibiotics in the poultry sector in Lebanon.

Acknowledgements

The authors would like to thank Taha Abdou, Mariam Yehya, Anas Al-Mir, Adel Al-Mir, Dr. Basel Halabieh, Veronique Metayer, Raquel Garcia Fierro and Wilco PM poultry slaughterhouse and processing plant workers for their assistance in sample collection and processing.

Conflict of Interest Statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Financial support

This work was supported by internal funding of the French Agency for Food, Environmental and Occupational Health & Safety (ANSES), and by a grant from the Hamidi Medical Center in Tripoli, Lebanon. H.A.M. was supported by fellowships from Association AZM & Saadeh, Lebanese Association for Scientific Research (LAsER) and Université de Lyon.

Author contribution statement

MoH and MO designed the experiments and supervised the sampling campaign. HAM performed the experiments. AD performed all WGS analyses. MaH, HAM and JYM analysed the data. MaH drafted the manuscript. All authors approved the final version of this manuscript.

4. Resistance to broad-spectrum cephalosporins, carbapenems and colistin in Lebanese food workers: a longitudinal study.

ESC, carbapenems and colistin are classified as critically important antibiotics, and resistance to these drugs have been recurrently reported worldwide, including in Lebanon. MDR *E. coli* have been identified in Lebanese hospitals, in hospital wastewater and in animals [200, 206, 242, 288]. Recently, Diab *et al.* reported a high-rise dissemination of ESBL-producing *Enterobacteriaceae* in livestock and in water sources in Lebanon. It is was learned as well that raw bovine milk and water estuaries were important reservoirs not only for CTX-M-15 producing isolates but also for different carbapenemases with a majority of OXA-48 enzymes [112, 207, 208].

In our previous studies conducted in the clinical and the poultry sectors, we highlighted the dissemination of *mcr-1*-positive *E. coli* isolates co-producing ESBL/AmpC genes. Globally, a high genetic diversity of *E. coli* STs was observed, with potential overlap as seen for ST1011, which was identified in both clinical and animal isolates. In addition to that, the *mcr-1* gene was located on IncX4 plasmids in human isolates, and on IncX4 and IncI2 plasmids in poultry isolates, suggesting potential intra- and inter-sector dissemination.

In Lebanon, a national AMR awareness campaign conducted in 2017 drew attention on the poor knowledge on the AMR crisis, but also on the absence of legislation on antibiotic consumption that can be easily obtained in pharmacy without prescription. This misuse is undoubtedly a major driver of AMR spread in the community. The conjunction between high proportions of AMR in Lebanon and the selection pressure of antibiotic use in the community prompted us to investigate AMR among healthy adults working in the food sector in North Lebanon. We thus performed one of the very few longitudinal studies outside the travelers population [289-294] by collecting fecal samples from 84 workers at two time points 6 months apart. Molecular determinants of ESC-, carbapenem- and colistin-resistance as well as transmission dynamics in the community were studied using whole-genome sequencing. For a single individual, we showed a very low proportion of ESBL *E. coli* long-term carriage, but we highlighted a high dynamic of acquisition and loss of resistant bacteria leading to an overall high prevalence of ESBL-positive workers. This study points out once more that strong political and health ambitions are now required in Lebanon to decrease the AMR burden at the One Health scale.

Article 3: to submit in Journal of Antimicrobial Chemotherapy.

**Novel insights into the dynamic of ESC-, carbapenem-, and colistin-resistant
Escherichia coli in the community in Lebanon**

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Running title:

Keywords: AMR, epidemiology, One Health, WGS, Lebanon

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Abstract

Resistance to Extended-Spectrum Cephalosporins (ESCs) in *Escherichia coli* has become a reliable indicator of the AMR burden in human medicine, but also in animals and the environment. Intestinal colonization with ESC-resistant (ESC-R) *E. coli* is of crucial concern since this phenomenon silently contributes to further ESC-E spread. However, ESC-R carriage in the community is poorly documented, notably in low and middle-income countries (LMICs). We performed a WGS-based longitudinal study on 84 healthy food workers in North Lebanon at two time points (0-6 months), and characterized the dynamics of ESC-R lineages in each individual using WGS-based approaches. Our results revealed a high global carriage rate of MDR *E. coli* (60.7%), as well as the emergence of different carbapenemases (OXA-181, OXA-244, NDM-3), and suggested that the rate of *mcr-1* gene is still below 10% in the community. Inside one individual, WGS characterization only evidenced one case of long-term carriage over the two time points. On the contrary, 14 workers carried two different ESBL *E. coli* at t0 and t6, showing a strong dynamic of acquisition/loss of ESBL-positive isolates. Clonal transfer was also exemplified between individuals from the same bakery, while plasmid dissemination between genetic backgrounds seemed much less frequent. This dynamic is permitted by the high prevalence of this resistance in the community, pinpointing the urgent need to decrease the global AMR burden at the One Health scale.

Introduction

Antimicrobial resistance (AMR) threat has emerged as a major international health issue of the 21st century. Notably, resistance to Extended-Spectrum Cephalosporins (ESCs) in *Escherichia coli* has become a reliable indicator of the AMR burden in human medicine, but also in animals and the environment [98]. Besides, human infections due to ESC-resistant *E. coli* (ESC-R) further treated with carbapenems (CPs) led to the emergence and spread of carbapenemase genes, including until endemicity in some geographical areas. Recently, plasmid-mediated resistance to colistin, a last-resort antibiotic against CP-resistant *E. coli* (CP-R), has been identified and is increasingly reported from human clinical isolates.

Intestinal colonization of human individuals with ESC-R is of crucial concern since this phenomenon silently contributes to further ESC-R spread within human communities. ESC-R carriers are at higher risk of developing ESC-R-associated infections, and some critical situations for increased ESC-R carriage were highlighted, such as international travels or hospitalization. ESC-R also persist for a variable period of time in patients after hospitalization. For instance, persistence of ESC-R carriage was shown to range from 3.4 to 13.4 months after hospital discharge in France [295], and lasted up to 59 months in the case of a Swedish nosocomial outbreak [296].

In the community, carriage of ESC-R is however poorly documented globally, with even fewer information originating from low and middle-income countries (LMICs). Also, contrary to healthcare settings where genomic characterization of ESC-R lineages is usually available, most surveys in the community report ESC-R prevalence data without further molecular insights [123, 297]. Until now, most longitudinal studies were performed on travelers, reporting global data on ESC-R carriage and persistence rates after return, but without tracing specific ESC-R lineages in definite individuals over time [289-294]. In this respect, long-term colonization by ESC-R has been exemplified in Japanese food handlers, highlighting the persistence of a clonal lineage of ESC-R isolates of serogroup O25[298]. Recently, Van den Bunt *et al.* investigated duration of ESC-R carriage in the Dutch community using whole-genome sequence (WGS) analysis, and identified travels and low-level hygiene as risk factors for long-term colonization [299].

Thus, we intended to fill in this knowledge gap by performing a WGS-based longitudinal study in food workers in North-Lebanon. Chamoun *et al.* reported 30.8% to 33.6% of ESC-E prevalence in 2011-2013 in 16 hospitals throughout the country [200, 288, 300] whereas data

in the Lebanese community are almost absent. Here, we evaluated the prevalence of ESC-R carriage in 84 healthy people working in three Lebanese pastries at two time-points (0-6 months), and characterized the dynamics of ESC-R lineages in each individual using WGS-based approaches. Besides, we also reported additional information on CP- and colistin-resistant (COL-R) *E. coli* lineages colonizing those people.

Materials and Methods

Ethics. This study received the approval of the Lebanese ethics committee (CE-EDST-3-2018).

Study design, bacterial isolation and identification. The longitudinal study was performed on 84 healthy adults working in three major pastries in Tripoli, North Lebanon. A standard questionnaire was completed by interviewing each worker, who had given informed consent, in order to obtain information about sex, age and the eventual use of antibiotics in the past three months. Those people were volunteers and asked to self-collect rectal swabs according to the study design, i.e. rectal swabs were collected twice at a 6 months' interval (t0 and t6). Resistant Enterobacterales were isolated on MacConkey agar (Bio-Rad, Hercules, CA, USA) supplemented with ceftazidime (2 mg/L) ertapenem (2 mg/L), and colistin (3,5 mg/L) (Sigma-Aldrich, St Louis, MO, USA), respectively. Selective plates were incubated at 37°C for 24 h. One presumptive *E. coli* colony was arbitrarily selected from each selective plate. Identification of isolates was performed using matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI TOF MS) (VITEK MS Version 3.0, bioMérieux, Marcy L'Etoile, France).

Antibiotic susceptibility testing and phenotypic characterization. Susceptibility testing was performed by the disc diffusion method on Mueller–Hinton agar according to the guidelines of the Antibiogram Committee of the French Society for Microbiology (CA-SFM) (www.sfm-microbiologie.fr). The *E. coli* ATCC 7624 strain was used as the quality control. A total of 16 beta-lactam (amoxicillin, piperacillin, ticarcillin, amoxicillin-clavulanic acid, piperacillin-tazobactam, ticarcillin-clavulanic acid, cefalotine, cefuroxime, cefotaxime, ceftiofur, ceftazidime, cefoxitin, cefepime, cefquinome, aztreonam, and ertapenem) and 14 non-beta-lactam (tetracycline, kanamycin, tobramycin, gentamicin, amikacin, apramycin, netilmicin, streptomycin, florfenicol, chloramphenicol, sulfonamides, trimethoprim, nalidixic acid, and enrofloxacin) antibiotics of both veterinary and human interest was tested. Minimum inhibitory

concentrations (MICs) was determined by broth microdilution for colistin, according to European Committee for Antimicrobial Susceptibility Testing (EUCAST).

Detection of resistance genes. The *bla*_{CTX-M} genes were detected using a CTX-M group specific multiplex PCR [301]. On CTX-M-negative ESBL isolates, the *bla*_{TEM} and *bla*_{SHV} genes were screened by PCR and sequenced.[302] The presence of the colistin resistance genes *mcr-1* to *mcr-5* was systematically screened by multiplex PCR [303]. The presence of the carbapenemase genes *bla*_{OXA-48-like}, *bla*_{NDM} and *bla*_{KPC} was detected by a specific PCR.[304]

Phylogeny and clonality. The detection of the major *E. coli* phylogenetic groups (A, B1, B2, or D) was performed as recently described by Doumith *et al.* [265]. PFGE was performed on all isolates using the restriction enzyme *Xba*I. Electrophoresis was conducted in a CHEF Mapper XP system using 6V/cm at 14°C for 24 h, with pulse times ramping from 10 to 60 sec using an angle of 120°C. PFGE results were interpreted according to international recommendations.

Whole-genome sequencing. DNA was extracted using the NucleoSpin Microbial DNA extraction kit (Macherey-Nagel, Hoerd, France) according to the manufacturer's instructions. Library preparation was performed using the Nextera XT technology and sequencing was performed on a NovaSeq instrument (Illumina, San Diego, USA). After sequencing, reads were quality trimmed and *de novo* assembled using Shovill 1.0.0 and the quality of assemblies was assessed using QUAST v4.5. STs, resistance genes and plasmid content were determined using the CGE online tools MLSTFinder 2.0.4, ResFinder 3.2 and PlasmidFinder 2.0.1 (<http://www.genomicepidemiology.org/>).

Phylogenetic analysis. The cgMLST (core genome multi-locus sequence type) was extracted from the whole genome sequence (WGS) data. The pan-genome was determined and core gene alignments were generated, for each collection, with Roary v. 3.11.0 [267] using a Protein BLAST identity of 80% and a core definition of 90%. In the first step all assemblies were annotated *de novo* with Prokka using default settings [268]. The Prokka annotations were provided to Roary as input; in turn, Roary produced a gene presence/absence matrix, a multi-FASTA alignment of core genes using PRANK v.0.140603 and a tree based on the presence and absence of accessory genes using FastTree v.2.1.8 [269, 270]. Subsequently, recombination was removed with gubbins v2.3.4 and a maximum likelihood tree was constructed from the core gene alignment produced by Roary using RAxML v.8.2.8 using 100 bootstrap repeats. Pairwise single nucleotide polymorphism (SNP) distances were calculated

from core genome alignments generated by Roary using snp-dists (<https://github.com/tseemann/snp-dists>). The SNP distance matrix is provided as Supplemental Table 1. The resulting tree for both analysis/collections were visualized using iTol v.5.5.1 (<http://itol.embl.de/itol.cgi>) [271].

Results

Prevalence of ESC-, CP- and COL-resistance at population level. Eighty-four people working in three Lebanese pastries (bakery A, n=53; bakery B, n=27; bakery C, n=4) in Tripoli, Lebanon, were enrolled in this study. Information on sex, age and antibiotic exposure could be recorded for 80/84 individuals (95%), with a distribution of 34 females and 46 males aged 17 to 62 years old. All workers were healthy at the time of first sampling in July 2018 (t0) when they voluntarily provided a rectal swab. At that time, 33 people (33/80, 41.3%) had received antibiotics in the past three months (19 from bakery A, 11 from bakery B and 3 from bakery C). In February 2019, 70 out of the initial 84 workers provided a second rectal swab (t6), of which 45 originated from bakery A, 23 from bakery B and 2 from bakery C. All presumptive *E. coli* isolated on selective plates were characterized according to their resistance phenotype, resistance gene(s), phylogroup and PFGE, so that only non-duplicate isolates were kept for further studies.

Globally, 29/84 individuals (34.5%) were colonized by an ESC-R at t0, compared to 36/70 individuals (51.4%) at t6. Fifteen workers (15/70, 21.4%) presented an ESC-R isolate at both t0 and t6 (**Table 8 and Table S2**). In bakery A, the proportion of ESC-R-positive individuals at t0 or t6 only was 15.1% (8/53) and 24.4% (11/45), respectively, while 10 individuals were ESC-R positive at both time-points. In bakery B, the proportion of ESC-R-positive individuals at t0 or t6 only was 22.2% (6/27) and 34.7% (8/23), respectively, while 5 individuals were ESC-R positive at both time-points. In bakery C, two individuals were ESC-R-positive, at t6 only. Regarding CP-R and COL-R, no individual was positive at both time-points. Five people (5/84, 6.0%) were colonized by a CP-R and five (6.0%) by a COL-R at t0 only while two individuals (2/70, 2.9%) were colonized by a CP-R and one (1/70, 1.4%) by a COL-R at t6 only (**Table 8 and Table S2**).

Table 8: Characteristics of ESC-R, CP-R and COL-R *E. coli* isolates.

Person	Bakery	T0	T6
24	A	ST1722/OXA-244,CTX-M-15	-
28	A	ST131/CTX-M-15 ; ST665/CMY-2	-
44	A	ST7937/CMY-42	-
46	A	ST34/CTX-M-15 ; ST131/CTX-M-15	-
49	A	ST405/CTX-M-15	-
76	A	ST7401/CTX-M-27	<i>withdrawn</i>
80	A	ST69/CTX-M-15	<i>withdrawn</i>
85	A	ST58/SHV-12	<i>withdrawn</i>
25	A	-	ST131/CTX-M-15
26	A	-	ST69/CTX-M-15 ; ST131/CTX-M-15
36	A	-	ST940/OXA-181, CTX-M-15
40	A	-	ST3172/CTX-M-15
41	A	-	ST10/DHA-1
48	A	-	ST1727/CTX-M-15 ; ST7629/CMY-2
52	A	-	ST131/CTX-M-27
56	A	-	ST484/CTX-M-15
59	A	-	ST120/CTX-M-15
62	A	-	ST4553/CTX-M-15
63	A	-	ST940/OXA-181, CTX-M-15
21	A	ST517/CTX-M-15	ST5271/CMY-2
37	A	ST10698 /CTX-M-15	ST120/CTX-M-15
38	A	ST10698 /CTX-M-15	ST120/CTX-M-15
42	A	ST58/CMY-2	ST62/CTX-M-15
51	A	ST69/CTX-M-15	ST69/CTX-M-15
53	A	ST131/CTX-M-15	ST120/CTX-M-15
54	A	ST6335*(recA)/CMY-2	ST131/CTX-M-15
57	A	ST131/CTX-M-15	ST131/CTX-M-15
58	A	ST48/CTX-M-15	ST7401/DHA-1, CTX-M-27
61	A	ST31/CTX-M-15	ST5529/CTX-M-15
3	B	ST206/VEB-1	-
10	B	ST4242/CTX-M-3 ; ST6836/NDM-5 ; ST1303/MCR-1.1	-
16	B	ST155/CTX-M-15	-
18	B	ST10/CTX-M-15	-
71	B	ST155/CTX-M-15	<i>withdrawn</i>
72	B	ST155/CTX-M-15	<i>withdrawn</i>
19	B	ST10/MCR-1.1	-
5	B	-	ST394/CTX-M-15

7	B	-	ST34/CTX-M-15 ; ST746/SHV-12
13	B	-	STnd (6/11/4/8/8/2/2) /CTX-M-15
14	B	-	ST69/CTX-M-15 ; <u>ST56/MCR-1.1</u>
15	B	-	ST1284/CMY-42, CTX-M-15
20	B	-	ST174/CTX-M-15
69	B	-	ST3172/CTX-M-15
1	B	ST8149/CTX-M-15 ; ST540/OXA-181 ; <u>ST10/MCR-1.1</u>	ST4553/CTX-M-15
8	B	ST1303/CMY-2 ; ST940/OXA-181 ; <u>ST10/MCR-1.1</u>	ST372/CTX-M-15
6	B	ST8149/CTX-M-15	ST361/CTX-M-15
9	B	ST648/CMY-42	ST2076/CTX-M-15
11	B	<u>ST1303/MCR-1.1</u>	ST1303/CTX-M-15
17	B	ST167/CMY-42	ST1193/CTX-M-15
67	C	-	ST5442*(adk) /CTX-M-15
79	C	ST46/OXA-181	ST361/CTX-M-42

Highlighted in grey: CP-R, underlined: COL-R

Finally, three workers (#1, #8, #10) were simultaneously ESC-R, CP-R and COL-R positive at t0, all three from bakery B. Among them, at t6, individual #10 recovered a full negative status while individuals #1 and #8 remained ESC-R-positive only.

Molecular determinants of ESC-, CP- and COL-resistance. WGS analysis was systematically carried out on all positive isolates. In all, 80 *E. coli* isolates were investigated, e.g. 67 ESC-R, 4 CP-R, 3 combining ESC-R and CP-R, and 6 COL-R isolates (**Table 8**). COL-R was systematically due to the presence of the *mcr-1.1* gene. No other *mcr* gene was detected. CP-R was due to *bla*_{OXA-181} in 5 isolates, and *bla*_{OXA-244} and *bla*_{NDM-5} in one isolate each. ESC-R was principally due to *bla*_{ESBL} (n=58) but also *bla*_{AmpC} (n=11) or both genes (n=2). Among ESBL genes, *bla*_{CTX-M-15} (n=53) was predominant by far, followed by *bla*_{CTX-M-27} (n=3), *bla*_{SHV-12} (n=2) *bla*_{CTX-M-3} (n=1) and *bla*_{VEB-1} (n=1), whereas AmpC genes included *bla*_{CMY-2} (n=6), *bla*_{CMY-42} (n=5) and *bla*_{DHA-1} (n=2). Occasionally, different bacterial morphologies on the selective plates led to the identification of ESC-R and/or CP-R and/or COL-R *E. coli* isolates within the same individual (**Table 8 and Table S2**). Also, five isolates simultaneously possessed combinations of ESBL/AmpC or ESBL/carbapenemase genes.

Associated resistance genes. Over the 80 tested isolates, 10 CTX-M-15-producing *E. coli* isolates presented no other acquired resistance gene and a fully susceptible phenotype (Table S2). On the contrary, 32 isolates presented genes conferring resistance to tetracycline, sulfonamides/trimethoprim and to at least one aminoglycoside. Among these 32 isolates, 14 additionally presented a resistance to fluoroquinolones.

The *tet* resistance genes were detected in 47 isolates (58.0%; 33 *tet*(A), 13 *tet*(B), one *tet*(A)+*tet*(L)), *dfr* genes (including 16 *dfrA14* and 14 *dfr17*) in 47 isolates (58.0%) and *sul* genes (including 18 *sul2* and 12 *sul1+sul2*) in 43 isolates (53.1%). Resistance to aminoglycosides was conferred by a high diversity (27 combinations) of *aad*, *ant* and *aph* genes. Associated phenotypic resistances were found to streptomycin (n=31), gentamicin (n=10), tobramycin (n=10) and kanamycin (n=9). None of the isolates presented resistances to netilmicin, apramycin and amikacin.

Four out of the six isolates presenting the *mcr-1* gene co-harboured *tet* (n=4), *sul* (n=4), *dfr* (n=4), *qnr* (n=3), *aph* (n=2) and *floR* (n=4) genes, while two isolates carried no associated resistances. Over the seven carbapenemase-producing isolates, three co-harboured a *bla*_{CTX-M-15} gene, while the presence of *tet* (n=6), *sul* and *dfr* (n=5) and aminoglycoside-resistance genes were also detected.

Population structure of *E. coli* isolates. A wide diversity of genetic backgrounds of *E. coli* was observed, with the detection of 47 different STs (**Figure 14**). Only seven STs were found in more than two occurrences, namely ST131 (n=9), ST10 (n=5), ST69 (n=5), ST120 (n=4), ST1303 (n=4), ST155 (n=3) and ST940 (n=3). The ST131 isolates displayed the *fimH* variant H30 (n=4, all carrying the *bla*_{CTX-M-15} gene) or H41 (n=5, including four *bla*_{CTX-M-15} and one *bla*_{CTX-M-27} producers). Isolate #52 carrying the *bla*_{CTX-M-27} gene did not belong to the emerging C1-M27 clade.

Persistence of the same phenotype (ESC-R or CP-R or COL-R) within the same individual between t0 and t6 was only observed for ESC-R. Indeed, 15 individuals were ESC-R-positive at both time-points, compared to 34 individuals who were ESC-R positive at t0 or t6. Among these 15 individuals, only two (2/15, 13.3%) displayed the same ST/ESC-R gene combination, i.e. workers #51 and #57 (bakery A) carrying a ST131/CTX-M-15- and a ST69/CTX-M-15-producing *E. coli*, respectively. WGS data of these isolates proved that only one SNP was found between the two ST69 isolates, so that they could be considered the same *E. coli* clone. On the

contrary, the two ST131 isolates within individual #51 differed by 6721 SNPs, were of a different *fimH* type (H30 and H41), and therefore from different *E. coli* lineages.

More globally, pairwise single nucleotide polymorphism (SNP) distances calculated from core genome alignments revealed a SNP distance matrix (**Figure 14**) that recurrently highlighted discrepancies or similarities between *E. coli* isolates of the same ST. Indeed, four *mcr-1*-positive *E. coli* isolates found at t0 in bakery B belonged to ST10 (n=3) and ST1303 (n=2), and WGS analysis revealed perfect identity (no SNP difference) between isolates of the same ST. On the contrary, two other *E. coli* of the ST1303 (ST1303/CMY-2 and ST1303/CTX-M-15) were also found in workers from the same bakery, including one in the same person (#11) harboring the ST1303/*mcr-1* isolate. However, ST1303/CMY-2 and ST1303/CTX-M-15 differed from ST1303/*mcr-1* by 1500 and 2200 SNPs differences, respectively, highlighting different clones. Likewise, ST940/OXA-181 *E. coli* (bakery A) presented no SNP difference at t6 in worker #36 and #63. Instead, another ST940/OXA-181 *E. coli* found in bakery B differed from 4621 SNPs with the two other ones. Other *E. coli* isolates differing from <10 SNPs were also identified in bakery A, i.e. ST10698 /CTX-M-15 in workers #37 and #38, ST120/CTX-M-15 in workers #37 and #38 and another lineage of ST120/CTX-M-15 in workers #53 and #59, and in bakery B, i.e. ST8149/CTX-M-15 in workers #1 and #6. Finally, two ST3172/CTX-M-15 *E. coli* were found identical (no SNP difference) in workers #40 (bakery A) and #69 (bakery B).

Tree scale: 100000

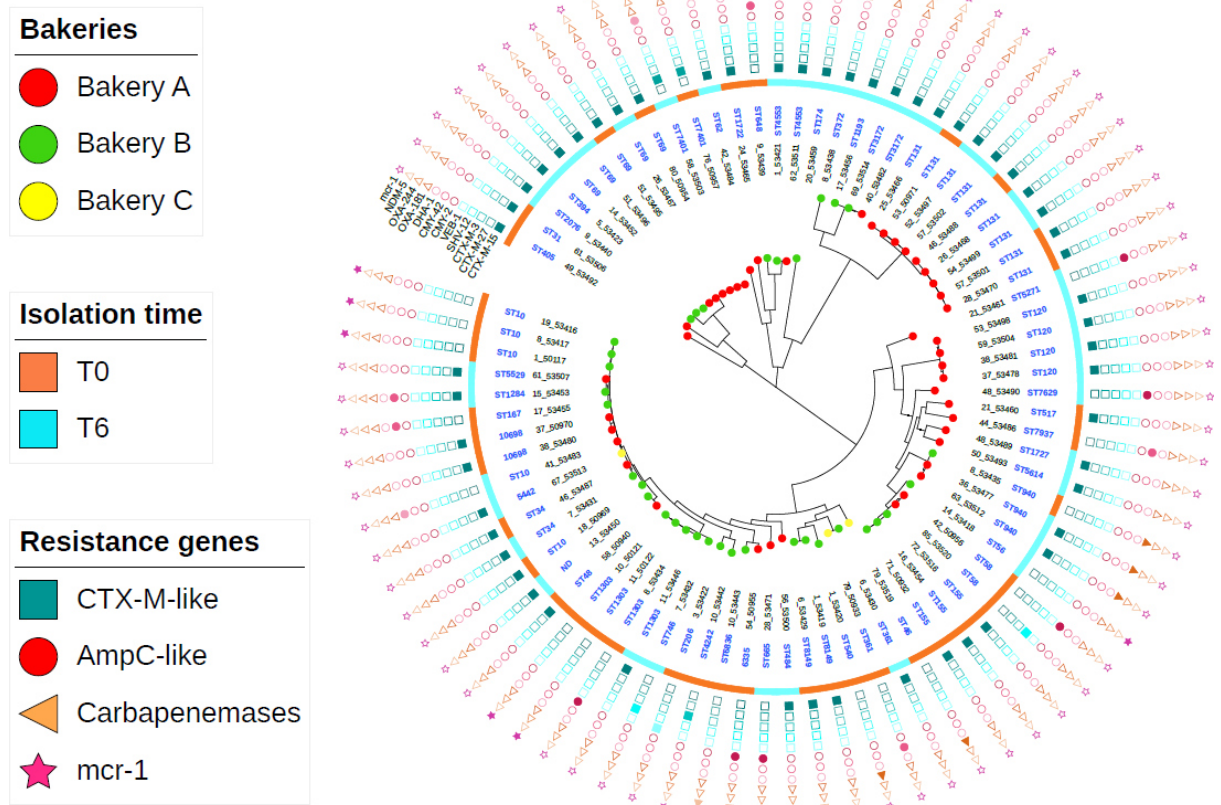


Figure 14: Maximum-likelihood phylogeny of human *E. coli* isolates. The phylogenetic tree was constructed based on nucleotide sequence alignments of the core genes. Metadata include bakery, STs, isolation time and resistance genes (ESC-R, CP-R and COL-R).

Plasmid content. All plasmid Inc types and subtypes were retrieved from Illumina sequences of the 81 *E. coli* isolates. No known Inc type was found in only nine *E. coli* isolates (Table S2). Among the others, the most frequent Inc type was by far IncF (n=56; 56/72, 77.8%), represented by the IncFII, IncFIA and/or IncFIB alleles. Among IncF plasmids, a wide diversity of IncF subtypes was found, as shown by the 37 different formula deduced from the DNA sequences (Table S2). In some cases, identical IncF formula were identified in *E. coli* isolates with no or a few SNP difference, highlighting the spread of a single clone. For example, F35:A6:B26 IncF subtype was detected in the two identical ST120/CTX-M-15-producing *E. coli* (workers #53 and #59, bakery A), whereas F36:A1:B66 IncF subtype was identified in ST69/CTX-M-15-producing *E. coli* from worker #51 at t0 and t6 (bakery B). However, identical IncF plasmid subtypes carrying the same ESBL/AmpC gene were also found in different *E. coli* backgrounds, such as: (i) F2:A-B- IncF subtype, which was carried by

ST62/CTX-M-15- and ST31/CTX-M-15-producing *E. coli* (workers #31 and #62, bakery A), (ii) F2:A-:B- IncF subtype, which carried *bla*_{CTX-M-15} in two *E. coli* isolates belonging to ST31 and ST62 (workers #42 and #61, bakery A), (iii) F1:A2:B20 IncF subtype, which carried *bla*_{CTX-M-27} in two *E. coli* isolates belonging to H30-ST131 and ST7401 (workers #52 and #58, bakery A), and (iv) F31:A4:B1 IncF subtype, which carried *bla*_{CMY-42} in two *E. coli* isolates belonging to ST1284 and ST167 (workers #15 and #17, bakery B) and (v) F29:A-:B10 IncF subtype, which was found bearing *bla*_{CTX-M-15} in eight *E. coli* isolates belonging to H41-ST131 (n=4), H30-ST131 (n=1), ST3172 (n=2) and ST405 (n=1) (workers #25, #26, #28, #40, #46, #49, #54, bakery A; #69, bakery B). In this last case, Illumina sequences suggest a chromosomal location of the CTX-M-15 in at least 5/8 isolates. All other situations will be further characterized using long-read sequencing (MinION, Oxford Nanopore).

Besides, the five *mcr-1*-positive *E. coli* isolates carried the IncX4 plasmid and the last one displayed an IncHI2 plasmid (Table S2). Also, the IncX3 plasmid was identified in all five OXA-181-producing *E. coli* isolates, and was the unique plasmid present in four of them. IncX3 plasmid was also the unique plasmid in the NDM-5-producing *E. coli*, similarly to IncI1 in the OXA-244-producing *E. coli*.

Discussion

We report here one of the rare longitudinal studies clarifying ESC-E carriage at the individual level in the community using WGS-based approaches. To our knowledge, it is also the largest genomic-based study of this type in Lebanon. Our data highlight a high global ESC-E carriage rate of 34.5% (t0) and 51.4% (t6) in the Lebanese community, in line with a 19.1-49.6% prevalence reported in other surveys in this country [116]. ESC-E dissemination has become intense in all sectors in Lebanon, imposing an important AMR burden on healthy human communities. There are still scarce and information on the occurrence of the *mcr*-mediated colistin-resistance in the Lebanese community [215, 300]. ESC-E carriage has been recurrently identified in Lebanese hospitals and hospital effluents, but also animals and environmental niches [200, 242, 288]. Most cattle farms (84%) were ESC-E positives in a recent study while 20.6% and 66.5% of fecal samples from poultry and swine, respectively grew on cefotaxime-supplemented media [206, 208, 246]. ESBL- and *mcr-1*-positive *E. coli* were also reported in broilers, farm workers and the farm surroundings [206]. In the environment, ESC-E and CP-E were identified in water from wells and springs (1.9% and 6.1%, respectively) intended for human consumption [207]. Almost half irrigation water samples (44%) from the two main agricultural regions of Lebanon displayed *mcr-1*-positive *E. coli*, some of which co-harboring ESBL and CP genes [264], while 49.1% of the river effluents were contaminated with ESC-E [232]. More recently, *mcr-1*-positive *E. coli* were also detected in hospitals, carried by IncX4 plasmids [226]. Sporadic cases of few multi-drug (MDR) resistant isolates were also reported, namely one OXA-181-producing ST940, one OXA-48-positive *E. coli* and nine ESBL-producing *E. coli* from a hospital in Beyrouth which belonged to ST131, ST648, ST405, ST73 and ST38 [212, 220, 305].

Dynamics of intestinal ESC-E colonization in the community reveals complex since it refers to a balance between true long-term carriage and successive decolonization/new acquisition rates. Here, at least 16/84 individuals (19%) were colonized with ESC-E at both time points (status was not known for five ESC-E positive subjects at t0, who were not tested at t6). However, only two individuals carried an ESC-E isolate with identical STs/ESBL types at the two time points, arguing for a most likely decolonization/recolonization scenario. The huge diversity of STs found in this study also supports the hypothesis of a polyclonal and dynamic colonization with ESC-E. Of note, several STs reported here were also identified for ESC-E

isolates in non-human sectors in Lebanon, such as ST69, ST457 and ST1303 in cattle, which may warrant further cross-sectorial phylogenomic comparisons.

In our study, 35 out of the 84 workers (41.7%) could be considered as incidental ESBL-carriers, displaying an ESBL-positive isolate at either t0 or t6. This was also the case for colistin- and carbapenem-resistant isolates, which never persisted over the two time points. Fifteen persons (17.9%) could be considered as long-term carriers displaying an ESBL-positive isolate at both t0 and t6, but only one person (1.2%) was a true long-term carrier, carrying the same clone over six months. The other 14 workers presented different ESBL-positive clones, suggesting an important dynamic of acquisition and loss of ESBL-positive *E. coli*. Interestingly, several clones that are known to circulate in hospital settings were detected here, suggesting that they are also successful in the community. This was the case for ST131, which was the only ST detected in nine different *E. coli*, but also for ST405, ST69 or ST940 (already detected as OXA-181-producer).

The diversity of the isolates found in one individual was much lower in the studies by Nakane *et al.* [298]. and van den Bunt *et al.* [299], most probably reflecting the much smaller reservoir of ESBL-positive isolates circulating in humans, animals and the environment in Japan and Sweden. Such a large diversity in ESBL-producing clones was also observed in UK among veterinarians, though on a smaller number of people, and was attributed to contacts with animals [306]. The Dutch study by van der Bunt *et al.* identified travel and low-level hygiene as risk factor [299], which can hardly be incriminated here. In our cohort of 84 Lebanese workers, 6 had been travelling recently, among which 4 were MDR carriers and declared a travel to Turkey, Saudi Arabia, Syria or Australia. Low-level hygiene was the second risk factor in the Netherlands, which cannot be estimated here. It is however worth to notice that 11 workers had access to water through an artesian well, where water is usually used without further potabilization treatment. In all, further studies in contexts of a high prevalence of ESBL-positive *E. coli* are needed to better understand the dynamics of transmission. Indeed, studies on travelers are important to pinpoint risk factors of acquiring MDR bacteria and estimate carriage persistence, but they do not reflect the reality of high MDR-prevalence countries, where AMR burden is never removed.

Transfer of bacteria between workers was proved since identical *E. coli* isolates (\leq SNPs) were found in different people from a same bakery at the same sampling time. In total, 10 transfer events were exemplified, eight between two different workers, and two between three different

workers. Clonal transfer is thus an important driver of the dissemination of MDR *E. coli*. This is a significant finding of this study which was made possible by the design of the study performed on people working together, while longitudinal studies are usually based on big cohorts of people with no epidemiological links. On the contrary, plasmid spread between different genetic backgrounds was more difficult to trace. ESBL, carbapenemase and colistin-resistance genes were not formally assigned to a precise plasmid, but information on Inc types are sufficient to build hypotheses on the low importance of plasmid transfer. Indeed, only a few known Inc types were detected, among which IncF was by far the most frequently identified. Among IncF plasmids, only two plasmids were detected at more than one occurrence in different genetic backgrounds, possibly indicating plasmid transfer. Transfer of IncX3 and IncX4 plasmids carrying *mcr-1* and *bla_{OXA-181}* respectively may also be suggested at one occasion each. The low number of situations where plasmid transfer may have occurred could indicate that such event is rare and that, in favorable environments, clonal transfer is preferred.

Another important issue in Lebanon is the availability of antibiotics in pharmacy without prescription and the poor antibiotic awareness [203]. In this study, 41.3% of the workers had been treated with antibiotics in the past three months, sometimes for questionable reasons (sore throat, flu, tendonitis, ...). Considering the global high circulation of MDR *E. coli* in Lebanon and the fact that antibiotic treatment is an important risk factor for the acquisition of ESBL-producing *E. coli* [Hassing, 2015 #1509], such an uncontrolled use of antibiotics undoubtedly promotes MDR persistence in the community.

Conclusion

We revealed a high global carriage rate of MDR *E. coli* (60.7%) and the emergence of different carbapenemases (OXA-181, OXA-244, NDM-3) and the *mcr-1* gene in the community in proportions that are still below 10%. Incidental carriage was observed in 41.7% and long-term carriage in 17.9% of the workers. A high genetic diversity was identified, suggesting an important dynamic of acquisition and loss of MDR *E. coli* in a context of high proportions in humans, animals and the environment. Our data also suggest the important role of clonal transfer in the dissemination of MDR *E. coli* and thus highlights the urgent need to decrease the AMR burden at the One Health scale.

Acknowledgements

The authors would like to thank Taha Abdou, Mariam Yehya, Nahla Ajaj, Nazih Lazkani, and all bakeries workers for their assistance in sample collection and processing

Conflict of Interest Statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Financial support

This work was supported by internal funding of the French Agency for Food, Environmental and Occupational Health & Safety (ANSES), and by a grant from the Hamidi Medical Center in Tripoli, Lebanon. H.A.M. was supported by a fellowship from Association AZM & Saadeh, Lebanese Association for Scientific Research (LASER) and Université de Lyon.

Chapter 3: Discussion and perspectives

It is relevant that, while writing this thesis, the spread of the novel coronavirus (2019-nCoV) pandemic continues to leave the world worried and paranoid about health issues. Even though the number of people being killed right now by antibiotic resistance dwarves the number of people at risk from covid-19, most people are unaware of AMR.

AMR threat is now emerging as one of the fundamental international healthcare crises of the 21st century. Moreover, the WHO has declared AMR as a top ten priority global health issue for 2019. However, and despite the global attention, the AMR crisis continues to grow, affecting our ability to combat human and animal diseases [307]. Recent investigations revealed a rapid and accelerating increase in AMR rates in many developing regions, including Lebanon. Besides these devastating health issues, AMR has a negative economic impact on communities, including Lebanese community, leading to huge annual charges almost similar to those of the global financial crisis that started in 2008 [196].

The true AMR issue in Lebanon remains unclear, owing to the lack of official national surveillance data and the lack of well-designed national studies. Moreover, available data are largely restricted to limited regional investigations. While Lebanon has been enrolled in the WHO's GLASS in 2017, its epidemiological data significance is still weak [300]. Moreover, Lebanon suffers from an insignificant attention to agricultural practices. Because of these evident gaps, antibiotic resistance rates are increasing and newly discovered antimicrobial resistant genes (such as *mcr* genes) are emerging quickly. In fact, numerous reports conducted at regional level documented that AMR represents a prominent public-health issue in the country [83, 203, 226]. Moreover, the influx of refugees coming from Syria and Iraq most probably contributed to the aggravation of the AMR issue in Lebanon [83, 308, 309]

A recent report revealed that MDR GNB have become widely prevalent in animals, food and the environment [116]. Two retrospective studies conducted in several Lebanese hospitals described a rise in the prevalence of common multidrug resistant (MDR) pathogens as follow: methicillin-resistant *Staphylococcus aureus* (28% in 2016 vs 23% in 2011), vancomycin-resistant *Enterococcus* (2% in 2016 vs 0% in 2011), carbapenem-resistant *Acinetobacter* (88% in 2016 vs 51% in 2011), carbapenem-resistant *Pseudomonas* (30% in 2016 vs 20% in 2011), and extended-spectrum beta-lactamase-producing *Enterobacteriaceae*

(34% in 2016 vs % 32% in 2011) [200, 300]. In the same context, other reports showed the emergence of clinical GNB isolates resistant to carbapenems, aminoglycosides, fluoroquinolones, and even colistin [216, 225], and Gram positive MDR bacteria [82, 310]. Moreover, a 12-month nationwide study identified the emergence of 3 XDR tuberculosis cases in Lebanon [308]. A pilot study also showed that around 50% of *Escherichia coli* isolates recovered from river effluents were resistant to third generation cephalosporins and fluoroquinolones, and carried several resistance determinants including *bla*_{CTX-M-14}, *bla*_{OXA-1}, *bla*_{SHV-12}, *bla*_{CMY-2}, *aac(6)-Ib*, *acc(3)-II*, *acc(3)-II*, and *Int-II* [232]. Another worrying example being a study carried out in three major Lebanese poultry farms showing that 98% of *Escherichia coli* isolates harbored plasmid-mediated colistin resistance gene (*mcr-1*) [247].

In line with these previous publications, data presented in this thesis work further highlighted the large diffusion of MDR *E. coli* in Lebanon at a One Health scale. We showed that 73.8% of the 32 Lebanese farms tested presented at least one *mcr-1*-positive colistin-resistant *E. coli*. We reported also for the first time the presence of the *mcr-1* gene in clinical human isolates and we performed the largest genomic-based study on healthy carriage of ESC-, carbapenem- and colistin-resistance in the Lebanese community underlining the transmission of ESBL-, *mcr-1*- and carbapenemase-producing *E. coli*. We reported a high genetic diversity among MDR *E. coli* in hospital and extra hospital settings in the country. We also strongly suggested that STs could spread from animals to humans, as showed by the occurrence of ST1011 both in poultry, where it is a major clone, and in a human patient in Tripoli.

Based on the aforementioned data, it is not surprising that all AMR studies conducted in Lebanon showed an alarming emergence and dissemination of antibiotic-resistant strains and AMR genes (ARGs) among different hosts including humans, animals and the environment [83, 116, 226].

To our knowledge, three main factors determine the high AMR rates in Lebanon: (i) the promotion of self-medication and self-prescription with antibiotics; (ii) the misuse of antibiotics as growth-promoting factors in livestock and poultry; and (iii) the lack of antibiotic stewardship programs and the inadequate infection control practices in most healthcare settings. Regrettably, multiple pre-disposing determinants promote self-medication risks such

as over-the-counter availability, low income, high cost of medical consultation and reliable diagnostic tests, low satisfaction with medical and veterinary practitioners, and low awareness regarding the efficiency of antimicrobials [201]. The situation in the veterinary field sadly mirrors the one in humans. A large number of brands that contained one or more antibiotics are readily and legally available in the Lebanese market without the requirement of a veterinarian's prescription [311]. Theoretically, these drugs must only be administered after veterinary prescription for the treatment of animal diseases, but practically they are excessively used on a long-term basis by farmers as growth promoters and prophylactic treatment. On the other hand, according to Médecins Sans Frontières, more than 1.5 million Syrians refugees have fled into Lebanon since the beginning of the Syrian civil war, making Lebanon the country hosting the largest percentage of refugees worldwide. Although the efforts of the Lebanese Ministry of Public Health in supporting healthcare facilities for refugees, the cost of medical care remains a barrier for a huge proportion of refugees [309]. Hence, the unnecessary use of antibiotics by resident communities in Lebanon is the main reason leading to strong selective pressure and driving the evolutionary response in the bacterial world. Although by Lebanese law, antibiotics are prescription medicines only, these wonder drugs are frequently supplied without medical prescription in the majority of Lebanese areas including higher and lower socio-economic regions. Furthermore, several cross-sectional studies showed poor knowledge and bad attitude of community pharmacists [312] and public [203] about antibiotic misuse and resistance. Unfortunately, based on poor knowledge and experience, these two population groups are now the main actors in the primary health care and in the prescription of antibiotics in Lebanon. In summary, the medical system in Lebanon suffers from the lack of medical education on antibiotic use for prescribers, the absence of standardized and evidence-based systems for antibiotic prescription, and the low efficacy of infection control measures and antibiotic stewardship programs in health care facilities. Moreover, a current investigation observed an alarming rate of errors in reporting cumulative antibiotic susceptibility data by clinical laboratories to epidemiologists and physicians of 9 Lebanese major hospitals [313]

Regional and national political actions, such as the implementation of National Action Plans, are now critical to preserve the antibiotic arsenal and to contain the threat of AMR in Lebanon. More cooperation is needed between stakeholders, policy-makers and health actors to tackle the problem of AMR, to create more impact on both medical and veterinary fields. At community level, extended awareness campaigns targeting the public and the community

pharmacists using a human centered and interactive approach are needed in order to reach high levels of agreement, and desired behavioral changes. At medical level, educating microbiologists, clinical pharmacists, physicians and veterinarians is the first step to reach our goals. Furthermore, a complete One Health approach program under a political commitment and the jurisdiction of the Ministries of Public Health and Agriculture should be implemented to: (i) establish a robust national AMR surveillance system; (ii) ensure the adequate use of antibiotics in human and animals, (iii) ban the use of antibiotics (or at least medically important drugs) as growth promoters in animal feeds, and (iv) prohibit the use of antibiotics without clinical or veterinary prescription. Finally, AMR national committee may require advanced training, and national and international support to coordinate all relevant stakeholders effectively.

In conclusion, the concerned scientific and community societies, as well as the Lebanese authorities should have the responsibility of addressing and following up on this important threat. The combination of all the interventions and initiatives, such as laws, follow-up policies, awareness and educational interventions, and official national surveillance on AMR and on knowledge and attitudes towards antibiotic misuse in the community and medical body, will definitely participate to reduce the use of antibiotics, and thus to minimize the development of AMR and to avoid public health and economic negative consequences.

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Annex



Short report

First Lebanese Antibiotic Awareness Week campaign: knowledge, attitudes and practices towards antibiotics

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ARTICLE INFO

Article history:

Received 12 June 2018

Accepted 8 July 2018

Available online 17 September 2018

Keywords:

Antibiotic awareness campaign

Antibiotic awareness week

Antibiotic resistance

Antibiotic misuse

Control of antibiotic use

Lebanon



SUMMARY

Antibiotic resistance (ABR) is a major global health threat that increases the risk of treatment failure and increases medical costs. One of the most common factors contributing to the spread of ABR is self-medication. The public, as well as workers in clinical and veterinary sectors, commit false practices towards appropriate antibiotic use, favouring the spread of resistance. As such, the first Lebanese Antibiotic Awareness Week campaign was initiated with a human-centred and interactive approach. The data showed a strikingly low level of antibiotic awareness. Cooperation between relevant stakeholders, policy-makers and health actors is crucial to control and overcome the problem of ABR.

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Introduction

Antibiotic resistance (ABR) is a major global health threat worldwide, and is spreading more rapidly than the

development of new agents [1]. The exceptional spread of multi-drug-resistant bacteria is of particular concern as this could lead to serious infections leaving patients with very limited, or no, therapeutic options.

Some countries have been able to contain the threat of ABR, but in most countries, particularly developing countries, the issue continues to grow [2]. The overuse and misuse of antibiotics in both the human and animal sectors has been a fundamental cause favouring the emergence, multiplication

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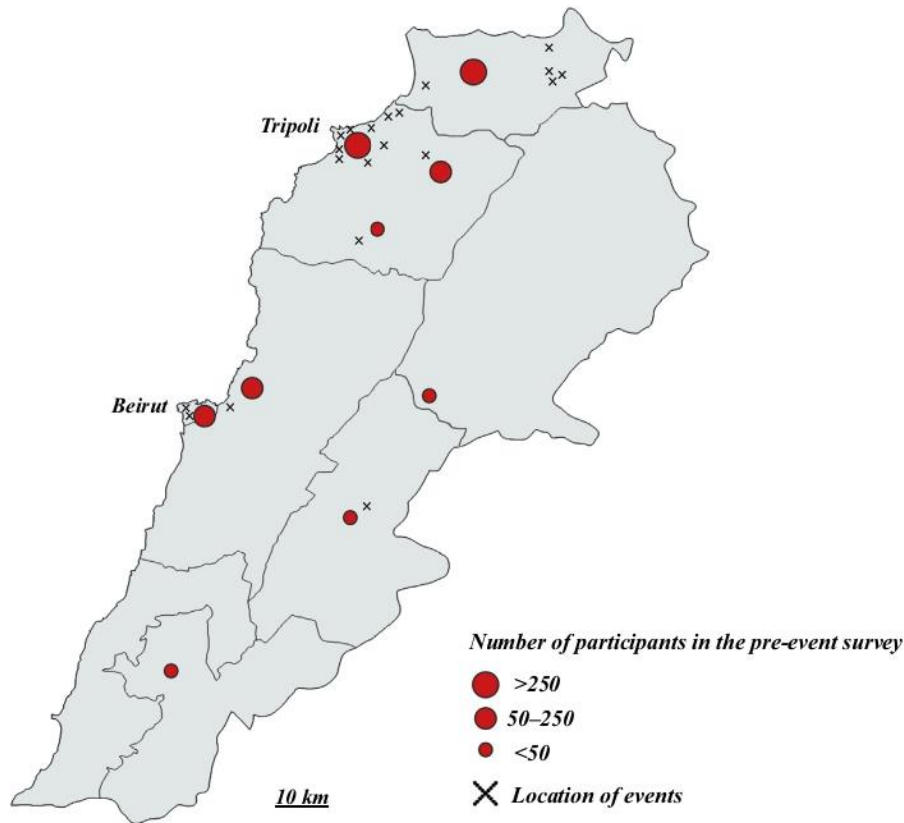


Figure 1. Geographic locations targeted in this study in Lebanon.

and spread of resistant strains. Worldwide, the most common factor contributing to the spread of ABR is self-medication [3]. The reasons for this behaviour are numerous, with the main reasons including expensive healthcare services, easy access to medication, smuggling and illegal sales of antibiotics, and lack of awareness about the concept of antibiotics, ABR and outcomes of overconsumption [4]. Hence, interventions to prevent and control the spread of ABR in the community and hospitals are needed urgently.

In Lebanon, numerous studies have investigated the epidemiology of certain types of resistance which seem to grow rapidly and uncontrollably [5,6]. Behaviours at individual level play a major role in the spread of resistant pathogens. Therefore, an initiative was planned in the form of an awareness campaign of multiple events in diverse regions and settings, mainly to target vulnerable populations. The Lebanese Association for Development initiated the first Lebanese Antibiotic Awareness Week campaign, synchronized with the World Health

Table I Beliefs regarding antibiotics and antibiotic resistance among resident communities in Lebanon (N = 1187)

Variables	Cluster 1			Cluster 2			Cluster 3		
	True	False	P-value (OR [95% CI])	True	False	P-value (OR [95% CI])	True	False	P-value (OR [95% CI])
N (%)	1367 (57.6)	1007 (42.4)		393 (33.1)	794 (66.9)		1572 (66.2)	802 (33.8)	
Sex									
Female	1066	852	0.001 (0.69 [0.55–0.86])	301	658	0.04 ^a (0.72 [0.53–0.99])	1271	647	0.75 (1.04 [0.83–1.3])
Male	259	143		78	123		263	139	
Age (years)									
>18	859	599	0.31 (1.1 [0.92–1.31])	221	508	0.007 ^a (0.7 [0.54–0.91])	993	465	0.016 ^a (1.26 [1.04–1.51])
≤18	432	330		146	235		480	282	
Education									
School	584	722	<0.001 ^a (0.29 [0.25–0.35])	211	442	0.52 (0.92 [0.72–1.18])	772	534	<0.001 ^a (0.48 [0.41–0.58])
University	783	285		182	352		800	268	

OR, odds ratio; CI, confidence interval.

^a Statistically significant.

Statistical analysis was performed using Epilnfo with Chi-squared tests to compare differences categorized by sex, level of education and age group. The tests were two-sided, with a type I error set at $\alpha = 0.05$.

Results and discussion

Despite the limited resources, this campaign was able to reach out to more than 10,000 individuals and spread the key messages to tackle the problem. The audience were supported and encouraged in active participation rather than passive attendance, and a variety of open questions were used that allowed participants to learn more about antibiotics and ABR. Moreover, a verbal commitment was made by all participants to stop using antibiotics without prescription.

The paper-based survey was administered to all the participants in the awareness lectures in the 20 different settings. In total, 1187 surveys were completed by people from different Lebanese regions (Figure 1), educational backgrounds, sexes and age groups. Each of these variables was correlated separately with the correctness of the answers.

This investigation revealed insufficient knowledge, poor attitudes and inadequate practices towards antibiotics among the population. The data showed a strikingly low level of awareness about the concept of antibiotics and their usage, and the issue of ABR (Table 1). Out of 1187 individuals who agreed to participate in the pre-event survey, only 48 (4%) answered all the questions correctly. Regarding the type of micro-organisms targeted by antibiotics, 58% of individuals believed that antibiotics worked on bacteria and should not be used on cold sores. Thirty-three percent of individuals had knowledge about the perception of ABR, and 51% of individuals were aware of the risk attributed to ABR. Overall, the highest percentages of correct responses by survey participants were to questions concerning the perception of misuses of antibiotics that lead to ABR (66%), and awareness about the magnitude of the issue of ABR (66%).

A logistic regression model was created to identify the risk factors associated with low awareness towards antibiotics and ABR among resident communities in Lebanon. Adults [odds ratio (OR) 1.1, 95% CI 1.01–1.78, $P = 0.04$], female sex (OR 1.2, 95% CI 1.08–1.33) and school level education (OR 2.1, 95% CI 1.93–3.4, $P < 0.001$) were significantly predictive of insufficient knowledge towards antibiotics in resident communities in Lebanon.

This study found that there is poor understanding of the concept and use of antibiotics, as well as inability to differentiate between the different types of germs, leading to confusion about the use of antibiotics to treat viral infections (e.g. cold). This confusion is probably attributed to the lack of proper communication by physicians prescribing antibiotics, and lack of awareness by educational settings (schools and universities) and governmental agencies. Younger participants seem to be more aware of the issue, and this might be attributed to the fact that they are more exposed to social media platforms where knowledge about antibiotics can be published. In addition, it is evident that university students are more likely to answer correctly because students that specialize in health receive in-depth knowledge, and students of other specializations can also receive some information

about the issue. Overall, the results of this study are consistent with other studies in Lebanon [8] and in nearby Middle Eastern countries such as Kuwait [7], Jordan [9] and Saudi Arabia [10], which also reported a significant lack of awareness about this global health issue.

To the best of the authors' knowledge, this campaign is the first national awareness initiative to target the problem of ABR in Lebanon. One of the main strengths of the campaign was the resilient management and sense of responsibility of the volunteers towards the issue and the community. Three important lessons were learned from this campaign: (i) even with limited resources, action can be implemented and impact can be made; (ii) there is high agreement of individuals regarding application of the recommendations and the behavioural changes needed when the approach is human-centred and interactive; and (iii) more cooperation is needed between stakeholders, policy-makers and health actors to control the problem of ABR, induce more impact and reach out to greater segments in both clinical and veterinary sectors.

In the authors' opinion, there is a need for more awareness interventions involving health and political stakeholders throughout the year, using not only methods that reach the target segments indirectly (e.g. media and online content), but also approaches that include direct contact and communication. This study has shown high agreement of individuals regarding application of the recommendations and the behavioural changes needed.

However, the main limitations remain those related to the healthcare system and socio-economic factors. In Lebanon, most people are of low socio-economic status, with only 45.9% of the population covered by the governmental health insurance. In addition, there is a lack of primary healthcare facilities. Moreover, due to the ongoing Middle Eastern conflicts, huge numbers of immigrant populations, including Syrian and Palestinian refugees displaced in Lebanon, face dire levels of poverty and vulnerability, and represent an ideal environment for the development and dissemination of infectious diseases. Thus, a remarkable percentage of the community cannot afford to see a medical doctor for a proper medical prescription of antibiotics under the worsening economic circumstances. To save money, people usually go to the pharmacist and take the antibiotic immediately. Pharmacists do not abide by the regulations and sell antibiotics without prescription.

In conclusion, a further movement needs to be initiated to take the change to a policy-based level. This includes, in addition to extended awareness campaigns targeting the public with various means, a pressing movement to ensure more surveillance of pharmacists and doctors by the Lebanese Government, as well as enhancing the coordination between healthcare facilities and the Ministry of Public Health to further control the spread of resistance in the hospital setting. An urgent intervention is needed in the animal sector which serves as a significant reservoir of resistant bacteria.

Acknowledgments

The authors wish to thank all Lebanese Antibiotic Awareness Movement team members: Aicha Awik, Ayate Zakaria, Bariaa Nafeh, Fidele Al Masri, Hana Al Mir, Iman Yassine, Lawahez Sablough, Mariam Benny, Mohammad Diab, Mona Hamze, Noor Kabbara, Riham Tamer, Roukaya Boustani, Sarah Dardari, and Sarah Ghazi, and Halima El Cheikh for her assistance in the

design of the figure. The authors also wish to thank all Lebanese civil associations and universities who helped in the success of the first Lebanese Antibiotic Awareness Week campaign.

Conflict of interest statement

None declared.

Funding sources

This study was supported by a grant from the Lebanese Association for Development. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Appendix A. Supplementary data

Supplementary data related to this article can be found at <https://doi.org/10.1016/j.jhin.2018.07.009>.

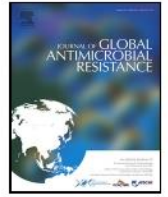
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Contents lists available at ScienceDirect

Journal of Global Antimicrobial Resistance

journal homepage: www.elsevier.com/locate/jgar

Review

Epidemiology of antimicrobial resistance in Lebanese extra-hospital settings: An overview

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ARTICLE INFO

Article history:

Received 20 September 2018

Received in revised form 7 November 2018

Accepted 26 November 2018

Available online 12 December 2018

Keywords:

Antimicrobial resistance

Epidemiology

Environment

Carbapenemase

ESBL

Lebanon

ABSTRACT

Antibiotics can truly be considered the symbol of the 20th century's miracle drugs. However, excessive and inappropriate use of antibiotics in human and veterinary medicine has led to repercussions in terms of mortality, morbidity, and financial and societal costs, especially with the lack of novel drugs to combat infections caused by antimicrobial-resistant pathogens. Unfortunately, antimicrobial resistance (AMR) has increased significantly worldwide in the past decades, particularly in developing third-world countries. Hence, various key organisations have made AMR the focus of highly visible reports, conferences and actions. Nevertheless, the majority of investigations were initiated in the developed world. In Lebanon, as in other developing countries, AMR remains responsible for significant morbidity and mortality. Whilst a large number of studies have targeted the AMR crisis in Lebanese clinical settings, its epidemiological situation in animals, foodstuffs and the environment is still unclear in this country. In this context, and in order to better survey, control and prevent the emergence of multidrug-resistant bacterial strains in Lebanese non-clinical settings, this work reviewed the available data on the epidemiology of antimicrobial-resistant bacteria isolated from Lebanese extra-hospital sources.

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1. Introduction

With the advent of the 90th anniversary of the discovery of penicillin, antimicrobial resistance (AMR) has become a major public-health issue of global concern and has emerged as one of the principal international healthcare crises of the 21st century [1]. In 2013, the US Centers for Disease Control and Prevention (CDC)

announced that the human race is now in the post-antibiotic era. The World Health Organization (WHO) global AMR surveillance report published in 2014 also warned that the AMR crisis is dire [1,2]. Recently, a high profile report warned that 700 000 annual deaths globally attributed to AMR in 2015 could climb to 10 million deaths in 2050 if nothing is done to reverse the trend [3]. Besides these serious issues, AMR has a negative economic impact on communities, leading to huge annual costs almost similar to those of the global financial crisis [4]. A recent study estimated the GDP loss due to AMR in the Middle East and North Africa (MENA) region, including Lebanon, to range between US\$2 billion and US\$159

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billion per year over 40 years [4]. Unfortunately, Lebanon, like the majority of developing countries, still suffers from high levels of AMR. In this context, and in order to better understand the epidemiology and transmission of AMR in Lebanon, the aim of the present review was to summarise recent findings on the epidemiology of antimicrobial-resistant Gram-negative bacteria isolated from the environment and from animals, mainly swine, cattle and poultry, in Lebanon, a small country in the MENA region sheltering a huge number of refugees and facing large social, health, economic, political and security challenges.

2. Desperate situation regarding antibiotics in Lebanon

Despite the implementation of consistent strategies and measures to prevent and control the emergence and spread of AMR in developed countries, this issue continues to grow in a large number of these countries [5]. However, the AMR issue is more complicated in developing countries, such as Lebanon, since numerous factors leading to the emergence and spread of multidrug-resistant (MDR) strains remain uncontrollable. The increasing availability of antibiotics in Lebanon is generally a good thing, however the misuse of these 'wonder drugs' has created and promoted the transmission of antimicrobial-resistant strains of deadly bacteria, particularly in poorer communities such as Syrian and Palestinian refugees and the vulnerable Lebanese population [2]. Besides the availability of antibiotics without prescription in pharmacies, various factors including (i) poor antibiotic awareness both among prescribers and the general public, (ii) extensive antibiotic use in humans, animals and agriculture, (iii) excessive use of antibiotics as growth-promoting factors in livestock, (iv) non-compliance with antibiotic therapy, (v) promotion of antibiotic self-medication, (vi) poor antibiotic stewardship and (vii) inadequate infection control are key drivers of AMR in Lebanon. Moreover, the availability of antibiotics for purchase on websites increases the circulation of counterfeit drugs and decreases the quality of care in the country [6]. Therefore, despite the outstanding progress to develop a national plan of action to combat the spread of AMR in Lebanon, AMR rates are at the higher end of levels reported globally [7]. Several cross-sectional studies showed a high prevalence of inappropriate antibiotic prescription in Lebanon, regardless of doctors' specialty [8,9]. Similarly, large amounts of antibiotics are used in the veterinary, livestock and agriculture fields without surveillance and control, causing a rapid increase in the prevalence of AMR and the use of last-resort antibiotic compounds both in clinical and veterinary fields in Lebanon. On the other hand, a cross-sectional nationwide study conducted last year, in synchronisation with the first Lebanese antibiotic awareness campaign, revealed very insufficient knowledge, poor attitudes and inadequate practices towards antibiotics among the resident populations in Lebanon [10].

3. Consequences of antibiotic misuse

It is well known that antibiotic use contributes to the selection of resistance by posing selective pressure on antimicrobial resistance genes (ARGs) and bacterial community composition in different environments. Humans and animals discharge in their faeces and urine a large proportion of the administered antibiotic either unchanged or as active metabolites, contaminating external environments. The presence of antibiotics, even at low concentrations, in environments and in treated animals and humans could allow for enrichment and selection of bacteria carrying multidrug resistance plasmids and thereby may contribute to the emergence, maintenance, multiplication and spread of antimicrobial-resistant bacterial pathogens of clinical importance [11,12]. Many studies have found a strong association between

administration of antibiotics in animals and the corresponding rate of resistance towards these antibiotics in bacterial strains isolated from cattle, poultry and pigs [13,14]. On the other hand, humans and animals may also be contaminated with antibiotics indirectly through food and water containing active antibiotic residues. Moreover, a population-based study found a strong association between virulence and AMR in bacterial communities [15]. Remarkably, a recent retrospective clinical study found that low antibiotic concentrations were detected in the urine of patients who had not consumed antibiotics that were capable of selecting antibiotic-resistant *Staphylococcus* species [16].

The exceptional spread of MDR and extensively drug-resistant (XDR) bacteria is highly worrisome as it could lead to serious infections leaving patients and animals with very limited or no therapeutic options [17]. Moreover, ARGs are often located on mobile genetic elements (MGEs), which can be easily transmitted between different hosts including humans, animals and the environment. Indeed, genetic similarities of such ARGs, MGEs and/or clones that are detected in humans, animals and the environment have repeatedly been reported worldwide [18,19]. Genes encoding extended-spectrum β -lactamases (ESBLs), carbapenemases and aminoglycoside-modifying enzymes as well as plasmid-mediated quinolone resistance genes are often located on MGEs, which can be transmitted both within and between different bacterial species and hosts. Unfortunately, all of these antimicrobial-resistant bacteria and genes are now widely disseminated among humans, animals and the environment, and their presence in animals and foodstuffs has also been regularly reported in Lebanon [20–25].

4. Epidemiology of antimicrobial resistance in non-clinical settings in Lebanon

Antibiotic misuse in humans is a fundamental issue favouring the emergence, multiplication and spread of resistant strains [12]. Although AMR is generally associated with uncontrolled use of antibiotics in human medicine, antibiotic abuse in the veterinary and agriculture sectors is also reported as a main driver for the rapid evolution of bacterial AMR [25,26]. Animals, food and the environment are generally considered a reservoir of MDR and XDR bacteria, particularly Enterobacteriaceae [24,27]. Nevertheless, the impact of animal reservoirs of AMR on human health has yet to be proven and remains debatable. New research studies suggest that sharing the same ARGs, MGEs and clones constitutes potential evidence of transmission of antimicrobial-resistant bacteria from animals to humans [28]. Bacterial strains carried by animals can be easily transferred to humans through direct contact or by handling or consumption of animal products [29]. Hence the importance of the implementation of a One Health approach to face the current public-health challenges, including the emerging AMR threat, and to provide a strategy to improve the lives of all species. Fortunately, surveillance of AMR in Lebanon has improved during the last years. Moreover, Lebanon has been enrolled in the WHO's Global Antimicrobial Resistance Surveillance System (GLASS) since April 2017. In this context, numerous epidemiological studies were conducted in the last decade in Lebanon that highlighted the worrying situation of AMR in the country and described high rates of methicillin-resistant *Staphylococcus aureus* (MRSA) and differentially resistant Gram-negative bacteria [7,30,31]. One nationwide study performed at 16 Lebanese tertiary-care centres between 2011–2013 reported a rise in the prevalence of common MDR pathogens including MRSA, vancomycin-resistant enterococci, ESBL-producing *Escherichia coli*, and carbapenem-resistant *Acinetobacter* and *Pseudomonas* spp. [7]. Another study performed at El Youssef Hospital Center in North Lebanon between 2015–2017 reported

Table 1
Epidemiology of antimicrobial resistance in non-clinical settings in Lebanon.

Investigation site	Targeted population	Type of sample	Year(s) of survey	No. of isolates		Presence of colistin resistance	Antibiotic resistance (%) among ESBL-producing and/or carbapenem-resistant strains							β-Lactam resistance genes (n)	Reference	
				ESBL-producing	Carbapenem-resistant		TET	NAL	ENR/CIP	TMP	SXT	GEN	STR			CHL
North and South Lebanon (search for MDR bacteria)	Healthy adult cattle	Stool	October 2012–February 2013	<i>Escherichia coli</i> (n=40)	0	No	83	30	18	75	– ^a	23	78	30	<i>bla</i> _{CTX-M-15} (24) <i>bla</i> _{SHV-12} (10) <i>bla</i> _{CTX-M-14} (2) <i>bla</i> _{CTX-M-15} + <i>bla</i> _{SHV-12} (3) <i>bla</i> _{CTX-M-14} + <i>bla</i> _{SHV-12} (1) [<i>bla</i> _{CTX-M} (83) <i>bla</i> _{TEM} (57) <i>bla</i> _{SHV} (9) <i>bla</i> _{CMY} (13) alone or in combination]	[24]
South Lebanon (search for MDR bacteria)	Swine	Stool	May 2017	<i>E. coli</i> (n=105) <i>Klebsiella pneumoniae</i> (n=2) <i>Escherichia fergusonii</i> (n=4)	0	<i>mcr-1</i> (n=23)	–	–	78	–	89	42	–	–	[<i>bla</i> _{CTX-M} (83) <i>bla</i> _{TEM} (57) <i>bla</i> _{SHV} (9) <i>bla</i> _{CMY} (13) alone or in combination]	[26]
North Lebanon	Animals (pigs, fowl and cattle)	Stool	2013	–	<i>Acinetobacter baumannii</i> (n=5) <i>Pseudomonas aeruginosa</i> (n=4) <i>E. coli</i> (n=1)	No	–	100	100	–	–	100	–	–	<i>bla</i> _{OXA-23} (4) <i>bla</i> _{TEM-2} (4) <i>bla</i> _{OXA-23} + <i>bla</i> _{OXA-58} (1)	[45]
Case report	Poultry (fowl)	Stool	December 2013	–	–	No	–	–	–	100	–	100	–	–	<i>bla</i> _{OXA-48} + <i>bla</i> _{CTX-M-14} + <i>bla</i> _{TEM-1} (1) <i>bla</i> _{TEM-135-like} (1)	[27]
Case report (Sidon)	Poultry	Rectal swab	14 August 2015	<i>E. coli</i> (n=1)	0	<i>mcr-1</i> (n=1)	–	–	100	100	100	100	–	–	<i>bla</i> _{TEM-135-like} (1)	[52]
Nationwide	Poultry	Rectal swabs	August–December 2015	<i>E. coli</i> (n=103) <i>K. pneumoniae</i> (n=5) <i>Enterobacter cloacae</i> (n=3) <i>E. fergusonii</i> (n=1)	0	No	–	–	62	–	68	71	–	–	<i>bla</i> _{CTX-M} (2) <i>bla</i> _{TEM} (36) <i>bla</i> _{SHV} (17) <i>bla</i> _{CTX-M} + <i>bla</i> _{TEM} (52) <i>bla</i> _{CTX-M} + <i>bla</i> _{TEM} + <i>bla</i> _{SHV} (5) [<i>bla</i> _{CMY} (152), alone or in combination with other genes]	[25]
Nationwide	Poultry	Faecal samples	September 2017–March 2018	–	–	<i>mcr-1</i> (n=88)	89	–	91	–	84	61	80	95	<i>bla</i> _{TEM-1} (84) <i>bla</i> _{CTX-M} (31)	[53]
Beqaa Valley	Agri-food environment	Vegetables; knife & board swabs; fresh produce; water	July–August 2013 and July 2014	– (100% of isolates were resistant to cefalotin)	–	No	–	–	–	–	–	–	–	–	–	[34]
North Lebanon	Food	Akkawi cheese	May–June 2011	<i>E. coli</i> (n=3) <i>K. pneumoniae</i> (n=2) <i>Rhanelia aquatilis</i> (n=1)	0	No	17	17	17	–	33	50	–	–	–	[35]
North Lebanon	Food	Raw bovine milk	September–November 2015	<i>E. coli</i> (n=7) <i>K. pneumoniae</i> (n=36)	<i>K. pneumoniae</i> (n=1)	No	–	–	–	–	–	–	–	–	<i>bla</i> _{CTX-M-15} (38) <i>bla</i> _{CTX-M-15} + <i>bla</i> _{SHV-12} (4) <i>bla</i> _{CTX-M-15} + <i>bla</i> _{OXA-48} (1)	[22]
North Lebanon (Tripoli)	Food	Raw meat	April–May 2010	<i>E. coli</i> (n=2)	0	No	100	–	50	–	–	0	–	–	–	[36]
Nationwide	Water	Sewage water	Spring 2016	<i>E. coli</i> (n=21)	0	No	71	–	81	–	81	43	–	–	<i>bla</i> _{CTX-M-15} (2) <i>bla</i> _{CTX-M-9} (1) <i>bla</i> _{CTX-M-15} + <i>bla</i> _{OXA-1} (3) <i>bla</i> _{CTX-M-15} + <i>bla</i> _{OXA-1} + <i>bla</i> _{SHV-12} (3) <i>bla</i> _{CTX-M-15} + <i>bla</i> _{OXA-1} + <i>bla</i> _{TEM-1} (1)	[39]

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Table 1 (Continued)

Investigation site	Targeted population	Type of sample	Year(s) of survey	No. of isolates		Presence of colistin resistance	Antibiotic resistance (%) among ESBL-producing and/or carbapenem-resistant strains							β-Lactam resistance genes (n)	Reference	
				ESBL-producing	Carbapenem-resistant		TET	NAL	ENR/ CIP	TMP	SXT	GEN	STR			CHL
North and South Lebanon (search for MDR bacteria)	Water	Dug wells; spring water; estuaries	March 2014–January 2015	<i>E. coli</i> (n=23) <i>K. pneumoniae</i> (n=4) <i>Citrobacter freundii</i> (n=1) <i>Citrobacter braakii</i> (n=1)	<i>E. coli</i> (n=3) <i>K. pneumoniae</i> (n=1)	No	67	58	48	58	–	33	42	15	<i>bla</i> _{CTX-M-15} + <i>bla</i> _{TEM-1} (2) <i>bla</i> _{CTX-M-15} + <i>bla</i> _{TEM-1} + <i>bla</i> _{SHV-12} (1) <i>bla</i> _{SHV-12} + <i>bla</i> _{OXA-1} (1) <i>bla</i> _{CTX-M-27} + <i>bla</i> _{TEM-1} (1) <i>bla</i> _{CTX-M-14} + <i>bla</i> _{OXA-1} (1) <i>bla</i> _{CTX-M-15} (24) <i>bla</i> _{CTX-M-14} (1) <i>bla</i> _{CTX-M-55} (1) <i>bla</i> _{SHV-12} (1) <i>bla</i> _{OXA-48} (1) <i>bla</i> _{OXA-244} (1) <i>bla</i> _{OXA-48} + <i>bla</i> _{CTX-M-14} (1) <i>bla</i> _{OXA-244} + <i>bla</i> _{CTX-M-14} + <i>bla</i> _{SHV-12} (1) <i>bla</i> _{CTX-M-15} + <i>bla</i> _{CMY-42} (2)	[23]
Beirut and North Lebanon	Water	Hospital wastewater	August 2011–June 2012	<i>E. coli</i> (n=45) <i>K. pneumoniae</i> (n=6)	<i>E. cloacae</i> (n=2)	No	–	–	–	–	53	41	–	–	[<i>bla</i> _{NDM-1} (2) <i>bla</i> _{CTX-M} (45) <i>bla</i> _{TEM} (19) <i>bla</i> _{SHV} (23) <i>bla</i> _{OXA} (29), alone or in combination]	[49]
Akkar District	Water	Dug wells	April–May 2010	<i>E. coli</i> (n=3) <i>C. freundii</i> (n=1)	0	No	75	75	50	–	–	67	–	–	–	[37]
Nationwide (search for <i>Acinetobacter</i> spp.)	Agri-food environment	Soil; water; vegetables; seawater fish; cow's milk; animals	September 2014–February 2015	0	<i>Acinetobacter calcoaceticus</i> (n=2)	No	–	–	0	–	–	–	–	–	<i>bla</i> _{OXA-72} (2)	[38,47]
Nationwide (search for <i>Acinetobacter</i> spp.)	Agri-food environment	Soil; animals; food	February 2012–October 2013	0	<i>A baumannii</i> (n=1) <i>Acinetobacter pittii</i> (n=1)	No	–	–	–	–	–	–	–	–	<i>bla</i> _{OXA-143} (1) <i>bla</i> _{OXA-24} (1)	[46]

ESBL, extended-spectrum β-lactamase; TET, tetracycline; NAL, nalidixic acid; ENR, enrofloxacin; CIP, ciprofloxacin; TMP, trimethoprim; SXT, trimethoprim/sulfamethoxazole; GEN, gentamicin; STR, streptomycin; CHL, chloramphenicol; MDR, multidrug-resistant.

^a Indicates not available.

that 35.7% and 1.1% of uropathogenic *E. coli* were ESBL-producers and ertapenem-resistant, respectively [32]. Another study also carried out in North Lebanon at Nini Hospital reported that 24.4% of cancer patients were colonised with carbapenem-resistant Gram-negative bacteria [33]. Despite the large number of published studies targeting the epidemiology of AMR among the human population in Lebanese hospital settings, the epidemiological situation of antibiotics in animals, food and the environment is still unclear. In the current review, the PubMed, ScienceDirect, Scopus and Google Scholar databases were searched for investigations published between 2008–2018 using the keywords ‘AMR’, ‘antibiotic resistance’, ‘ESBL’, ‘carbapenemase’, ‘mcr’, ‘Lebanon’, ‘extra-hospital’, ‘environment’, ‘animals’, ‘water’ and ‘vegetables’. The retrieved studies were first screened using title and abstract; and all eligible studies were retained for full-text assessment. Only indexed original research papers were included in the review (Table 1). According to our records; very few investigations have targeted the AMR issue in Lebanese non-clinical settings. However; during the last decade; the prevalence of ESBL-; AmpC β-lactamase- and carbapenemase-producing Gram-negative bacteria has become widely reported in animals; food and the environment in Lebanon. Interestingly; the majority of investigations were conducted either in overcrowded urban regions or in rural regions with high agricultural production (Fig. 1). Recently; Diab et al. reported the first ESBL-producing *E. coli* in animals in the country [24]. The same paper also described massive colonisation of the Lebanese bovine sector with ESBL-

producing *E. coli*; with a prevalence of 23% among healthy adult cattle and 84% among randomly selected cattle farms [24]. In contrast to worldwide findings; the enzymes responsible for the ESBL phenotype found in Lebanon were mostly CTX-M-15; followed by other ESBL types such as SHV-12; CTX-M-9; CTX-M-14; CTX-M-27 and CTX-M-55 [22–24,27,39]. Globally; CTX-M-1 is the most identified enzyme responsible for the ESBL phenotype in livestock; followed by CTX-M-14 [19,40,41]. However; recent data highlighted the presence of the *bla*_{CTX-M-15} gene in non-clinical contexts [23,39]. In Lebanon as well as worldwide; CTX-M-15 remains the most widespread ESBL disseminated both in hospitals and the community [41,42]. Interestingly; multilocus sequence typing (MLST) and pulsed-field gel electrophoresis (PFGE) results revealed high genetic diversity of CTX-M-15-producing strains circulating in Lebanon. The isolated *E. coli* strains belonged to ST10; ST58; ST69; ST155; ST617 and ST1303; which have been observed both in humans and animals; and to ST162; ST196; ST218; ST457; ST515; ST540; ST746; ST1140; ST1294; ST1303; ST2325; ST2450; ST4252; ST4623; ST5204; ST5442 and ST5728; which have no common history [24]. However; none of the isolated strains belonged to ST131; ST405 or ST648 clones; which are the most responsible for the spread of CTX-M-15 in humans. Similarly; water contamination by ESBLs and carbapenemases is potentially a major risk to public health in Lebanon. A recent nationwide study reported a high diversity of *E. coli* STs in sewage water in Lebanon; including ST38; ST90; ST617; ST4144; ST4608; ST6222; ST6450; ST6470; ST6480 and ST6894

Pheno-type	Bacterial host	Antibiotic resistance gene	Symbol	Origin(s)
CST-R	E	<i>mcr-1</i>	◆	
ESBL	E	<i>bla</i> _{CTX-M-9}	☀	
	E	<i>bla</i> _{CTX-M-14}	■	
	E	<i>bla</i> _{CTX-M-15}	▲	
	E	<i>bla</i> _{CTX-M-27}	☀	
	E	<i>bla</i> _{CTX-M-55}	★	
	E	<i>bla</i> _{SHV-12}	●	
	E	<i>bla</i> _{TEM-135-like}	⬢	
	Carbapenemase	E	<i>bla</i> _{OXA-48}	☆
E		<i>bla</i> _{OXA-244}	▼	
E		<i>bla</i> _{NDM-1}	▴	
P		<i>bla</i> _{VIM-2}	⬢	
A		<i>bla</i> _{OXA-23}	×	
A		<i>bla</i> _{OXA-24}	◻	
A		<i>bla</i> _{OXA-58}	▶	
A		<i>bla</i> _{OXA-72}	○	
A		<i>bla</i> _{OXA-143}	◇	

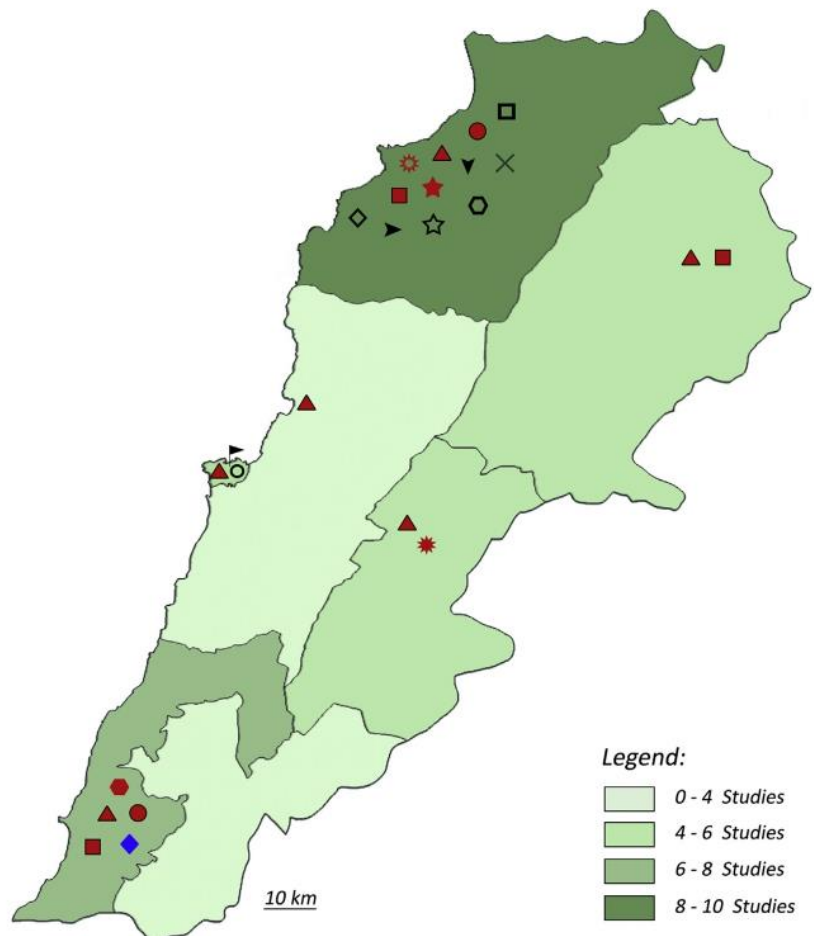


Fig. 1. Geographical distribution of reported extended-spectrum β-lactamases (ESBLs), carbapenemases and *mcr-1* genes and their corresponding producing Gram-negative bacteria and sources in Lebanon. CST-R, colistin-resistant; E, Enterobacteriaceae; P, *Pseudomonas aeruginosa*; A, *Acinetobacter* spp.

[39]. Another study conducted in North and South Lebanon also described very similar findings associated with high ESBL levels (59%) in water sampled from estuaries [23]. As described above; CTX-M-15 was the predominant ESBL identified in water sources including estuaries; wells and springs. None the less; this study described for the first time the presence of *E. coli* isolates belonging to ST131 and ST405 clones that are commonly reported in clinical settings [43]. Interestingly; well and spring water in Lebanon; particularly in rural areas; is directly intended for human and animal consumption without any further treatment. In addition; estuary water is commonly used to water animals and/or irrigate crops. Moreover; a cross-sectional study conducted on poultry farms at a national level revealed the presence of STs common to both humans and animals; particularly ST155 [25]. Surprisingly; none of the aforementioned studies reported the occurrence of *bla*_{CTX-M-1}; which is mainly associated with animal sources. All of these findings are in agreement with the proposition that livestock; poultry and water in Lebanon are inhabited by genetically diverse *E. coli* carrying and disseminating *bla*_{CTX-M-15} plasmids in different genetic backgrounds and imply an association with clinical isolates [44].

Regarding carbapenemases, Al Bayssari et al. published the first description of *bla*_{VIM-2}-carrying *Pseudomonas aeruginosa* and *bla*_{OXA-23}-carrying *Acinetobacter baumannii* strains isolated from Lebanese livestock and poultry [45]. Moreover, they also detected the co-occurrence of *bla*_{OXA-23} and *bla*_{OXA-58} in the same strain of *A. baumannii* isolated from fowl. Similarly, Rafei et al. identified a *bla*_{OXA-143}-harbouring *A. baumannii* and a *bla*_{OXA-24}-harbouring *Acinetobacter pittii*, both from horses [46]. In addition, two *Acinetobacter calcoaceticus* strains carrying the *bla*_{OXA-72} carbapenemase gene have also been isolated from vegetables [47]. Furthermore, a case report showed the presence of *E. coli* OXA-48 clone ST38 in a Lebanese fowl (*Gallus domesticus*). This latter represents an emerging human clone in the Mediterranean basin region but has also been identified as an OXA-244-producer in estuaries water in Lebanon [23,27,43]. Furthermore, an OXA-48-producing *Klebsiella pneumoniae* ST530 was recently detected in raw bovine milk [22]. In addition, *E. coli* and *K. pneumoniae* strains harbouring *bla*_{OXA-48} were also reported in estuary water, confirming the diversity of bacterial species producing OXA-48 and their probable endemic status in Lebanon [20,22,27,48]. Likewise, a recent study reported for the first time NDM-1-producing *Enterobacter cloacae* in Lebanon [49]. The NDM-1 enzyme was previously detected in Lebanon among *A. baumannii* and *A. pittii* isolated from clinical samples, but not in Enterobacteriaceae [50,51]. Besides, Gram-negative bacteria producing ESBLs and carbapenemases are co-resistant to other veterinary and clinically-licensed antibiotics (Table 1), making the AMR situation worse.

On the other hand, a case report published in January 2018 showed the emergence of an *mcr-1* plasmid-mediated colistin-resistant *E. coli* strain isolated from Lebanese poultry [52]. The isolated strain belonged to ST515, which was lately observed in Lebanese livestock [24]. In the same year, the same research group reported 23 colistin-resistant *E. coli* strains harbouring the *mcr-1* gene isolated from pigs [26]. Of the 23 colistin-resistant strains, only 4 were ESBL-producers whilst the remaining strains were susceptible to the majority of β -lactams [26]. A nationwide study carried out between September 2017 and March 2018 in three main poultry farms in Lebanon revealed widespread resistance to colistin, with an alarming percentage of *E. coli* isolates harbouring the *mcr-1* gene (98%) [53].

The level of colistin usage in farms and veterinary medicine remains unclear. However, unpublished data have revealed the heavy use of this compound both in humans and animals in Lebanon. Hence, further studies including a large number of human, animal and environmental samples are urgently required.

In conclusion, the AMR issue is not only confined to clinical settings but is also disseminated in the community. Animals as well as foodstuffs and the environment are significant reservoirs of AMR in Lebanon. Although few epidemiological studies have been conducted, the available data are highly worrisome indicating that MDR and XDR bacteria are ubiquitous in Lebanon. Besides, it is suggested that the dissemination of resistant clones in the community may have a zoonotic origin since the same clones were found both in clinical and non-clinical samples in Lebanon. Moreover, the presence of antimicrobial-resistant Gram-negative bacteria in the environment suggests potential contamination either by human or animal activities and represents a prominent public-health issue in Lebanon. Hence the importance of a One Health concept based on whole-genome sequencing surveillance data of microbes in humans, animals and the environment in order to address the AMR issue by developing comprehensive and integrative measures and effective preventive and control strategies. Moreover, awareness movements involving stakeholders, policy-makers and health actors need to be initiated in order to stop using antibiotics without prescription and even to ban antibiotic use in the veterinary sector.

Acknowledgment

The authors would like to thank Halima El Cheikh for assistance in the design of the figure.

Funding

HAM was supported by PhD fellowships from the Azm & Saade Association and the Lebanese Association for Scientific Research (LASeR).

Competing interests

None declared.

Ethical approval

Not required.

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Table S1: Epidemiological and molecular characteristics of all isolates.

Strain	Farm	Animal number	ST	Phylogeny	MIC colistin mg/L	mcr gene	ESBL gene	pAmpC gene	mcr-1-carrying plasmid
50092	F1	1	1011	D	8	<i>mcr-1.1</i>	<i>bla</i> CTX-M-3	<i>bla</i> CMY-2	IncX4
50097	F1	1	6856	A	8	<i>mcr-1.1</i>		<i>bla</i> CMY-2	IncX4
50098	F1	2	4162	B1	8			<i>bla</i> CMY-2	-
50099	F1	2	93	A	4	<i>mcr-1.1</i>	<i>bla</i> CTX-M-65		IncI2
50100	F1	3	93	A	4	<i>mcr-1.1</i>	<i>bla</i> CTX-M-65		IncI2
49988	F2	4	744	A	8	<i>mcr-1.1</i>			ND
50032	F2	5	4550	A	4				-
50037	F2	6	93	A	4	<i>mcr-1.1</i>	<i>bla</i> CTX-M-65		IncI2
50041	F2	6	388	D	8	<i>mcr-1.1</i>			IncX4
50042	F2	7	359	B1	8	<i>mcr-1.1</i>	<i>bla</i> CTX-M-15		IncX4
50043	F2	8	1011	B1	8	<i>mcr-1.1</i>	<i>bla</i> CTX-M-3	<i>bla</i> CMY-2	IncX4
50046	F2	9	457	D	4	<i>mcr-1.1</i>	<i>bla</i> CTX-M-15		IncX4
50050	F2	10	1011	B2	8	<i>mcr-1.1</i>	<i>bla</i> CTX-M-3	<i>bla</i> CMY-2	IncX4
50051	F2	11	93	A	8	<i>mcr-1.1</i>	<i>bla</i> CTX-M-65		IncI2
50053	F2	6	359	B1	4	<i>mcr-1.1</i>		<i>bla</i> CMY-2	IncX4
50057	F2	11	10	B1	4			<i>bla</i> CMY-2	-
50058	F2	12	1011	A	8	<i>mcr-1.1</i>	<i>bla</i> CTX-M-3	<i>bla</i> CMY-2	IncX4
49987	F3	13	744	A	8	<i>mcr-1.1</i>	<i>bla</i> CTX-M-14		IncI2
50020	F3	13	115	B1	8			<i>bla</i> CMY-2	-
50093	F3	14	1011	D	8	<i>mcr-1.1</i>		<i>bla</i> CMY-2	IncX4
50095	F3	15	752	A	8	<i>mcr-1.1</i>			IncX4
50096	F3	15	93	A	64	<i>mcr-1.1</i>	<i>bla</i> CTX-M-65		IncI2
50033	F4	16	1421	A	8	<i>mcr-1.1</i>	<i>bla</i> SHV-12		IncX4
50035	F4	16	93	A	8	<i>mcr-1.1</i>	<i>bla</i> CTX-M-65		IncI2
50036	F4	17	1011	A	8	<i>mcr-1.1</i>			IncHI2
50034	F5	17	6844	A	8	<i>mcr-1.1</i>		<i>bla</i> CMY-2	IncI2
50038	F5	18	6/31/83*/28/1/1/2	A	4	<i>mcr-1.1</i>		<i>bla</i> CMY-2	IncI2
50039	F5	19	6115	D	8	<i>mcr-1.1</i>			IncX4
50040	F5	17	1011	A	8	<i>mcr-1.1</i>	<i>bla</i> CTX-M-3	<i>bla</i> CMY-2	IncX4
50059	F6	20	93	A	8	<i>mcr-1.1</i>	<i>bla</i> CTX-M-65		IncI2
50069	F6	21	6856	A	4	<i>mcr-1.1</i>		<i>bla</i> CMY-2	IncX4
50088	F6	22	93	A	8	<i>mcr-1.1</i>	<i>bla</i> CTX-M-65		IncI2
50075	F8	23	4162	B1	8			<i>bla</i> CMY-2	-
50076	F8	24	354	A	32	<i>mcr-1.1</i>	<i>bla</i> CTX-M-3	<i>bla</i> CMY-2	IncX4
50077	F8	25	93	A	8	<i>mcr-1.1 (2 copies)</i>	<i>bla</i> CTX-M-65		IncI2
50072	F9	26	1011	A	4	<i>mcr-1.1</i>			IncX4
50073	F9	27	1638	B1	16	<i>mcr-1.1</i>			IncX4
50074	F9	28	6/4/159*/44/112/1/17 (SLV ST1011)	D	16	<i>mcr-1.1</i>		<i>bla</i> CMY-2	IncI2
50079	F9	29	2705	A	16	<i>mcr-1.1</i>	<i>bla</i> CTX-M-3	<i>bla</i> CMY-2	IncX4

50080	F9	27	1638	A	16				-
50070	F10	30	48	A	4	<i>mcr-1.1</i>	<i>blaCTX-M-64</i>		IncX4
50071	F10	31	744	A	4				-
50064	F11	32	93	A	8	<i>mcr-1.1</i>	<i>blaCTX-M-65</i>		Incl2
50091	F11	33	616	B1	8				-
50061	F12	34	744	A	4	<i>mcr-1.1</i>	<i>blaCTX-M-14</i>		Incl2
50062	F12	34	616	A	8				-
50063	F12	35	48	B1	8				-
49953	F14	36	665	A	8			<i>blaCMY-2</i>	-
49962	F14	37	8748	A	4				-
49965	F15	38	6/29*/4/18/11/8/6	B1	4				-
49966	F16	39	155	B1	4				-
50010	F18	40	744	A	4	<i>mcr-1.1</i>			IncX4
50012	F18	41	155	D	4	<i>mcr-1.1</i>			IncX4
50002	F19	42	1011	B1	8	<i>mcr-1.1</i>	<i>blaCTX-M-3</i>	<i>blaCMY-2</i>	IncX4
50003	F19	43	1011	D	4	<i>mcr-1.1</i>		<i>blaCMY-2</i>	IncX4
50004	F20	44	1196	D	4	<i>mcr-1.1</i>			IncX4
50006	F20	45	117	D	4			<i>blaCMY-2</i>	-
50007	F20	46	117	D	4			<i>blaCMY-2</i>	-
50008	F20	46	10	D	4	<i>mcr-1.1</i>			IncX4
50009	F20	47	64/27/1/1/8/8/6	A	4	<i>mcr-1.1</i>			IncX4
49996	F21	48	1196	B1	8	<i>mcr-1.1</i>			IncX4
49997	F21	49	206	B1	8	<i>mcr-1.1</i>			IncX4
49998	F21	49	93	A	4	<i>mcr-1.1</i>	<i>blaCTX-M-65</i>		Incl2
49999	F21	50	93	A	4	<i>mcr-1.1</i>	<i>blaCTX-M-65</i>		Incl2
50000	F21	50	398	B1	4	<i>mcr-1.1</i>	<i>blaCTX-M-65</i>		IncX4
50021	F22	51	48	B1	16	<i>mcr-1.1</i>			IncX4
50023	F22	52	1626	A	4	<i>mcr-1.1</i>			IncX4
49973	F23	53	93	A	8	<i>mcr-1.1</i>	<i>blaCTX-M-65</i>		Incl2
49972	F24	54	57	A	4		<i>blaCTX-M-65</i>		-
49974	F25	55	1140	D	8	<i>mcr-1.1</i>			Incl2
50016	F26	56	1140	D	8	<i>mcr-1.1</i>			IncX4
50019	F26	57	93	A	16	<i>mcr-1.1</i>	<i>blaCTX-M-65</i>		Incl2
50054	F26	56	10	B1	8	<i>mcr-1.1</i>			IncX4
49975	F29	58	1140	A	16	<i>mcr-1.1</i>			Incl2
49979	F29	59	155	B1	16			<i>blaCMY-2</i>	-
49981	F29	60	1638	B1	16	<i>mcr-1.1</i>	<i>blaCTX-M-3</i>		Incl2
49983	F29	58	624	D	8	<i>mcr-1.1</i>		<i>blaDHA-1</i>	Incl2
49984	F29	61	10/1509*/4/8/8/8/2	A	8	<i>mcr-1.1</i>			IncX4
50017	F30	62	8492	B1	16				-
49976	F31	63	10/11/154/10/7/8/2	A	16	<i>mcr-1.1</i>			IncX4
49982	F31	65	1140	A	16	<i>mcr-1.1</i>			Incl2
49977	F32	66	212	D	8				-

49978	F31	64	1140	D	16				
49980	F32	67	4162	B1	8			<i>bla</i> CMY-2	-

Table S2: Characteristics of all *E. coli* isolates.

Individual Number	Bakery	Inclusion criteria	T0 or T6 positive	Strain	ST	Cephalosporins	Carbapenems	Colistin	Inc content	IncF formula
1	B	COL-R	t0	50117	10	-		<i>mcr-1.1</i>	IncFII, IncX4, IncR	F2:A-B-
1	B	ESC-R	t0	53419	8149	<i>bla</i> CTX-M-15		-	IncFIB	F-A:B53
1	B	CP-R	t0	53420	540	-	<i>bla</i> OXA-181	-	IncFIB, IncX3	F-A:B53
1	B	ESC-R	t6	53421	4553	<i>bla</i> CTX-M-15		-	none	no FII
3	B	ESC-R	t0	53422	206	<i>bla</i> VEB-1		-	IncFIB	F-A:B53
5	B	ESC-R	t6	53423	394	<i>bla</i> CTX-M-15		-	IncFII, IncFIB, IncB/O/K/Z	F68*:A-B12*
6	B	ESC-R	t0	53429	8149	<i>bla</i> CTX-M-15		-	IncFIB	F-A:B53
6	B	ESC-R	t6	53430	361	<i>bla</i> CTX-M-15		-	none	no FII
7	B	ESC-R	t6	53431	34	<i>bla</i> CTX-M-15		-	IncY	no FII
7	B	ESC-R	t6	53432	746	<i>bla</i> SHV-12		-	IncX3	no FII
8	B	COL-R	t0	53417	10	-		<i>mcr-1.1</i>	IncFII, IncX4, IncR	F2:A-B-
8	B	ESC-R	t0	53434	1303	<i>bla</i> CMY-2		-	IncFIB, IncX4	F24:A-B16
8	B	CP-R	t0	53435	940	-	<i>bla</i> OXA-181	-	IncX3	no FII
8	B	ESC-R	t6	53438	372	<i>bla</i> CTX-M-15		-	none	F35:A-B-

9	B	ESC-R	t0	53439	648	<i>bla</i> CMY-42		-	IncFII, IncFIA, IncFIB, IncIlg	F1:A2:B33
9	B	ESC-R	t6	53440	2076	<i>bla</i> CTX-M-15		-	IncFII, IncFIB	F77:A-:B-
10	B	COL-R	t0	50121	1303	-		<i>mcr-1.1</i>	IncX4	no FII
10	B	ESC-R	t0	53442	4242	<i>bla</i> CTX-M-3		-	IncFII, IncFIB	F2:A-:B-
10	B	CP-R	t0	53443	6836	-	<i>bla</i> NDM-5	-	IncX3	no FII
11	B	COL-R	t0	50122	1303	-		<i>mcr-1.1</i>	IncX4	no FII
11	B	ESC-R	t6	53446	1303	<i>bla</i> CTX-M-15		-	IncFII, IncY	F82:A-:B-
13	B	ESC-R	t6	53450	ND (6/11/4/8/8/2/2)	<i>bla</i> CTX-M-15		-	none	no FII
14	B	COL-R	t6	53418	56	-		<i>mcr-1.1</i>	IncHI2	no FII
14	B	ESC-R	t6	53452	69	<i>bla</i> CTX-M-15		-	Incl1	no FII
15	B	ESC-R	t6	53453	1284	<i>bla</i> CMY-42, <i>bla</i> CTX-M-15		-	IncFIA, IncFIB, IncIlg	F31:A4:B1
16	B	ESC-R	t0	53454	155	<i>bla</i> CTX-M-15		-	none	F44:A-:B-
17	B	ESC-R	t0	53455	167	<i>bla</i> CMY-42		-	IncFIA, IncFIB, IncX1, IncIlg	F31:A4:B1
17	B	ESC-R	t6	53456	1193	<i>bla</i> CTX-M-15		-	IncFIA, IncFIB	F-:A1:B10
18	B	ESC-R	t0	50969	10	<i>bla</i> CTX-M-15		-	IncY	no FII
19	B	COL-R	t0	53416	10	-		<i>mcr-1.1</i>	IncFII, IncX4, IncR	F2:A-:B-
20	B	ESC-R	t6	53459	174	<i>bla</i> CTX-M-15		-	IncFII, IncB/O/K/Z	F53:A-:B-
21	A	ESC-R	t0	53460	517	<i>bla</i> CTX-M-15		-	IncFIB	F-:A-:B15
21	A	ESC-R	t6	53461	5271	<i>bla</i> CMY-2		-	IncFIB, IncHI2, IncB/O/K/Z	F24:A6*:B8
24	A	CP-R	t0	53465	1722	<i>bla</i> CTX-M-15	<i>bla</i> OXA-244	-	Incl1	no FII
25	A	ESC-R	t6	53466	131 (H30)	<i>bla</i> CTX-M-15		-	IncFII, IncFIB	F29:A-:B10

26	A	ESC-R	t6	53467	69	<i>bla</i> CTX-M-15		-	IncFIA, IncFIB, IncI1	F36*:A4:B1
26	A	ESC-R	t6	53468	131 (H41)	<i>bla</i> CTX-M-15		-	IncFII, IncFIB	F29:A-:B10
28	A	ESC-R	t0	53470	131 (H41)	<i>bla</i> CTX-M-15		-	IncFII, IncFIB	F29:A-:B10
28	A	ESC-R	t0	53471	665	<i>bla</i> CMY-2		-	IncFII, IncFIB, IncI1, IncI2	F16:A-:B1
36	A	CP-R	t6	53477	940	<i>bla</i> CTX-M-15	<i>bla</i> OXA-181	-	IncX3	no FII
37	A	ESC-R	t0	50970	10698	<i>bla</i> CTX-M-15		-	IncFIB	F19:A-:B9
37	A	ESC-R	t6	53478	120	<i>bla</i> CTX-M-15		-	IncFII, IncFIA, IncFIB, IncB/O/K/Z, IncX4	F4:A6:B10*
38	A	ESC-R	t0	53480	10698	<i>bla</i> CTX-M-15		-	IncFIB	F19:A-:B9
38	A	ESC-R	t6	53481	120	<i>bla</i> CTX-M-15		-	IncFII, IncFIA, IncFIB, IncB/O/K/Z, IncX4	F4:A6:B8*
40	A	ESC-R	t6	53482	3172	<i>bla</i> CTX-M-15		-	IncFII, IncFIB	F29:A-:B10
41	A	ESC-R	t6	53483	10	<i>bla</i> DHA-1		-	IncFII	F98:A-:B-
42	A	ESC-R	t0	50956	58	<i>bla</i> CMY-2		-	IncFII, IncFIB, IncHI2	F2:A-:B1
42	A	ESC-R	t6	53484	62	<i>bla</i> CTX-M-15		-	IncFII	F2:A-:B-
44	A	ESC-R	t0	53486	7937	<i>bla</i> CMY-42		-	IncFII, IncFIA_1, IncFIB, IncIg	F48:A1:B49
46	A	ESC-R	t0	53487	34	<i>bla</i> CTX-M-15		-	IncFII, IncI1	F40:A-:B-
46	A	ESC-R	t0	53488	131 (H41)	<i>bla</i> CTX-M-15		-	IncFII, IncFIB, IncY	F29:A-:B10
48	A	ESC-R	t6	53489	1727	<i>bla</i> CTX-M-15		-	IncFIB	F24:A-:B1
48	A	ESC-R	t6	53490	7629	<i>bla</i> CMY-2		-	IncHI2, IncY	no FII
49	A	ESC-R	t0	53492	405	<i>bla</i> CTX-M-15		-	IncFII, IncFIB	F29:A-:B10
50	A	ESC-R	t6	53493	5614	<i>bla</i> CTX-M-15		-	IncB/O/K/Z	no FII

51	A	ESC-R	t0	53495	69	<i>bla</i> CTX-M-15		-	IncFII, IncFIA, IncFIB, IncX1	F36:A1:B66
51	A	ESC-R	t6	53496	69	<i>bla</i> CTX-M-15		-	IncFII, IncFIA, IncFIB, IncX1	F36:A1:B66
52	A	ESC-R	t6	53497	131 (H30)	<i>bla</i> CTX-M-27		-	IncFII, IncFIA, IncFIB	F1:A2:B20
53	A	ESC-R	t0	50971	131 (H30)	<i>bla</i> CTX-M-15		-	IncFIA, IncFIB	F22*:A1:B20
53	A	ESC-R	t6	53498	120	<i>bla</i> CTX-M-15		-	IncFII, IncFIA, IncB/O/K/Z, IncX1, IncX3	F35:A6:B26
54	A	ESC-R	t0	50955	6335*(<i>recA</i>)	<i>bla</i> CMY-2		-	IncFIA, IncFIB, IncI1	F18:A5:B1
54	A	ESC-R	t6	53499	131 (H41)	<i>bla</i> CTX-M-15		-	IncFII, IncFIB	F29:A-:B10
56	A	ESC-R	t6	53500	484	<i>bla</i> CTX-M-15		-	IncI1	F59:A-:B-
57	A	ESC-R	t0	53501	131 (H41)	<i>bla</i> CTX-M-15		-	IncFII, IncFIB	F2:A-:B10
57	A	ESC-R	t6	53502	131 (H30)	<i>bla</i> CTX-M-15		-	IncFII, IncFIA, IncX4	F2:A1:B66
58	A	ESC-R	t0	50940	48	<i>bla</i> CTX-M-15		-	IncFII IncQ1,	F7:A-:B-
58	A	ESC-R	t6	53503	7401	<i>bla</i> DHA-1, <i>bla</i> CTX-M-27		-	IncFII, IncFIA, IncFIB, IncX4	F1:A2:B20
59	A	ESC-R	t6	53504	120	<i>bla</i> CTX-M-15		-	IncFII, IncFIA, IncB/O/K/Z, IncX1, IncX3	F35:A6:B26
61	A	ESC-R	t0	53506	31	<i>bla</i> CTX-M-15		-	IncFII	F2:A-:B-
61	A	ESC-R	t6	53507	5529	<i>bla</i> CTX-M-15		-	IncFIA, IncFIB, IncX1	F36:A1:B20
62	A	ESC-R	t6	53511	4553	<i>bla</i> CTX-M-15		-	none	no FII
63	A	CP-R	t6	53512	940	<i>bla</i> CTX-M-15	<i>bla</i> OXA-181	-	IncX3	no FII
67	C	ESC-R	t6	53513	5442*(<i>adk</i>)	<i>bla</i> CTX-M-15		-	none	no FII
69	B	ESC-R	t6	53514	3172	<i>bla</i> CTX-M-15		-	IncFII, IncFIB	F29:A-:B10
71	B	ESC-R	t0	50932	155	<i>bla</i> CTX-M-15		-	none	no FII

72	B	ESC-R	t0	53516	155	<i>bla</i> CTX-M-15		-	none	no FII
76	A	ESC-R	t0	50957	7401	<i>bla</i> CTX-M-27		-	IncFII, IncFIA, IncFIB	F1:A2:B20
79	C	ESC-R	t6	50933	361	<i>bla</i> CMY-42		-	IncFII, IncFIB, IncIg	F1:A-B49
79	C	CP-R	t0	53519	46	-	<i>bla</i> OXA-181	-	IncX3	no FII
80	A	ESC-R	t0	50954	69	<i>bla</i> CTX-M-15		-	IncFII, IncFIB, IncX4, IncY	F1:A-B33
85	A	ESC-R	t0	53520	58	<i>bla</i> SHV-12		-	IncFII, IncFIB, IncX3	F2:A-B1

