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Multi-Drug Resistant Organisms in Lebanese Livestock

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AVANT PROPOS

Le format de présentation de cette thèse correspond à une recommandation à la spécialité Pathologie Humaine, Maladies infectieuses, à l'intérieur du Master des Sciences de la Vie et de la Santé qui dépend de l'Ecole Doctorale des Sciences de la Vie de Marseille.

Le candidat est amené à respecter les règles qui lui sont imposées et qui comportent un format de thèse utilisé dans le Nord de l'Europe et qui permet un meilleur rangement que les thèses traditionnelles. Par ailleurs, la partie introduction et bibliographie est remplacée par une revue envoyée dans un journal afin de permettre une évaluation extérieure de la qualité de la revue et de permettre à l'étudiant de commencer le plus tôt possible une bibliographie sur le domaine de cette thèse.

Par ailleurs, la thèse est présentée sur article publié, accepté, ou soumis associé d'un bref commentaire donnant le sens général du travail. Cette forme de présentation a paru plus en adéquation avec les exigences de la compétition internationale et permet de se concentrer sur des travaux qui bénéficieront d'une diffusion internationale.

Professeur Didier RAOULT

RÉSUMÉ

De nos jours, l'épidémiologie des bactéries multi-résistantes aux antibiotiques a évolué et ne se limite plus aux milieux hospitaliers. En effet, les animaux dont ceux utilisés dans la production alimentaire sont désormais considérés comme d'importants réservoirs de bactéries multi-résistantes, notamment des Bacilles à Gram négatif sécréteurs de bêta-lactamases et/ou résistant à la colistine. L'émergence de ces bactéries multi-résistantes chez les animaux est due principalement à l'utilisation excessive d'antibiotiques en tant que prophylaxie et facteurs de croissance. De plus, certains antibiotiques utilisés chez les animaux le sont également chez les humains tels que la colistine. Le transfert d'organismes multi-résistants aux antibiotiques provenant d'animaux vers les humains est un problème majeur pouvant entrainer de graves infections. La transmission zoonotique se fait principalement par contact direct / indirect mais aussi par voie environnementale. Au Liban, plusieurs études ont été menées dans les hôpitaux et ont montré une prévalence élevée de bactéries multi-résistantes. En revanche, ces études sont rares dans le milieu vétérinaire. Le but de ce travail de thèse est de décrire l'épidémiologie des organismes multi-résistants dans les animaux d'élevage destinés à la consommation au Liban. Pour cela, nous avons tout d'abord déterminé 1) la prévalence nationale du portage intestinal de bactéries résistantes aux béta lactamines chez les poulets 2) la présence d'une relation entre les organismes multi-résistants chez les poulets et leur milieu environnant direct et en 3) la prévalence des organismes multi-résistants chez les porcs. Le typage des bactéries par MLST, le transfert de plasmides par conjugaison et le séquençage du génome entier ont été utilisés pour décrire la prévalence des organismes multi-résistants et les mécanismes de résistance chez les souches isolées de poulet, de porc, d'éleveur et de l'environnement. Nous pouvons ainsi conclure que les élevages de poulets et de porcs sont de puissants réservoirs de gènes de résistance BLSE et mcr-1 au Liban. La dissémination de la résistance semble être polyclonale et liée à la propagation de plasmides porteurs de gènes de résistance. Par conséquent, l'utilisation de la colistine en médecine vétérinaire au Liban doit être interdite.

Mots-clés: poulets, cochons, mcr-1, ESBL, environnement, agriculteurs.

ABSTRACT

Nowadays, the epidemiology of multi-drug resistance has changed and is no more confined to the hospital settings. Food producing animals are increasingly regarded as potent reservoirs of multi-drug resistant organisms i.e. beta lactamase producers and colistin-resistant Gram-negative bacilli. The emergence of multi-drug resistance in animals is thought to be mainly driven by the overuse of antibiotics as growth promoters and prophylaxis. The dissemination of multi-drug resistant organisms in animals is sparked by the concern of being transferred to humans where they can be candidates for infections with limited therapeutic options. The zoonotic transmission of resistant organisms from animals to humans occurs mainly via direct/indirect contact but also via environmental routes. In Lebanon, several studies were conducted in hospitals and showed a high prevalence of multi-drug resistance; unlikely, these studies are scarce in animals. The aim of this thesis research was thus to describe the epidemiology of multi-drug resistant organisms in Lebanese Livestock via 1) Determination of the nationwide prevalence of multi-drug resistance in poultry in terms of intestinal carriage, 2) Determination if any link exists between the prevalence of multi-drug resistant organisms in chicken and the surrounding environment and 3) Determination of the prevalence of multi-resistant organisms in pigs. Multi-locus sequence typing, conjugation experiments and whole genome sequencing were used to describe the prevalence of multi-drug resistant organisms and the corresponding mechanisms of resistance in the isolated strains from chicken, pigs, farmers and environment. Chicken and swine farms showed to be potent reservoirs of ESBL and mcr-1 genes in Lebanon. The dissemination of multi-drug resistance appears to be multi-clonal and related to the spread of plasmid carrying resistance genes. Colistin use in veterinary medicine in Lebanon should be banned.

Keywords: Chicken, pigs, mcr-1, ESBL, environment, farmers

Introduction

In the 1940s, the discovery of antibiotics was considered as one of the medicine's major achievements that saved millions of lives (1). However, in the past twenty years, bacterial resistance has increased and reduced the efficiency of many antibiotics frequently used in the clinical settings (2). Antibiotic resistance in bacteria can be intrinsic or acquired. Acquired resistance can occur either through sequential mutations within the bacterial cell genome or via the acquisition of resistance genes from another bacterium, the so-called "horizontal gene transfer" (3). The mechanisms of antibiotic resistance in bacteria are manifested by alterations of the antibiotic's target, activation of efflux pumps, changes in the outer membrane permeability or via the secretion of hydrolyzing enzymes (4). Nowadays, vancomycin-resistant Enterococci (VRE), methicillin-resistant Staphylococcus aureus (MRSA), multi-drug resistant Pseudomonas aeruginosa and Acinetobacter baumannii, extended spectrum beta-lactamase producing Enterobacteriaceae (ESBL-PA), carbapenem-resistant Enterobacteriaceae (CRE) and colistin resistant Gram-negative bacilli are among the most common organisms where multi-drug resistance is encountered (5). The over-usage of antibiotics appears to be the main driven for the rapid evolution of resistance in bacteria. Antibiotic overuse creates a selective pressure that favors the proliferation of resistant strains over the susceptible ones provoking thus their dissemination (6).

Nowadays, the animal intestinal microbiota is considered as a potent reservoir of multi-drug resistant organisms as well as an epicenter for gene resistance (7). Antibiotics in livestock are not only administered for therapeutic purposes but are rather also given as growth promoters and for prophylaxis (8). The European centre for disease Prevention and control/ European Food Safety Authority/European Medicines Agency (ECDC/EFSA/EMA) joint report found that the average consumption of antibiotics in animals exceeded the one in humans: 152 mg/kg versus 124mg/kg in 2014 respectively. In this same report, univariate analysis showed a significant correlation between E. coli resistance in the animal/human sectors and fluoroquinolones consumption and between tetracyclines and polymyxins and resistant E. coli in animals (9). ESBL, ampC and carbapenemase producers as well as colistin resistant Gram-negative bacilli are currently frequently detected in wild type animals, pets and Livestock (10).

In Lebanon, several studies were conducted in the clinical settings and showed an elevated prevalence of ESBL and carbapenemase producing Gram-negative bacilli. One study done at the American University of Beirut Medical Centre reported that between 2008 and 2011, 2.45% of Klebsiella pneumoniae and 1.07% of Escherichia coli strains were ESBL producers

as well as ertapenem resistant (11). Another study in the north showed that during 2009-2012, 28% and 9% of the bacteremia episodes in febrile neutropenic patients were caused by third-generation cephalosporin and carbapenem resistant Gram-negative bacilli, respectively (12). In animals as well as in the environment, studies addressing multi-drug resistance are scarce in Lebanon. In the environment, Rafei et al reported the detection of Acinetobacter baumannii in 7% of water samples, 3% of milk samples, 14% of cheese samples, 8% of meat samples and 8% of animal samples (13). VIM-2 producing Pseudomonas aeruginosa, OXA-23/OXA-58 A. baumannii as well as OXA-48 carrying E. coli strains were previously detected in animals in this country (14, 15). More recently, Diab et al showed a high prevalence of CTX-M-15 producing E. coli isolates in Lebanese cattle (16). The epidemiology of ESBL/ampC producers and more importantly colistin-resistant Gram-negative bacilli remains unknown in livestock and the surrounding environment in Lebanon. Hence the aim of this PhD research work was to describe the epidemiology of multi-drug resistant organisms in Lebanese livestock at the nationwide level via:

1) Determination of the nationwide prevalence of ESBL/ampC producing Gram-negative bacilli in Lebanese chicken farms in terms of intestinal carriage.
2) Investigating if any link exists between multi-drug resistant organisms in poultry and the ones in farmers and the surrounding environment.
3) Determination of the prevalence of ESBL/ampC producers and mcr-1 Gram-negative bacilli in the main swine farms located in Lebanon.

This manuscript is divided into six main chapters.

Chapter I involves a systematic review and a mini review. The first one **"Article 1"** presents an extensive examination of the current literature on the epidemiology of ESBL, ampC and carbapenemase producing Gram-negative bacilli as well as colistin resistant ones in animals of the region surrounding the Mediterranean Basin. This review is beneficial in that it shows the driver of multi-drug resistance emergence in this area of the world. In addition it sheds the light on the countries where insufficient data are available regarding the spread of multi-drug resistant organisms and the level of antibiotic consumption. The second one, mini review **"Article 2"** describes the impact of colistin use on the worldwide emergence and dissemination of colistin resistance in animals especially the one mediated by mcr colistin resistance genes. The risk of transmission of colistin resistant Gram-negative bacilli from animals to humans was also discussed.

In **Chapter II** we describe the epidemiology of multi-drug resistant organisms in Lebanese Livestock in terms of intestinal carriage. "**Article 3"** includes the prevalence of ESBL and

ampC producing Gram-negative bacilli in chicken farms distributed over the seven districts of Lebanon. "**Article 4"** reports the first detection of an mcr-1 positive E. coli strain in Lebanese poultry. "**Article 5"** shows the prevalence of ESBL/ampC producing Gram-negative bacilli in the main swine farms located in Lebanon. In addition, it outlines the first detection of mcr-1 in pigs of this country. "**Article 6"** describes the dissemination of ESBL/ampC producers and especially mcr-1 E. coli strains in chicken, farmers and environment in the same farm where the first detection of mcr-1 was reported by our team two years ago.

In **Chapter III** we describe the genomic analysis of a colistin hetero-resistant Enterobacter cloacae strain that was isolated from chicken in Lebanon. This strain presented with an elevated colistin MIC up to 1024µg/ml and was an ampC producer harbouring the MIR-20 ampC beta lactamase. Using whole genome sequencing and qPCR, the mechanism of colistin hetero-resistance in this isolate was explored **"Article 7"**.

Chapter IV included a collaborative study in which colistin and carbapenem resistant Klebsiella pneumoniae strains were isolated from clinical samples in Algeria **"Article 8"**.

Chapter V involves the description of a Lachnoclostridium nov. species. The strain was isolated from the urine sample of a patient in Marseille **"Article 9"**.

Chapter VI is devoted to the work achieved in Lebanon during M2 and 1st year PhD studies. **"Article 10"** describes the dynamic of beta-lactamase-producing enterobacteriaceae carriage among elderlies in two nursing homes located in the north of Lebanon over a four month period. In this study, we described the first detection of an OXA-48 producing E. coli strain isolated from a community setting in Lebanon. **"Article 11"** describes the dynamic of multi-drug resistant organisms in Lebanese elderlies and their impact on bacterial fitness. **"Article 12"** describes the fitness cost achieved by competing different species of sensitive and ESBL strains isolated from nursing home residents in Lebanon

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Chapter I

Review Papers

Description of the prevalence of Beta-lactamase and Colistin Resistant Gram-negative bacilli in animals worldwide.

Introduction

Gram-negative bacilli are common inhabitant of the human and animals' intestinal tract (1). During the past twenty years, resistance in these organisms have increased and reduced the efficacy of commonly prescribed antibiotics such as beta-lactams, aminoglycosides and fluoroquinolones (2). The main mechanism of beta-lactam resistance encountered nowadays in Gram-negative bacilli is the production of ESBLs, ampC beta lactamases and carbapenemases (2). Genes encoding these enzymes are often localized on plasmids carrying resistance genes to other non beta-lactam antibiotics (3). Furthermore, colistin resistance has recently emerged in these organisms. Colistin resistance in Gram-negative bacilli occurs either via the acquisition of mcr colistin resistance genes or via chromosomal mutations that mediates the modification of the lipid A moiety in the lipopolysaccharide chain (4). In this chapter, we aim to 1) shed the light on the current distribution of multi-drug resistant organisms in the animal sector of the Mediterranean 2) provide an updated view on the effect of colistin use in animals and the corresponding emergence of mcr colistin resistant Gram-negative bacilli in animals worldwide.

In the first review paper **Article 1** entitled **"Prevalence and emergence of ESBLs, carbapenemases and colistin resistant Gram-negative bacteria in animals of the Mediterranean basin"** we describe the epidemiology of ESBL and carbapenemase producers in addition to colistin resistance in animals of the region surrounding the Mediterranean basin. The Mediterranean basin is a region of the world that compromises a wide diversity of populations. It includes five Asian countries (Israel, Lebanon, Syria, Cyprus and Turkey), eleven European countries (Greece, Albania, Montenegro, Bosnia, Herzegovina, Croatia, Slovenia, Italy, Monaco, France and Spain) and five African countries (Morocco, Algeria, Tunisia, Libya and Egypt).

Studies involving chicken, cattle, pigs, pets and wild type animals in the aforementioned nations were all included. The types of antibiotics in each country were also included. CTX-M group 1 followed by SHV-12 and CTX-M group 9 were the most ESBL types prevailing in animals of the Mediterranean region. On the other hand, the spread of carbapenemase producers and mcr strains remains limited. Antibiotic prescription in veterinary medicine is not controlled in this area of the world. Tetracyclines, aminoglycosides, fluoroquinolones and polymyxins are often administered as therapeutics, prophylaxis and growth promoters. This review paper is now in the interactive review forum in Frontiers in Microbiology, manuscript reference number 373411.

In the mini-review **Article 2** entitled "**Colistin use in animals: a two side weapon against multi-drug resistant organisms"**, we summarize the impact of colistin use in animals in terms of emergence of resistance in Gram-negative bacilli. Colistin previously abandoned in the human medicine in view of its toxicity inside the human body was always prescribed in animals many decades ago. Available data on the level of colistin consumption, in addition to the corresponding distribution of mcr plasmid mediated colistin resistant isolates in the Asian, European, African and American countries in the animal sector were included. In addition, the risk of mcr colistin resistant Gram-negative bacilli transmission to humans was also discussed.

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Article 1

Prevalence and emergence of Extended-spectrum Cephalosporin-, carbapenem- and Colistin- resistant Gram negative bacteria of Animal Origin in the Mediterranean basin.

Iman Dandachi*, Selma Chabou*, Ziad Daoud, Jean-Marc Rolain

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Prevalence and Emergence of Extended-spectrum Cephalosporin-, Carbapenem- and Colistin- Resistant Gram Negative Bacteria of Animal Origin in the Mediterranean Basin

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Abstract

In recent years, extended ESBL and carbapenemase producing Gram negative bacteria have become widespread in hospitals, community settings and the environment. This has been triggered by the few therapeutic options left when infections with these multi-drug resistant organisms occur. The emergence of resistance to colistin, the last therapeutic option against carbapenem-resistant bacteria, worsened the situation. Recently, animals were regarded as potent antimicrobial reservoir and a possible source of infection to humans. Enteric Gram negative bacteria in animals can be easily transmitted to humans by direct contact or indirectly through the handling and consumption of undercooked/uncooked animal products. In the Mediterranean basin, little is known about the current overall epidemiology of multi-drug resistant bacteria in livestock, companion and domestic animals. This review describes the current epidemiology of ESBL, carbapenemase producers and colistin resistant bacteria of animal origin in this region of the world. The CTX-M group 1 seems to prevail in animals in this area, followed by SHV-12 and CTX-M group 9. The dissemination of carbapenemase producers and colistin resistance remains low. Isolated multi-drug resistant bacteria were often co-resistant to non beta-lactam antibiotics, frequently used in veterinary medicine as treatment, growth promoters, prophylaxis and in human medicine for therapeutic purposes. Antibiotics used in veterinary medicine in this area include mainly tetracycline, aminoglycosides, fluoroquinolones and polymyxins. Indeed, it appears that the emergence of ESBL and carbapenemase producers in animals is not related to the use of beta-lactam antibiotics but is, rather, due to the co-selective pressure applied by the over usage of non-beta-lactams. The level of antibiotic consumption in animals should be, therefore, re-considered in the Mediterranean area especially in North Africa and western Asia where no accurate data are available about the level of antibiotic consumption in animals.

Background

Antimicrobial resistance is an emerging and rapidly evolving phenomenon. This phenomenon is currently observed in all bacterial species including clinically important Gram negative bacilli (GNB) (Rubin and Pitout 2014). Gram negative bacilli, "enterobacteriaceae and non-fermenters" are normal inhabitants of the human intestinal microflora (Vaishnavi 2013); they are responsible for the most common hospital and community acquired infections. Antibiotic resistance in GNB is mediated by target drug modification (Lambert 2005), changes in bacterial cell permeability (Delcour 2009) and, most importantly, the production of hydrolyzing enzymes, namely beta-lactamases. The most common beta-lactamases which are now widespread include the extended spectrum beta-lactamases (ESBL) (SHV, TEM, OXA and CTX-M types), AmpC beta-lactamases, and carbapenemases (MBL, KPC and class D oxacillinases) (Giedraitiene et al. 2011)(Poirel et al. 2011). These enzymes provide the bacterium with resistance towards the majority of therapeutic options available in the clinical market. Furthermore, resistance determinants of these enzymes are often located on plasmids carrying resistance genes to other non-beta-lactam antibiotics, thus further limiting treatment options (Guerra, Fischer, Helmuth 2014)**.**

The emergence of colistin resistance in GNB is another concern. Colistin belongs to the polymyxin group of polypeptide antibiotics (Olaitan, Morand, Rolain 2014). Previously abandoned due to its nephrotoxicity and neurotoxicity, it is now in use once again and is considered to be the last resort antimicrobial agent against carbapenem resistant GNB (Kempf et al. 2013). Colistin resistance can be mediated either by the acquisition of the plasmid mediated "mcr" gene or by chromosomal mutations that lead to modification of the lipid A moiety of lipopolysaccharide (LPS), which is considered the primary target of colistin in Gram negative bacilli (Baron et al. 2016).

It is currently known that, in addition to the human intestinal microflora, resistant GNB can be found in water, soil and fecal animal matter (Verraes et al. 2013)**.** In fact, there is increasing evidence that animals constitute a potent reservoir of resistant GNB (Ewers et al. 2012)**.** This is mainly due to the over- and misuse of antibiotics in veterinary medicine (Guerra, Fischer, Helmuth 2014): antibiotics are not only prescribed for treatment but are also administered for disease prevention and growth promotion (Economou and Gousia 2015)**.** Although studies have shown that the direct threat of resistant GNB to human health is still controversial (Olsen et al. 2014)**,** the wide dissemination of these resistant organisms is worrying due to their ease of transmission (Rolain 2013) and their high potential contribution

to the spread of bacterial resistance across all ecosystems (Pomba et al. 2017). In this review, we attempt to describe the epidemiology of ESBL, AmpC and carbapenemase producing GNB of animal origin in the Mediterranean region. Colistin resistance in GNB in the same area is also described. The Mediterranean basin is a region of the world that compromises a wide diversity of populations. It includes five Asian countries (Cyprus, Israel, Lebanon, Syria, and Turkey), eleven European countries (Albania, Bosnia, Croatia, France, Greece, Herzegovina, Italy, Monaco, Montenegro, Slovenia and Spain) and five African countries (Algeria, Egypt, Libya, Morocco and Tunisia).

Distribution of ESBLs and AmpC producers in animals

Chicken and food of poultry origin

Poultry production is a complex system in the food and agricultural industry. It includes breeding chickens for meat and eggs. Chickens are kept either as a "breeding flock" or as a "broiler flock" for human consumption. Along with eggs, broilers are traded and transported across different countries around the world (Dierikx et al. 2013). This trade results in a vulnerable system that can be hacked by multi-drug resistant organisms (MDRO), i.e., once a MDRO is introduced into the production chain, it can be transferred internationally. This is why the dissemination of ESBL and AmpC-producing GNB, recently extensively reported in chicken farms (Blaak et al. 2015) is worrying, as these can contribute to not only local but global dissemination of antimicrobial resistance (Dierikx et al. 2013). Studies have shown that the carriage of ESBL and AmpC producers in chicken is persistent (Huijbers et al. 2016). ESBL and AmpC producers are isolated from grandparent breeding stock (Nilsson et al. 2014), broiler chickens (Reich, Atanassova, Klein 2013), retail meat (Choi et al. 2015) and at the slaughterhouses (Maciuca et al. 2015).

In the Mediterranean basin, the first detection of ESBL in chicken dates back to 2000 in Greece, when a CTX-M-32 harboring *Salmonella enterica* was isolated from poultry end products (Politi et al. 2005). Since then, many studies have reported the emergence of ESBL in poultry in the Mediterranean area. In Italy for instance, the first ESBL reported was a case of SHV-12 detected in *Salmonella* spp (Chiaretto et al. 2008). *Salmonella infantis* species harboring CTX-M-1 were later isolated in 2011 from broiler chicken flocks. These strains led to human infection in Italy in 2013-2014 (Franco et al. 2015). In both studies, isolated strains were co-resistant to non beta-lactam antibiotics, notably nalidixic acid, sulfonamide, trimethoprim and tetracyclines. According to the European Food Safety Authority and the

European Centre for Disease Prevention and Control recent report, S. infantis is the fourth most common serovar detected in humans in the European Union and that is mostly being observed in the turkey and broiler chain. In this report, it has been stated that this serovar has been able to extensively disseminate along the broiler production chain (EFSA 2017). Indeed it has been suggested that the consumption of contaminated chicken meat is among the most common sources of salmonellosis in humans (Antunes et al. 2016). Furthermore, in Italy, opportunistic pathogen such as Escherichia coli isolates producing CTX-M-32, CTX-M-1 and SHV-12 type beta-lactamases were also reported (Giufre et al. 2012). These strains were retrieved from flocks which had no prior treatment with cephalosporins. It is proposed that the prescription of other antimicrobials such as enrofloxacin and tylosin is responsible for the co-selection of the aforementioned resistant organisms (Bortolaia et al. 2010). Reports on chicken feces (Giufre et al. 2012), broiler chicken samples and retail chicken meat (Ghodousi et al. 2016) showed that these latter carried E. coli producing CTX-M-grp-1, CTX-M-grp-2 and CTX-M-grp-9 enzymes in Italy. The co-existence of these enzymes with AmpC beta-lactamases was also reported, including CTX-M-1/CMY-2 (Accogli et al. 2013) and CIT-like/CTX-M (Ghodousi et al. 2015) in E. coli of poultry origin. CTX-M and AmpC beta-lactamase producers in the Italian poultry belong mostly to the A and B phylogroups with the genes being carried mainly on IncI1 plasmids. In France, the only report from poultry was the detection of two CTX-M-1-producing E. coli isolates (Meunier et al. 2006). CTX-M-1 was linked to the insertion sequence ISEcp1 (Meunier et al. 2006). This insertion sequence has been previously described as being a potent contributor to the mobilization and insertion of blaCTX-M genes (El Salabi, Walsh, Chouchani 2013). Although no studies described the emergence of ESBL in the Slovenian animal sector, one study reported the presence of CTX-M-1 and SHV-12-producing in Slovenian raw chicken meat samples sold on the Swiss market (Zogg et al. 2016).

In Spain, the Spanish Veterinary Antimicrobial Resistance Surveillance Network (VAV) monitored antimicrobial resistance of Salmonella enterica in healthy broilers in 2003-2004: two CTX-M-9 producers were isolated (Riano et al. 2006). During the same period, ESBL-producing E. coli were also detected (Mesa et al. 2006)(Moreno et al. 2007). Indeed, it seems that early monitoring systems often targeted resistance in Salmonella species, as these are common causative agents of human infections of food of animal origin (Antunes et al. 2016). Thereafter, as bacterial resistance became widely disseminated in all environments (Stoll et al. 2012), researchers began to think of poultry as a reservoir of resistance in enteric organisms. For instance, Egea et al. found that the prevalence of retail poultry meat colonized

by CTX-M and/or SHV producing E. coli increased from 62.5% in 2007 to 93.3% in 2010 (Egea et al. 2012). During these three years, a significant increase was observed at the level of A0 and D1 phylogroups. Egea et al suggested that the rise of meat colonization is muli-clonal since only 2 strains from the main phylogroup detected in this study showed genetic relatedness by PFGE typing. Thus, it appears that the diffusion of ESBL producers in retail chicken meat is related rather to successful spread of one or several plasmids carrying the blaCTX-M and blaSHV genes (Egea et al. 2012). Apart from E. coli, ESBL production in the poultry production system in Spain was also detected in Klebsiella pneumoniae, Enterobacter cloacae, Proteus mirabilis and Serratia fonticola (Ojer-Usoz et al. 2013). In parallel, CMY-2 is the only AmpC beta-lactamase type reported in E. coli originating from chicken in this country (Blanc et al. 2006) (Sola-Gines et al. 2015b) (Cortes et al. 2010). Apart from chicken, one study in Spain reported the detection of CTX-M-1, CTX-M-9, CTX-M-14 harboring E. coli strains in flies surrounding chicken farms (Sola-Gines et al. 2015a). For instance, the detection of ESBL producers in flies reflects on one side the contamination status of the farm housing environment; and on the other side, it contributes to the colonization of other broilers with ESBL producing E. coli strains (Sola-Gines et al. 2015a).

In Turkey, the first ESBL production in animals was detected in K. pneumoniae and Klebsiella oxytoca in 2007-2008 (Gundogan, Citak, Yalcin 2011). In 2012-2014, E. coli producing CTX-M-1, CTX-M-3, CTX-M-15, CTX-M-8 as well as SHV-5 and SHV-12 were identified in raw chicken meat samples in different areas across the country (Pehlivanlar Onen et al. 2015)-(Tekiner and Ozpinar 2016). The A, D1 and D2 were the most common phylogroups detected. In the same aforementioned study, ESBL was also detected in E. cloacae, Citrobacter werkmanii and K. pneumoniae (CTX-M-1) (Tekiner and Ozpinar 2016). Similarly, CMY-2 type beta-lactamase was detected in E. coli (Pehlivanlar Onen et al. 2015) as well as in E. cloacae (Tekiner and Ozpinar 2016). In Lebanon, CTX-M type beta-lactamase followed by CMY AmpC beta-lactamase appear to dominate the Lebanese chicken farms (Dandachi et.al 2018). MLST typing of CTX-M positive E. coli strains revealed the presence of different sequence types across the territory. Furthermore, a significant resistance of ESBL producers toward gentamicin was observed. The spread of ESBL producers in Lebanon could be attributed in part to the co-selective pressure applied by the heavy usage of gentamicin in the veterinary sector as previously reported (Dandachi et.al 2018). In Israel, only one study showed the presence of CTX-M-producing E. coli of A, B and D phylogroups in liver samples of dead broiler chickens and ready-to-market chicken meat (Qabajah, Awwad, Ashhab 2014).

Concerning Africa, ESBL was first detected in E. coli strains isolated from foods of poultry origin in Tunisia in 2006. These harbored SHV-5, CTX-M-8, CTX-M-14 and CTX-M-1 type beta-lactamases (Jouini et al. 2007). It appears that in this country, blaCTX-M-1 and blaCMY-2 are the dominant genes responsible for ESBL and AmpC production in E. coli isolated from chicken samples (Ben Sallem et al. 2012) (Ben Slama et al. 2010). This is in addition to blaCTX-M-15, blaCTX-M-14 (Maamar et al. 2016) and blaCTX-M-9 that were detected in E. coli isolated from the fecal samples of dead/diseased chickens (Grami et al. 2014). ESBL genes in Tunisia appear to be located on various plasmids carried by different E. coli phylogroups. These include mainly IncI1 followed by IncF and IncFIB (table 2). blaCTX-M as well as CMYgenes in Tunisia were found to be also associated to the ISEcp1 insertion sequence. Furthermore, apart from the CMY gene, AmpC production in E. coli strains in this country was found to be also mediated via mutations in the promoter region of the chromosomal AmpC gene (Ben Slama et al. 2010). In Algeria, CTX-M-like enzymes were detected in E. coli (Mezhoud et al. 2015) (Chabou et al. 2017) as well as in other species such as ST15 Salmonella Heidelberg (Djeffal et al. 2017). In their study, Djeffal et al reported the detection of the same sequence type "ST15" of Salmonella spp isolated from both chicken and human. This emphasizes on the hypothesis that the poultry production system could constitute a potent contributor to the diffusion of multi-drug resistant Salmonella in the human population (Djeffal et al. 2017). In parallel, blaSHV-12 and CMY-2 genes were detected in E. coli strains recovered from slaughtered broilers' intestinal swabs (Belmahdi et al. 2016).

In Egypt, E. coli producing CTX-M-15 and CMY-2 were initially reported from blood samples from the hearts of septicemic broilers in 2011 (Ahmed, Shimamoto, Shimamoto 2013). CTX-M-15 and CTX-M-14 were further detected in E. coli, K. pneumoniae, K. oxytoca and Enterobacter spp isolated from chicken carcasses in the north of Egypt (Abdallah et al. 2015)(Ahmed and Shimamoto 2015). E. coli isolates harboring SHV-12 have also been reported in Egypt; although they originated from liver and heart samples of chickens affected with colibacillosis (El-Shazly et al. 2017) (figure 1). Similarly to other countries in the Mediterranean basin, ESBL producers in the Egyptian poultry sector belong mainly to the A and B1 phylogroups with the blaCTX-M genes being associated with ISEcp1 (table 2).

Cattle and sheep

Cattle and sheep are essential members of the human food and agricultural system. For humans, cattle and sheep serve as a source of meat and milk. In agriculture, their feces are commonly used as manure for artificial fertilization (Nyberg et al. 2014). As it is now widely recognized that animals' intestines are a normal habitat for wild type and resistant micro-organisms (Nelson, Rogers, Brown 2013), it has been suggested that if resistant bacteria contaminated animal manures are used without prior treatment, there is a potential risk of transmitting this resistance to the surrounding environment and to the human population (Hruby et al. 2016). This transmission may occur through irrigation and drinking water without treatment (Hruby et al. 2016) or through animals grazing on contaminated lands (Bagge, Lewerin, Johansson 2009).

In France, the first identification of an ESBL producer in cattle dates back to 2004 when E. coli strains harboring CTX-M-1 and CTX-M-15 were isolated from cows (Meunier et al. 2006). E. coli producing the CTX-M-15 type ESBL were later isolated from the fecal sample of a dead calf (Valat et al. 2012) and from the feces of cattle located in 10 different geographical areas in France (Madec et al. 2012). In the aforementioned study, CTX-M-15 was carried on IncI1 plasmids but also on F31:A4:B1/IncFII and F2:A–:B–/IncFII plasmids which has been extensively reported in humans (Madec et al. 2012). Although CTX-M-15 appears to be dominant in French cattle, other ESBL types were also reported in E. coli (Hartmann et al. 2012) and Klebsiella species (Dahmen et al. 2013b)(Haenni et al. 2014) such as CTX-M-1, CTX-M-14, CTX-M-9, CTX-M-2, CTX-M-32, CTX-M-57, CTX-M-3 (Dahmen et al. 2013b)(Haenni et al. 2014) and TEM-71(Hartmann et al. 2012). These latter were carried by E. coli strains of different sequence types such as ST23, ST58, ST10, ST45, ST88, ST2210, ST2212-ST2215, ST2497 and ST2498 (table 1); no epidemic clones such as ST101 were detected. Moreover, two studies in France detected AmpC-producing E. coli in calves. In both, AmpC beta-lactamase production was suggested as being due to highly conserved mutations in the promotor/attenuator region and to an over-expression of the chromosomal AmpC gene, respectively (Haenni et al. 2014)(Haenni, Chatre, Madec 2014). In sheep, only one study was conducted in France in which one CTX-M-1 E. fergusonii and three K. pneumonia harboring both blaCTX-M-15 and DHA genes were detected (Poirel et al. 2013). The three K. pneumoniae were co-resistant to nalidixic acid, sulfonamides, trimethoprim-sulfamethoxazole and tetracycline and belonged to the same sequence type ST274. In Spain, ESBL-producing Gram-negative bacilli were isolated from beef samples collected from different geographical locations (Doi et al. 2010)(Ojer-Usoz et al. 2013). In

Italy, Stefani et al. reported the isolation of five Klebsiella ozaenae harboring CTX-M-1, CTX-M-1/TEM-24 and CTX-M-15 ESBL types from cattle (Stefani et al. 2014).

In Turkey, a study conducted in 2007-2008, showed the presence of ESBL-producing K. pneumoniae and K. oxytoca in raw calf meat (Gundogan, Citak, Yalcin 2011). Later on, CTX-M-3 and CTX-M-15 harboring E. coli were isolated from beef samples sold in a market in the south of Turkey (Conen et al. 2015). Recently, a study conducted by Tekiner et al. reported the isolation of ESBL-producing E. coli, E. cloacae and Citrobacter brakii from raw cows' milk collected from different cities of Turkey. In these areas, CTX-M-1 was dominant (Tekiner and Ozpinar 2016). In Lebanon the situation differs, in that unlike Turkey but similarly to other Mediterranean countries, blaCTX-M-15, blaSHV-12 and blaCTX-M-14 are the dominant ESBL genes prevailing in E. coli in the Lebanese cattle (Diab et al. 2016). In this latter study, various sequence types were detected. Of special interest is the detection of ST10. ST10 was heavily reported in the literature as being shared between animal and human isolates all over the world: Chile (Hernandez et al. 2013), Denmark (Huijbers et al. 2014), Vietnam (Nguyen et al. 2015), Germany (Belmar Campos et al. 2014). Indeed, it has been suggested that ST10 became associated with the production and dissemination not only of CTX-M-type ESBLs but also of mcr-1 in animals, humans and environment (Monte et al. 2017). In Israel, Adler et al. reported the identification of CTX-M-1/CTX-M-9 and SHV-12 beta-lactamase producing E. coli and K. pneumoniae strains respectively, which were isolated from cattle farms situated in the main farming locations across the country (Adler et al. 2015).

In Egypt, SHV-12 (Ahmed et al. 2009) in addition to CTX-M-1/15 and CTX-M-9 were detected in E. coli strains isolated from cattle (Braun et al. 2016). On study targeting raw milk samples reported the detection of SHV-12 /CTX-M-3, in addition to CMY-2-producing E. coli strains (Ahmed and Shimamoto 2015). In Tunisia, E. coli strains producing CTX-M-1and TEM-20 were isolated from beef and sheep situated in different areas across the country (Jouini et al. 2007)(Ben Slama et al. 2010). Furthermore, blaCTX-M-15 was detected in an ST10 E. coli isolate recovered from the milk sample of cattle affected with mastitis (Grami et al. 2014). Similarly, In Algeria, Yaici et al reported the detection of four ST1284 E. coli strains carrying CTX-M-15, CMY-42 and NDM-5 in raw milk samples (Yaici et al. 2016).

Swine

Meat from pigs is used by humans for consumption and their feces are used as manure for land fertilization. Studies have shown that antibiotics are usually detected in higher

concentrations in pig manures compared to that of other farm animals (Hou et al. 2015). This finding reflects high and uncontrolled antimicrobial usage in swine farms (Woolhouse et al. 2015). Heavy antibiotic usage creates a selective pressure that contributes to the emergence and spread of bacterial resistance; in this regard, pigs are suggested as a potential source of resistant bacteria.

Reports concerning the prevalence of ESBL of swine origin in the Mediterranean area are very scarce with the majority being reported from Spain where a blaSHV-12 positive Salmonella enterica was isolated in the early 2000s (Riano et al. 2006). Furthermore, CTX-M-grp-9 (Doi et al. 2010) (Ojer-Usoz et al. 2013), SHV-5 and CTX-M-grp-1 carried by A phylogroup E. coli strains and SHV-12 carried by B1 E. coli and blaSHV-5 were detected (Cortes et al. 2010) (Blanc et al. 2006). One study conducted in 13 different Spanish provinces found seven AmpC-producing E.coli. In these cases, AmpC production was due to a mutation in the promoter region of the chromosomal AmpC gene (Escudero et al. 2010). In Italy, TEM-52, CTX-M-1, CTX-M-15 and CTX-M-1/TEM-201 carrying E. coli were reported in pigs (Stefani et al. 2014). Franco et al. reported also the presence of Salmonella infantis carrying CTX-M-1 in swine (Franco et al. 2015). In France, only one study conducted at the beginning of the 21th century reported the detection of CTX-M-1-producing E. coli strains in pigs (Meunier et al. 2006). Similarly to what is widely observed in the Mediterranean basin, the CTX-M-1 was associated with the insertion sequence ISEcp1(Meunier et al. 2006). In Algeria, CTX-M-15 harboring E. coli and K. pneumoniae strains were isolated in 2014 from wild boars (Bachiri et al. 2017). MLST typing showed the K. pneumoniae belongs to the ST584 while on the other hand several sequence types (ST617, ST131, ST648, ST405, ST1431, ST1421, ST69, ST226) were observed among E. coli strains (Bachiri et al. 2017). The aforementioned study was the only one to investigate the epidemiology of ESBL-producing Gram-negative bacilli in the African and Asian countries lining the Mediterranean Sea.

Companion animals

Unlike food producing animals, companion animals are not used as consumption source of human food, nor are their feces used as manure for land fertilization. Instead, these animals are kept for the individual's protection, entertainment and company. The number of companion animals has significantly increased in modern society in recent decades (Pomba et al. 2017). Despite regular close contact with people, little attention has been given to the prevalence of antimicrobial resistance in these animals (Scott Weese 2008). The close contact

between companion animals such as dogs, cats and horses and their owners makes the transmission of resistant organisms more likely to occur (Dierikx et al. 2012). As such, it is essential to investigate the prevalence of resistant bacteria in companion animals as well as to identify the possible risk factors for the transmission of resistant organisms to humans (Rubin and Pitout 2014).

In the Mediterranean basin, the first detection of ESBL in companion animals was in Spain where an E. coli harboring SHV-12 was isolated from a dog with a urinary tract infection (Teshager et al. 2000). Subsequently, between 2008 and 2010, three strains carrying CMY-2 (one ST2171 E. coli and two P. mirabilis) were recovered from dogs infected with respiratory, urinary tract and skin and soft tissue infections, respectively (Bogaerts et al. 2015). In all three strains, the CMY-2 genes were associated with the ISEcp1. More recently, one K. pneumoniae and one E. cloacae producing CTX-M-15/DHA and SHV-12, respectively, were isolated from the fecal swabs of healthy dogs in this same country (Gonzalez-Torralba et al. 2016).

In Italy, a study conducted by Donati et al. on 1555 dog samples of clinical cases and necropsy specimens with suspicious bacterial infections, between the center and the north of Italy found two K. oxytoca harboring SHV-12/DHA-1 and 11 K. pneumoniae carrying the following genes: blaCTX-M-15 (six strains), blaCTX-M-15/DHA-1, blaCTX-M-15/SHV-28, blaCTX-M-1/SHV-28 and blaCTX-M-1 (Donati et al. 2014). In this same study, 429 cats' samples were also investigated revealing the presence two K. oxytoca producing CTX-M-9 and four K. pneumoniae producing CTX-M-15 (two isolates), CTX-M-15/ DHA-1 and SHV-28/CMY-2 beta-lactamases (Donati et al. 2014). The beta-lactamase and AmpC genes in K. oxytoca strains isolated from dogs and cats were located on different plasmid types: IncL/M versus IncHI2 respectively. This is unlike the K. pneumoniae strains where the blaCTX-M-15 was localized on the same plasmid IncR and both strains in dogs and cats shared the same ST340. ST15 and ST101 were also common between dogs and cats in this study. ST15 and ST101 are among the most international clones carrying ESBL as well as carbapenemase genes which became highly detected recently worldwide (Donati et al. 2014). Another study conducted reported the detection of CTX-M-1-producing K. pneumoniae was further reported from a dog with urinary tract infection and an E. coli carrying the CMY-2 type beta-lactamase associated to ISEcp1 also in a diseased cat with a urinary tract infection (Bogaerts et al. 2015). Infections in pets with E. coli strains carrying CTX-M-14 (three isolates), CTX-M-15, CTX-M-1 and CTX-M-14/CMY-2 (two isolates) were also reported in Italy (Nebbia et al. 2014). The strains also showed different sequence types and phylogroups (A "ST3848,

ST3847", B2 "ST131, ST155, ST555, ST4181", B1 "ST602") emphasizing that apparently the dissemination of ESBL and AmpC beta-lactamase producers is most likely due to the successful spread of various plasmids carrying these resistance genes (Nebbia et al. 2014). In France, the highest number of studies addressing the prevalence of extended-spectrum-cephalosporin resistance in companion animals in the Mediterranean was conducted. In dogs, CTX-M-grp 1 (CTX-M-1, CTX-M-15, CTX-M-3, CTX-M-32) and CTX-M-grp 9 in addition to CMY-2 and TEM-52 prevail in E. coli (Poirel et al. 2013) (Dahmen et al. 2013a) (Haenni et al. 2014) (Bogaerts et al. 2015) (Melo et al. 2017). These genes were mostly carried on IncI1, IncFII and IncHI2 plasmid types and were harbored by strains of different sequence types and phylogroups. Furthermore, K. pneumoniae isolated from dogs showed to produce the CTX-M-15, CTX-M-32, SHV-12 and DHA-1 have been reported (Poirel et al. 2013) (Haenni et al. 2014). In parallel, P. mirabilis showed to produce CMY-2, DHA-16, VEB-6 and CTX-M-15 have been described (Schultz et al. 2017) and E. cloacae the CTX-M-15, CTX-M-14, CTX-M-3 and SHV-12 have been identified (Haenni et al. 2016). In addition, CTX-M-15 and CMY-2 were also decribed in K. oxytoca and Salmonella enterica, respectively isolated from dogs in this same country (Poirel et al. 2013)(Haenni et al. 2014). On the other hand, in cats, the following distribution was observed: in E. coli (CTX-M-1, CTX-M-15, CTX-M-32, CTX-M-3, CTX-M-14) (Poirel et al. 2013)(Haenni et al. 2014) (Melo et al. 2017), in K. pneumoniae (CTX-M-15/DHA) (Poirel et al. 2013), in E. cloacae (CTX-M-15, SHV-12) (Haenni et al. 2016), in P. mirabilis (CMY-2) and in Proteus rettgeri (CTX-M-1) (Schultz et al. 2017). The dissemination of extended-spectrum-cephalosporin resistance in companion animals in France necessitates studies addressing the risk factors responsible for the acquisition of these strains in pets as well as novel approaches to control the spread of resistance in these animals. Furthermore, the contribution of the pet animals to the spread of resistance in the common population in France should be also investigated. Moreover, France is the only Mediterranean country in which studies reporting ESBL and/or AmpC-producing bacteria in horses are available. Between 2010 and 2013, E. cloacae harboring CTX-M-15, CTX-M-1 and SHV-12 were isolated from clinical samples of horses. These genes were located on IncHI2 and IncP plasmids and were harbored by strains of various sequence types such as ST127, ST372, ST145, ST114, ST135, ST118, ST268, ST107 (Haenni et al. 2016). Later on, VEB-6 carrying P. mirabilis were isolated from healthy horses (Schultz et al. 2017). In Greece, CMY-2 carried on IncI1 plasmid and harbored by ST212 E. coli strains were isolated from diseased canines in 2011 (Vingopoulou et al. 2014). More recently, a study conducted in Greek households revealed the detection of extended-

spectrum-cephalosporin-resistant E. coli isolates. The strains presented with different sequence types including the human pandemic ST131 clone which suggests a possible from humans to animals and vice-versa (Liakopoulos et al. 2018).

In Egypt, CTX-M beta-lactamases have been detected in E. coli recovered from cats' rectal swabs. In this same study, CTX-M-producing E.coli, K. pneumoniae and P. mirabilis were isolated from dogs (Abdel-Moein and Samir 2014). In Algeria, only one study reported the detection of E. coli strains carrying blaCTX-M-1, blaCTX-M-15 in cats and blaCTX-M-1, blaCTX-M-15, blaSHV-12 in dogs (Yousfi et al. 2016b). In Tunisia, CTX-M-1 carrying E. coli were isolated from cats; while from dogs CTX-M-1, CTX-M-15 and CMY-2-producing E. coli were detected (Sallem et al. 2013) (Grami et al. 2013). CTX-M-1 was mostly carried on IncI1 plasmid where as CTX-M-15 on IncFII (Grami et al. 2013). The blaCTX-M-1 and CMY-2 genes were also found associated with the ISEcp1. Indeed it appears that the insertion sequence ISEcp1 might be also responsible for the dissemination of CMY-2 AmpC genes apart from the blaCTX-M ones.

Wild Birds and domestic animals

Besides companion and food producing animals, scattered reports exist on the isolation of ESBL from domestic animals such as wild birds and dromedaries in the Mediterranean. For instance, CTX-M-producing E. coli was isolated from wild birds in Algeria (Meguenni et al. 2015), Turkey (Yilmaz and Guvensen 2016), blaCTX-M-1 in addition to blaCTX-M-15 carrying E. cloacae in France (Bonnedahl et al. 2009). Furthermore, in France, CTX-M-1 and CTX-M-15 were detected in ST93, ST124 and ST10 E. coli strains recovered from tawny owls/rock pigeons and domestic geese, respectively. In addition, a CTX-M-15/DHA-producing ST274 K. pneumoniae was isolated from a hedgehog living in the same city (Poirel et al. 2013). Rooks carrying CTX-M-14 type ESBL in E. coli have been described in Italy and Spain (Jamborova et al. 2015). Furthermore, in Spain, E. coli and K. pneumoniae harboring CTX-M-14, CTX-M-1, CTX-M-32, CTX-M-9, CTX-M-15, CTX-M-14b, CTX-M-3, and CTX-M-8 were recovered from the fecal samples of gulls (Stedt et al. 2015). In rabbits, CMY-2-producing E. coli and CTX-M-14, CTX-M-9-producing E. cloacae were isolated (Blanc et al. 2006)(Mesa et al. 2006). More recently, blaCTX-M-1 was identified in E. coli isolated from the fecal sample of a deer living in the Los Alcornocales natural park in southern Spain (Alonso et al. 2016). In Algeria, blaCTX-M-15 and blaCTX-M-9 genes were detected in E. coli isolated from the gut and gills of fish caught in the Mediterranean across Bejaia city (Brahmi et al. 2016). In this study, it has been suggested that the presence of beta-

lactamase producers is due to contamination of the fish from river water and the rising amount of untreated waste that is released into the Mediterranean Sea from the agricultural as well as the industrial operations (Brahmi et al. 2016). These findings emphasizes on the importance of the natural environment in the dissemination of resistance from humans to animals and vice versa. Furthermore, Bachiri et al. also reported the detection of CTX-M-15-producing ST584 K. pneumoniae in Barbary macaques situated in national parks in the north of Algeria (Bachiri et al. 2017). In both Tunisia and Egypt, CTX-M beta-lactamases were detected in E. coli and Pseudomonas aeruginosa recovered from dromedaries and camels, respectively (Ben Sallem et al. 2012) (Elhariri et al. 2017). In Croatia, the only study investigating the prevalence of ESBL in animals was conducted in 2009-2010 in mussels caught in the Adriatic Sea. In this study, 18 Aeromonas species carrying SHV-12, CTX-M-15, FOX-2 and PER-1 were identified (Maravic et al. 2013).

Prevalence of carbapenemase producers in livestock and domestic animals

Carbapenems are beta-lactam antibiotics often considered as the last resort antimicrobial agent against multi-drug resistant organisms (Temkin et al. 2014). Carbapenems are active against ESBL and AmpC-producing Gram negative bacilli. Due to the wide dissemination of multi-drug resistant organisms, these antimicrobials recently became heavily used in human medicine. As a result, the emergence of carbapenem resistance has accelerated and it is now a normal phenomenon encountered in hospital settings and, to a lesser extent, community settings. The production of hydrolyzing enzymes called “carbapenemases” is one of the mechanisms by which carbapenem resistance is mediated in Gram negative bacilli. These include a) class A carbapenemases (KPC, GES, SME, IMI, NMC-A) b) class B metallo beta-lactamases “MBL” (NDM, VIM, IMP and TMB) and c) class D oxacillinases (Martinez-Martinez and Gonzalez-Lopez 2014).

In the Mediterranean basin, in Egypt, OXA-48 and OXA-181 carbapenemases were detected in E. coli strains recovered from dairy cattle farms (Braun et al. 2016). In the poultry production system, one study reported the isolation of K. pneumonia and K. oxytoca harboring NDM metallo beta-lactamases (Abdallah et al. 2015). Another study described the identification of K. pneumoniae carrying OXA-48, NDM and KPC type carbapenemases. Isolated strains were recovered from the liver, lungs and trachea of broiler chicken (Hamza, Dorgham, Hamza 2016). In Algeria, NDM-1 and NDM-5 were observed, respectively, in ST85 Acinetobacter baumannii and ST1284 E. coli originating from raw milk in the west and north of the country (Chaalal et al. 2016) (Yaici et al. 2016). In E. coli, NDM-5 was located

on an IncX3 plasmid (Yaici et al. 2016). In broilers, OXA-58 was identified (Chabou et al. 2017) while in pigeons, in addition to OXA-58 and OXA-23 were detected (Morakchi et al. 2017). In terms of companion animals, NDM-5 and OXA-48-producing E. coli were reported from healthy dogs Algeria (Yousfi et al. 2015) (Yousfi et al. 2016a). The NDM-5 was harbored by an E. coli strain having the same sequence type ST1284 previously described in cattle (Yaici et al. 2016) (Yousfi et al. 2015). OXA-48 was further detected in healthy and diseased cats in the same city (Yousfi et al. 2016a). Furthermore, in this same country, two A. baumannii producing OXA-23 were isolated from fish (Brahmi et al. 2016). In Lebanon, A. baumannii with different sequence types (ST294, ST491, ST492, ST493) were detected in a horse's mouth carrying OXA-143 (Rafei et al. 2015), and in pigs and cattle carrying OXA-23(Al Bayssari et al. 2015a). Furthermore, in cattle, a VIM-2-producing P. aeruginosa was isolated (Al Bayssari et al. 2015a). In fowl, Bayssari et al. reported the detection of OXA-23 and OXA-58 harboring A. baumannii and OXA-48-producing E. coli as well as VIM-2 producing P. aeruginosa (Al Bayssari et al. 2015b). VIM-2 producers in fowl and cattle were of different sequence types suggesting the presence of plasmid that is mediating the spread of this resistance gene. In France, OXA-23-producing Acinetobacter species were described in cows and dogs (Poirel et al. 2012) (Herivaux et al. 2016). Melo et al reported the detection of OXA-48 located on an IncL plasmid and carried by an ST372 E. coli strain from dogs in France (Melo et al. 2017). In contrast, in Spain, only one study reported the isolation of a VIM-1-producing ST2090 K. pneumoniae from a dog's rectal swab (Gonzalez-Torralba et al. 2016) (Figure 2).

Clonal relationship of beta-lactamase producers and plasmid types of beta-lactamase genes isolated from all animal sources.

The different phylogroups and sequence types of beta-lactamase and mcr-1 positive strains as well as the type of plasmids carrying ESBL, AmpC, carbapenemase and mcr-1 genes detected in all animal sources in the Mediterranean region are summarized in table 2. In this area of the world, it appears that multi-drug resistance in the veterinary sector is mediated by the spread of different phylogroups and sequence types with the main ones being A, B and D phylogroups (table 2). The detection of ST10 in CTX-M producers in poultry, cattle, pets and domestic animals in Algeria, Tunisia, Lebanon and France is of special interest. ST10 was often described in the literature as being common to ESBL E. coli strains of human and avian origin worldwide such as in Germany (Belmar Campos et al. 2014), Denmark (Huijbers et al.

2014), Vietnam (Nguyen et al. 2015) and Chile (Hernandez et al. 2013). ST10 was suggested as being associated with the spread of CTX-M ESBL types and mcr-1 genes in humans, animals and environments (Monte et al. 2017). Another distinct finding is the detection of ST101 in dogs and cats in Italy. ST101 is an international sequence types frequently detected in pigs (El Garch et al. 2017), broilers (Sola-Gines et al. 2015) as well as in the clinical settings. In several countries, ST101 was associated to NDM-1 E. coli strains isolated from the clinical settings of Germany, Canada, Australia, UK and Pakistan (Yoo et al. 2013) implying thus that ST101 is a candidate for the zoonotic transmission to the human population.

More deeply speaking, ESBL and AmpC encoding genes were mostly carried on conjugative IncI1, IncFIB, IncN and IncK plasmids (table 1). ISEcp1 was the most common insertion sequence associated with the CTX-M ESBL types with the main ones being blaCTX-M-1 and blaCTX-M-15 genes. ISEcp1 has been previously described as a potent contributor to the mobilization and insertion of blaCTX-M genes worldwide (El Salabi, Walsh, Chouchani 2013). As for the carbapenemase encoding genes, these latter were found to be carried by IncX3 and IncL plasmids detected in E. coli strains isolated from cattle, swine and dogs in Algeria, Italy and France, respectively. Overall, the detection of a variety of sequence types and phylogroups in ESBL and AmpC producers isolated from animals of all origins within and among countries's animals suggests that the dissemination of multi-drug resistance in the Mediterranean is multi-clonal and related rather to the diffusion of conjugative plasmids carrying beta-lactamase genes.

Prevalence of colistin resistance in livestock and domestic animals

Polymyxin E (colistin) and polymyxin B are polycationic antimicrobial peptides that are considered as the last-line antibiotic treatment for multi-drug resistant (MDR) Gram-negative bacterial infections (Olaitan and Li 2016). From the 1960s until the 1990s, colistin was considered as an effective treatment for MDR-GNB (Olaitan et al. 2014). However, due its nephrotoxicity within the human body, the clinical use of this antimicrobial was abandoned (Olaitan and Li 2016). Recently, the emergence of carbapenem resistance in clinically important bacteria such as P. aeruginosa, A. baumannii, K. pneumoniae and Escherichia coli, necessitated the re-introduction of colistin into clinical practice as a last-resort treatment option (Olaitan and Li 2016).

Colistin is not only administered in humans, its use has been also described in veterinary medicine. Indeed, it has been suggested that the uncontrolled use of colistin in animals has played an important role in the global emergence of colistin-resistant bacteria (Collignon et al. 2016). The World Health Organization recently added polymyxins to the list of critically important antibiotics used in food producing animals worldwide (Collignon et al. 2016). The main use for colistin in animals includes the treatment of gastrointestinal infections caused by E. coli in rabbits, pigs, broilers, veal, beef, cattle, sheep and goats; and, in particular, gastrointestinal infections caused by E. coli (Poirel, Jayol, Nordmann 2017). Colistin is mainly administered orally using different formulations such as premix, powder and oral solutions (Catry et al. 2015). In European countries, several epidemiological studies reported the use of colistin in veterinary medicine. In fact, Kempf et al. reported that colistin is mainly used to inhibit infections caused by E. coli, a Gram-negative bacillus known as a common causative agent of diarrhea, septicemia and colibacillosis in animals (Kempf et al. 2013). In Spain, Casal et al. revealed that colistin is among the most frequent administered drug for the treatment of digestive diseases in pigs (Casal et al. 2007).

Epidemiologically speaking, the worldwide prevalence of resistance to polymyxins accounts for 10% of Gram-negative bacteria with the highest rates being observed in Mediterranean countries and Southeast Asia (Al-Tawfiq, Laxminarayan, Mendelson 2017). For many years, colistin resistance was thought to be mainly mediated by chromosomic mutations, with no possibility of horizontal gene transfer. However, the emergence of the mcr-1 plasmid mediated colistin resistance gene (Liu et al. 2016) has thoroughly altered the view of colistin resistance as a worldwide problem (Baron et al. 2016). The current epidemiology of colistin resistance is poorly understood.

In the Mediterranean area (figure 2), the first detection of mcr-1 was in an E. coli strain isolated from chickens in Algeria (Olaitan et al. 2016). This same isolate was further detected in sheep in another region of this country in 2016 (Chabou et al. 2017). In Tunisia, Grami et al. reported a high prevalence of multi-clonal E. coli carrying the mcr-1 gene in three chicken farms imported from France (Grami et al. 2016). Isolated strains were found to co-harbor the blaCTX-M-1 ESBL gene along with mcr-1 on an IncHI2/ST4 plasmid (table 1) (Grami et al. 2016). Apart from colistin resistance, these strains were also co-resistant to tetracyclines, quinolones, fluoroquinolones, trimethoprim and sulfonamides (Grami et al. 2016). The co-existence of ESBL and mcr-1 genes on the same plasmid facilitates the dissemination of colistin resistant strains by the co-selective pressure applied via the use of colistin as well as possibly the utilization of non beta-lactam antibiotics. Molecular analysis targeting the co-

localization of ESBL and mcr genes along with the ones mediating resistance toward non beta-lactams is however warranted in order to validate this hypothesis. Also in Tunisia, two colistin resistant E. coli strains positive for mcr-1 and harboring the CMY-2 gene were recently detected in chicken. Both strains shared the same sequence type "ST2197" in addition to their PFGE patterns. The mcr-1 gene in these latter was associated with the ISApl1 and was carried by IncP plasmid while the CMY-2 gene was located on an IncI1 plasmid type (Maamar et al. 2018). Furthermore, in this same country, a recent study revealed the absence of mcr-1 and mcr-2 positive Gram-negative bacilli in camel calves in southern Tunisia (Rhouma et al. 2018). Likewise, in Egypt, mcr-1 was detected in E. coli isolated from diseased chickens as well as from cows displaying subclinical mastitis (Khalifa et al. 2016) (Lima Barbieri et al. 2017). The emergence of mcr-1 in Egypt can be related to the use of colistin in animal agriculture, and its ready application as a therapeutic agent for colibacillosis as well as other infections, in rabbits and calves (Lima Barbieri et al. 2017). In Southeast Asia, Dandachi et al. reported the detection of the mcr-1 plasmid mediated colistin resistance gene in E. coli in poultry in the south of Lebanon (Dandachi et al. 2018). This strain had a sequence type of ST515 that was not reported before in mcr-1 E. coli strains of poultry origin (Dandachi et al. 2018).

Of the European countries bordering the Mediterranean, Spain was the first to report the detection of mcr-1 in E. coli and Salmonella enterica isolated from farm animals (Quesada et al. 2016). This could be related to the fact that Spain is one of the countries were colistin is extensively used in veterinary medicine (de Jong et al. 2013). More recently, mcr-1 co-existing with mcr-3 on the same non mobilizable IncHI2 plasmid was detected in an E. coli strain recovered from cattle feces in a slaughterhouse (Hernandez et al. 2017). In France, as part of routine surveillance by the French agricultural food sector, mcr-1 was identified in four Salmonella spp isolated from sausage, food of poultry origin and boot swabs taken from broiler farms (Perrin-Guyomard et al. 2016) (Webb et al. 2016). E. coli harboring mcr-1 was also isolated in France from pig, broiler and turkey samples (Haenni et al. 2016). Haenni et al. reported the identification of unique IncHI2/ST4 plasmid co-localizing mcr-1 and ESBL genes in an E. coli strain isolated from French veal calves (Haenni et al. 2016). In Italy, Carnevali et al. reported the detection of mcr-1 in Salmonella spp strains isolated from poultry and pigs (Carnevali et al. 2016). Subsequently, mcr-1 was further detected in E. coli of swine origin. In the aforementioned report, mcr-1 was co-existent with the carbapenemase OXA-181 in the same bacterium and was carried on an IncX4 plasmid type (Pulss et al. 2017). In the Mediterranean basin, likewise ESBL producers, mcr positive strains belong to

606 different phylogroups and appear to be not clonally related; however, they were not
607 associated to a common plasmid or an insertion sequence type. This questions the molecular
608 mechanism by which the mcr genes are being disseminating in this region of the world. More
609 molecular work is warranted in this area especially that mcr genes are often located on
610 plasmids carrying ESBL and/or carbapenemase genes.
611
612 **Antibiotic use in animals and potential impact on public health**
613 For many years, the use of antibiotics in the veterinary medicine has increased animal health
614 via lowering mortality and the incidence of infectious diseases (Hao et al. 2014). However, in
615 view of the heavy dissemination of resistant organisms namely ESBL, AmpC and
616 carbapenemase producers in addition to the emergence of colistin resistance in livestock and
617 animals with frequent contacts with human; the efficiency of antibiotic administration to
618 animals has been reconsidered. Indeed, antibiotic use in animals is not controlled, in that
619 these latter are not only prescribed for treatment, but are also given for prophylaxis and as
620 growth promoters (Economou and Gousia 2015). In its recent publication, the world health
621 organization recommended a reduction but an overall restriction of the use of medically
622 important antibiotics for prophylaxis and growth promotion in farm animals (WHO 2017
623 2017). According to the world health organization list of Critically Important Antimicrobials
624 for Human Medicine (WHO CIA list), these include mainly extended spectrum
625 cephalosporins, macrolide, ketolides, glycopeptides and polymixins (WHO CIA 2017 2017).
626 The control of antibiotic use in the veterinary sector aims to reduce the emergence of
627 resistance in addition to preserving the efficacy of important classes for treatment in the
628 human medicine.
629 In the Mediterranean region, tetracyclines, aminoglycosides, sulfonamides, fluoroquinolones
630 and polymixins are the most common antimicrobial classes prescribed in the veterinary sector
631 (table 1). The usage level of each antibiotic class in addition to its real purpose of
632 administration apart from treatment is limited and not well understood in this area of the
633 world. In fact, it is nowadays accepted that the over-use of antibiotics in animals is the main
634 driven for the dissemination of multi-drug resistance (Barton 2014). As shown in table 1,
635 ESBL, AmpC and carbapenemase producers are often co-resistant to non beta-lactam
636 antibiotics with the most common being gentamicin, streptomycin, tetracycline,
637 trimethoprim-sulfamethoxazole, nalidixic acid and ciprofloxacin. One study conducted in
638 healthy chicken in Tunisia showed the presence of tetA, tetB, sul1 and sul2 on the same
639 plasmids carrying the blaCTX-M genes (Maamar et al. 2016). Another study in Egypt,

reported the detection of tetB, qnrB2, qnrA1, aadA1 on the same gene cassette along with the blaCMY-2 AmpC beta-lactamase gene (Ahmed, Shimamoto, Shimamoto 2013). In Italy, strA/B, tetD, qnrB, aadA1, sulI genes were associated with the blaCTX-M and blaSHV ESBL genes types in companion animals (Donati et al. 2014). Furthermore, in this same country, aminoglycoside modifying enzymes (aadA1, aadA2), quinolone resistance genes (qnrS1), florfenicol/chloramphenicol resistance gene (floR), in addition to tetracycline and sulfonamide resistance genes (tetA, sul1, sul2, sul3) were found associated with OXA-48/181 and OXA-48/181/ CMY-2 /mcr-1 positive E. coli strains isolated from pigs (Pulss et al. 2017). In Salmonella enterica, Franco et al reported the detection of a megaplasmid harboring the blaCTX-M-1 ESBL gene along with tetA, sulI, dfrA1 and dfrA14 conferring thus additional resistance towards tetracycline, sulfonamide and trimethoprim (Franco et al. 2015). Beta-lactamase producing Gram-negative bacilli appear thus to be selected by the co-selective pressure applied by the use of non beta-lactam antibiotics in livestock and companion animals. Surveillance studies addressing the types, purpose and level of antibiotic classes' administration in animals of the Mediterranean region are warranted in order to develop approaches that control the use of antibiotics while preserving animal's health. This is especially in Syria, Cyprus, Albania, Montenegro, Bosnia, Herzogovina, Monacco, Morocco and Libya where even no data exists on the prevalence and epidemiology of multi-drug resistant organisms in animals.

The spread of multi-drug resistant organisms of animal origin is sparked by the concern of being transmitted to humans; these latter can then be causative agents for infections with limited therapeutic options (Bettiol and Harbarth 2015). The transfer of resistant organisms from animals to humans can occur either via direct contact or indirectly via the consumption of under/uncooked animals products (Dahms et al. 2014). Recent studies have also highlighted the importance of the farms surrounding environment in the transmission chain. Air (von Salviati et al. 2015), dust (Blaak et al. 2015), contaminated waste waters (Guenther, Ewers, Wieler 2011) and soil fertilized with animal manures (Laube et al. 2014) are all potential sources from which resistant organisms can be transferred to the general population. In their study, Olaitan et al, demonstrated the transfer of a colistin resistant E. coli strain from a pigs to its owner (Olaitan et al. 2015). This was documented by both strains (in the pig and its owner) having the same sequence types and sharing the same virulence as well as same PFGE patterns (Olaitan et al. 2015). The increased risk of ESBL fecal carriage in humans with frequent contact with broilers has been further taken as an evidence of transmission (Huijbers et al. 2014). Furthermore, sharing the same sequence types, virulence and PFGE

patterns in addition to common plasmids/ESBL genes are all proofs for the possible transfer of resistant organisms and/or genes from the veterinary sector to the human population (Leverstein-van Hall et al. 2011). In Algeria, Djeffal et al reported the detection of a common sequence type (ST15) in Salmonella spp producing ESBL isolated from both humans and avian isolates (Djeffal et al. 2017). In Egypt, Hamza et al showed an abundance of carbapenemase genes namely blaOXA-48, blaKPC and blaNDM in chicken, drinking water and farm workers suggesting a possible transmission of carbapenemase encoding genes from broilers to farmers and the surrounding environment (Hamza, Dorgham, Hamza 2016). Another study conducted in Italy reported the spread of a multi-drug resistant clone of "Salmonella enterica subsp. enterica serovar Infantis" that was first detected in 2011 in broiler farms and few years later led to human infections most likely via transmission from the broiler industry (Franco et al. 2015). In Spain, common blaCTX-M-grp1 and blaCTX-M-grp9 ESBL genes were detected in retail meat as well as in E. coli strains isolated from infected and colonized patients in the same region (Doi et al. 2010). In France, Hartmann et al showed a clonal relationship among CTX-M carrying E. coli strains in cattle and farm cultivated soils (Hartmann et al. 2012). Another study in cattle, demonstrated that CTX-M-15 harboring plasmids in non-ST131 E. coli strains are highly similar to those detected in humans suggesting thus a multi-clonal plasmidic transmission of multi-drug resistant organisms from livestock to the humans (Madec et al. 2012). The detection of common genes and sequence types among animals and humans and the surrounding environment emphasizes the need to have a global intervention measures to avoid the dissemination of multi-drug resistance in the one health concept.

Conclusion

Antimicrobials have been used in veterinary medicine for more than 50 years. The use of antibiotics proved to be crucial for animal health by lowering mortality and incidence of diseases, in addition to controlling the transmission of infectious agents to the human population. Recently, the dissemination of ESBL, carbapenemase and colistin resistant Gram negative bacteria in food producing animals brought into question the real efficacy of antibiotic administration in animals in terms of treatment, prophylaxis and growth promotion. Indeed, the emergence of MDR in food producing animals has been suggested to be largely linked to the over and misusage of antibiotics in veterinary medicine. The level of antibiotic consumption in animals varies between countries. Although, cephalosporins are not often

prescribed in veterinary medicine, the use of other non-beta-lactams could account for the co-selection of multi-drug resistant bacteria. As shown in Table 1, ESBL and carbapenemase producers were frequently co-resistant to aminoglycosides, tetracyclines and fluoroquinolones, with these latter being mostly used in the veterinary field. Furthermore, the aforementioned antibiotics are classified by the World Health Organization as critically important antibiotics for human medicine that should be restricted in the animal field (Collignon et al. 2016). That said, the direct public health effect of the transmission of MDR bacteria from animals to humans is still controversial. Several studies have demonstrated a direct link of transmission between these two ecosystems. Resistant bacteria once transmitted to humans can be further selected by the over-use of antimicrobial agents in the clinical and community settings. This spread will promote the global dissemination of bacterial resistance across all ecosystems. The level of antibiotic consumption in animals in the European countries lining the Mediterranean is available in the European Surveillance of Veterinary Antimicrobial Consumption report (EMA/ESVAC, 2014), however this is not the case for the countries in North Africa and western Asia, where no accurate data are available. Therefore, surveillance studies investigating the levels of antibiotic prescription should be conducted in these areas. Antimicrobial prescriptions in animals should be re-considered and controlled to limit the spread of bacteria which are cross resistant to the antibiotics used in human medicine. In addition, a risk assessment of other factors contributing to the emergence of antimicrobial resistance in animals should be conducted in future studies. Poor sanitary conditions, overcrowding and poor infection control practices in animals are all possible contributors to the robust emergence of MDR in food-producing animals.

Conflict of Interest Statement

No conflicts of interest or financial disclosure for all authors.

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Authors' contributions

ID and SC wrote the review paper. ZD and JMR corrected the manuscript. All authors approved and revised the final version of the manuscript.

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1349 **Table 1.** Non Beta-lactam resistance in MDR of animal origin versus antibiotic consumption in the Mediterranean Basin
1350

Country	Animal host	Species (number)	blagene Type (number)	Non beta-lactam Resistance	Antibiotic usage	Reference
Algeria	Poultry	E. coli (17)	CTX-M (17)	CMX,NAL,SXT	Unknown	(Mezhoud et al. 2015)
	Poultry	E. coli (16)	CTX-M (2), SHV (14), CMY (4)	AMK, CIP, KAN, NAL, STR, TOB		(Belmahdi et al. 2016)
	Poultry	Salmonella spp (11)	CTX-M (11)	CIP		(Djeffal et al. 2017)
	Cattle	A. baumannii (1)	NDM (1)	CIP		(Chaalal et al. 2016; Yaici et al. 2016)
	Cattle	E. coli (4)	NDM (4), CTX-M (4), CMY (4),			(Yaici et al. 2016)
	Birds	E. coli (11)	CTX-M (11)	CIP, NAL, NEO SXT, TET,		(Meguenni et al. 2015)
	Birds	A. baumannii (4)	OXA (4)			(Morakchi et al. 2017)
	Dogs	E. coli (1)	NDM (1)	FLU, TET		(Yousfi et al. 2015)
	Dogs	E. coli (15)	CTX-M (13), SHV (3)	CIP, GEN, NAL, SUL, SXT, TET, TMP, TOB		(Yousfi et al. 2016b)
	Dogs	E. coli (3)	CTX-M (1), CMY (1), NDM (1), OXA-48 (2)	GEN, CIP, NAL, SXT, TEM, TOB,		(Yousfi et al. 2016a)
	Cats	E. coli (2)	CMY (1), OXA-48 (2)	CIP, GEN, NAL, SXT, TEM, TOB		(Yousfi et al. 2016a)
	Cats	E. coli (5)	CTX-M (5)	CIP, NAL, SUL, SXT, TET, TMP, TOB		(Yousfi et al. 2016b)
	Fish	E. coli (22)	CTX-M (16), TEM (6)	AMK, CIP, CMX, GEN, KAN, NAL, NET, OFX		(Brahmi et al. 2016)
	Fish	A. baumannii (2)	OXA-23 (2)	CIP, GEN, KAN, SXT		(Brahmi et al. 2016)
	Macaques	K .pneumoniae (7)	CTX-M (7)	CIP, FOS, GEN, SXT		(Bachiri et al. 2017)
	Wild Boars	E. coli (30)	CTX-M (30)	AMK, CIP, FOS, GEN, SXT, TET		(Bachiri et al. 2017)
		K .pneumoniae (10)	CTX-M (10)			
Tunisia	Poultry	E. coli (13)	CTX-M (12), CMY (1)	CIP, CHL, GEN, NAL, SXT, SUL, STR, TET	Streptomycin, Tetracycline, Sulphonamides, Trimethoprim	(Ben Slama et al. 2010) (Ben Sallem et al. 2012)
	Poultry	E. coli (67)	CTX-M (42), CMY (24)	AMK, GEN, NAL, NOR, SXT, TET		(Mnif et al. 2012)
	Poultry	E. coli (16)	CTX-M (16)	NAL, SXT, STR, SUL, TET		(Kilani et al. 2015)
	Poultry	E. coli (7)	CTX-M (7)	NAL, STR, TET, SUL, TMP		(Grami et al. 2013)
	Poultry	E. coli (10)	CTX-M (8), TEM (1), CMY (2)	NAL, SXT, SUL, TET, STR		(Ben Sallem et al. 2012)
	Poultry	E. coli (48)	CTX-M (35), CMY (13)	AMK, CIP, GEN, MIN, NAL, SXT, TET		(Maamar et al. 2016)
	Poultry	E .coli (5)	CTX-M (4), SHV (1)			(Jouini et al. 2013)

Table 1. Continued

Country	Animal host	Species (number)	blagene Type (number)	Non beta-lactam Resistance	Antibiotic usage	Reference
	Cattle	E. coli (1)	CTX-M (1)	GEN, TOB, TET		(Grami et al. 2014)
	Beef	E. coli (1)	CTX-M (1)	CIP, NAL, SXT, SUL, TET		(Ben Slama et al. 2010)
	Beef	E. coli (5)	CTX-M (5)	CHL, GEN, STR, SUL, SXT, TET, TOB		(Jouini et al. 2013)
	Sheep	E. coli (3)	CTX-M (5), TEM (1)	CIP, GEN, NAL, SXT, SUL, STR, TET		(Ben Slama et al. 2010)
	Dogs	E. coli (6)	CTX-M (6)	CHL, ENR. GEN, KAN, NAL, NET, SUL, STR, TET, TMP, TOB		(Grami et al. 2013)
	Dogs	E. coli (6)	CTX-M (5), CMY (1)	CIP, NAL, SXT, STR, SUL, TET		(Sallem et al. 2013)
	Cats	E. coli (1)	CTX-M (1)	NAL, STR, SUL, TET, TMP,		(Grami et al. 2013)
	Cats	E. coli (8)	CTX-M (8)	CIP, KAN, NAL, STR, SXT, SUL, TET		(Sallem et al. 2013)
	Dromedaries	E. coli (1)	CTX-M (1)	SUL, TET		(Ben Sallem et al. 2012)
Egypt	Poultry	E. coli (18)	CTX-M (7), CMY (11)	CHL, CIP, KAN, NAL, SPX, STR, SXT, TET	Fluoroquinolones, Tetracyclines, Aminoglycosides, Cefotaxime	(Ahmed, Shimamoto, Shimamoto 2013)(Ahmed, Shimamoto, Shimamoto 2013) (Dahshan et al. 2015)
	Poultry	E. coli (9)	CTX-M (2), SHV (1), TEM (1), CMY (1)	CIP, CMX, DOX, GEN, STR		(El-Shazly et al. 2017)
	Poultry	K .pneumoniae (15)	NDM (15), KPC (14), OXA (12)	-		(Hamza, Dorgham, Hamza 2016)
	Poultry	K. pneumoniae (11), K. oxytoca (1)	NDM (12)			(Abdallah et al. 2015)
		E. coli (8)	CTX-M (8)			
		K. pneumoniae (40)	CTX-M (40)			
		K. oxytoca (2)	CTX-M (2)			
		Enterobacter spp (9)	CTX-M (9)			
	Cattle	E. coli (112)	CTX-M (106), OXA (6)	FOS, FLU, CMX, CHL, MLS, TET	Tetracycline, quinolones	(Braun et al. 2016)
	Cattle	E. coli (8)	CTX-M (2), SHV (5), CMY (1)	,NAL, SXT, STR, TET		(Ahmed et al. 2009)
	Beef	E. coli (4)	CTX-M (1), SHV (1), CMY (2)	CHL, CIP, GEN, KAN, NAL, SPX, STR, SXT, TET	Fluoroquinolones	(Ahmed and Shimamoto 2015)
	Cats	E. coli (5)	CTX-M (5)			(Abdel-Moein and Samir 2014)

Table 1. Continued

Country	Animal host	Species (number)	blagene Type (number)	Non beta-lactam Resistance	Antibiotic usage	Reference
	Dogs	E. coli (11)	CTX-M (11)			(Abdel-Moein and Samir 2014)
		K. pneumoniae (3)	CTX-M (3)			
		P. mirabilis (1)	CTX-M (1)			
Palestine	Cattle	E. coli (287)	CTX-M (287)	SXT, STR, TET	Chlortetracycline, doxycycline, Norfloxacin, Cephalexin, Ceftiofur, Sulfa agents, Gentamicin, Monensin	(Adler et al. 2015)
		K. pneumoniae (4)	SHV (4)	CHL, CIP, GEN		
	Poultry	E. coli (9)	CTX-M (9)			(Qabajah, Awwad, Ashhab 2014)
Lebanon	Poultry	E. coli (217), K. pneumoniae (8), P. mirabilis (3), E. albertii (2), E. fergusonii (1), E. cloacae (3),	CTX-M, CMY	CIP, GEN, SXT	Gentamicin, Tetracyclines	(Dandachi et al. 2018a)
	Cattle	E. coli (27)	CTX-M (27)	CHL, ENR, GEN, KAN, NAL, STR, SUL, TET, TMP	Penicillin G - Streptomycin, Ampicillin, Amoxicillin Oxytetracycline, Gentamicin,	(Diab et al. 2016) (Gundogan, Citak, Yalcin 2011)
	Fowl	A.baumannii (1)	OXA-48 (1)	AMK, GEN, TOB	Unknown	(Al Bayssari et al. 2015b)
	Horse	A.baumannii (1)	OXA-143 (1)			(Rafei et al. 2015)
	Rabbit	A. pitii (1)	OXA-24 (1)			
Turkey	Poultry		CTX-M (60), SHV (4), CMY (18)	CHL, KAN, NAL, STR, SUL, TET, TMP	Tetracycline, Quinolones	(Politi et al. 2005) (Pehlivanlar Onen et al. 2015)
	Cattle	E. coli (3)	CTX-M (2), CMY (1)	NAL, SXT, STR, TET		
	Poultry	E. coli (15)	CTX-M (15)			(Tekiner and Ozpinar 2016)
	Cattle	E. coli (19)	CTX-M (19)			
Croatia	Mussel	Aeromonas. Caviae (25)	CTX-M (11), SHV (11), FOX (3)		Tetracycline, Amphenicol, Penicillins, Sulfonamides, Trimethoprim, Fluoroquinolones, Aminoglycosides, Polymixins	(Maravic et al. 2013) (EMA/ESVAC, 2014)
		A. Hydrophila (8)	CTX-M (8), SHV (2)			
Greece	Poultry	Salmonella enteric (2)	CTX-M (2)	CHL, KAN, STR, SUL, TMP, TET	Unknown	(Politi et al. 2005)
	Dogs	E. coli (8)	CMY (8)	FLU		(Vingopoulou et al. 2014)

Table 1. Continued

Country	Animal host	Species (number)	blagene Type (number)	Non beta-lactam Resistance	Antibiotic usage	Reference
Slovenia	Poultry	E. coli (6)	CTX-M (2), SHV (4)	GEN, NAL, STR, SUL	Ceftiofur	(Chiaretto et al. 2008)
Italy	Poultry, Cattle, Swine				Tetracyclines, Amphenicol, Penicillins, $3^{rd}/4^{th}$ Cephalosporins, Sulfonamides, Trimethoprim, Macrolides, Lincosamides, Fluoroquinolones, Aminoglycosides, Polymixins, Pleuromutilins, Tylosin, Flumequine,	
	Poultry	E. coli (8)	CTX-M (7), SHV (1),	CIP		(Giufre et al. 2012)
	Poultry	E. coli (60)	CTX-M (45), CIT-like (15)	CIP, GEN, SXT, TET		(Ghodousi et al. 2015)
	Poultry	E. coli (67)	CTX-M (24), SHV (43)	CIP, NAL, SUL, TMP, TET		(Bortolaia et al. 2010)
	Poultry	Salmonella spp (12)	SHV (12)	GENT, NAL, SUL, STR, TET		(Chiaretto et al. 2008)
	Poultry	Salmonella infantis (30)	CTX-M (30)	CIP, NAL, SUL, TMP, TET		(Franco et al. 2015)
	Swine	Salmonella infantis (2)	CTX-M (2)			
	Cattle	K. ozaenae (5)	CTX-M (5), TEM (1)			(Stefani et al. 2014)
	Swine	E. coli (15)	CTX-M (10), TEM (7)			
	Dogs	K. oxytoca (2)	SHV (2), DHA (2)	CIP, GEN, KAN, STR, SUL, TET, TMP		(Donati et al. 2014)
		K. pneumoniae (11)	CTX-M (11), SHV (5), DHA (1)	CIP, GEN, KAN, NAL, TET, TMP		
	Dogs	K. pneumoniae (1)	CTX-M (1), SHV (1)	CIP, LEV		(Bogaerts et al. 2015)
		E. coli (1)	CMY (1)	CIP, LEV		
	Cats	K. oxytoca (2)	CTX-M (2)	CIP, SUL, TMP, TET		(Donati et al. 2014)
		K. pneumoniae (4)	CTX-M (2), SHV (2), DHA (1), CMY (1)	CIP, KAN, NAL, SUL, TET, TMP		
	Cats	E. coli (7)	CTX-M (7), CMY (2)	CHL, ENR, GEN, NAL, NIT, SPX, STR, SUL, TET, TMP.		(Nebbia et al. 2014)
France	Poultry, Cattle, Swine				Tetracycline, Amphenicol, Penicillins, $1^{st}/2^{nd}/3^{rd}/4^{th}$ Cephalosporins, Sulfonamides, Trimethoprim, Macrolides, Lincosamides, Fluoroquinolones, Aminoglycosides, Polymixins, Pleuromutilins	(EMA/ESVAC, 2014)

Table 1. Continued

Country	Animal host	Species (number)	blagene Type (number)	Non beta-lactam Resistance	Antibiotic usage	Reference
	Cattle	E. coli (26)	CTX-M (21), TEM (5)	CHL, GENT, SXT		(Hartmann et al. 2012)
	Cattle	E. coli (3)	CTX-M (3)	CHL, ENR, FFC, GEN, KAN, NAL, STR, SUL, TET, TMP		(Meunier et al. 2006)
	Cattle	A. baumannii (9)	OXA-23 (9)	FOS, KAN, TET		(Poirel et al. 2012)
	Cattle	E. coli (9)	CTX-M (9)	CHL, ENR, GEN, KAN, NAL, NET, OFX, STR, SUL, TET, TOB, TMP		(Madec et al. 2012)
	Cattle	E. coli (5)	CTX-M (5)	APR, CHL, ENR, GEN, KAN, NAL, NET, OFX, STR, SUL, TET, TOB, TMP		(Dahmen et al. 2013b)
		K. pneumoniae (1)	CTX-M (1)			
	Sheep	K. pneumoniae (3)	CTX-M (3), DHA (3)	NAL, SUL, SXT, TET		(Poirel et al. 2013)
		E. fergusonii	CTX-M (1)			
	Veal calves	E. coli (147)	CTX-M (147)	APR, CHL, ENR, FFC, GEN, KAN, NAL, NET, SUL, STR, TET, TOB, TMP		(Haenni et al. 2014)
		K. pneumoniae (3)	CTX-M (2), SHV (1)	FLU, SUL, STR, TET, TMP		
	Swine	E. coli (3)	CTX-M (3)	CHL, NAL, STR, SUL, TET, TMP		(Meunier et al. 2006)
	Dog	E. cloacae (11)	CTX-M (10), SHV (1)	FLU, GEN, KAN, QUI, TET, SUL, STR, TMP		(Haenni et al. 2016)
	Dog	E. coli (47)	CTX-M (47), CMY (24)	CHL, GEN, KAN, STR, TOB ENR, FFC, NAL, NET, OFX, SUL, TET, TMP		(Haenni et al. 2014)
	Dog	E. coli (9)	CTX-M (8), TEM (1)	GEN, SUL, TET		(Poirel et al. 2013)
		K. pneumoniae (8)	CTX-M (8), DHA (1)	GEN, NAL, SUL, SXT, TET		
		K. oxytoca (2)	CTX-M (2)			
	Dog	P. mirabilis (14)	CTX-M (1), CMY (7), DHA (2), VEB (6)	APR, CHL, ENR, GEN, KAN, NAL, NET, STR, SUL, TOB, TMP		(Schultz et al. 2017)
	Dog	A. baumannii (2)	OXA-23 (2)	CIP, SXT		(Herivaux et al. 2016)
	Dog	E. coli (3)	CMY (2), OXA-48 (1)	GEN, NAL		(Melo et al. 2017)
	Cat	A. baumannii (1)	OXA-23 (1)	GEN, NAL, SUL, STR, TET		(Ewers et al. 2016)
	Cat	K. pneumoniae (3)	CTX-M (3), DHA (3)	NAL, SUL, SXT, TET	Unknown	(Poirel et al. 2013)
		E. coli (3)	CTX-M (3)	GEN, SUL, TET	Unknown	

Table 1. Continued

Country	Animal host	Species (number)	blagene Type (number)	Non beta-lactam Resistance	Antibiotic usage	Reference
	Cat	P. mirabilis (1)	CMY (1)	ENR, NAL, SUL, TMP		(Schultz et al. 2017)
		P. rettgeri (1)	CTX-M (1)	ENR, NAL, SUL, TMP		
	Cat	E. coli (2)	CTX-M (2)	STR, TMP		(Melo et al. 2017)
	Cat	E. cloacae (11)	CTX-M (10), SHV (1)	FLU, GEN, KAN, QUI, SUL, STR, TET, TMP		(Haenni et al. 2016)
	Companions	E. coli (19)	CTX-M (19)	CIP, NAL, SUL, STR, TET		(Dahmen et al. 2013a)
	Hedgehog	E. coli (1)	CTX-M (1), DHA (1)	NAL, SUL, SXT, TET	Unknown	(Poirel et al. 2013)
	Tawny Owl	E. coli (1)	CTX-M (1)			
	Domestic goose	E. coli (1)	CTX-M (1)			
	Rock Pigeon	E. coli (1)	CTX-M (1)			
	Horse	E. cloacae (14)	CTX-M (8) , SHV (6)	FLU, GEN, KAN, QUI, SUL, STR, TET, TMP		(Haenni et al. 2016)
	Horse	P. mirabilis (14)	VEB (2)	ENR, CHL, KAN, NAL, NET, SUL, STR, TOB, TMP	Unknown	(Schultz et al. 2017)
Spain	Poultry, Cattle, Swine				Tetracycline, Amphenicol, Penicillins, $3^{rd}/4^{th}$ Cephalosporins, Sulfonamides, Trimethoprim, Macrolides, Lincosamides, Fluoroquinolones, Quinolones, Aminoglycosides, Polymixins, Pleuromutilins	(Abreu et al. 2014) (EMA/ESVAC, 2014)
	Poultry	E. coli (64)	CTX-M (44), SHV (6), TEM (2), CMY (13)	CHL, CIP, FUR, GEN, KAN, NAL, SUL, SXT, TET, TOB, TMP		(Blanc et al. 2006)
	Poultry	S. enterica (2)	CTX-M (1), SHV (1)	NAL, SXT, STR, SUL, TET,		(Riano et al. 2006)
	Poultry	E. coli (116)	CTX-M (116)	CIP, NAL, SXT		(Abreu et al. 2014)
	Poultry	E. coli (11)	CTX-M (6), SHV (2), CMY (2)	CHL, CIP, FFC, GEN, KAN, NAL, STR, SUL, TET, TMP		(Sola-Gines et al. 2015)
	Poultry	E. coli (50)	CTX-M (40), CMY (10)	NAL		(Cortes et al. 2010)
	Poultry	E. coli (62)	CTX-M (20), SHV (42)	CIP, NAL		(Egea et al. 2012)
	Swine	E. coli (20)	CTX-M (20)			(Sola-Gines et al. 2015)
	Swine	S. enteric (1)	SHV (1)	SUL, STR, TET		(Riano et al. 2006)
	Swine	E. coli (39)	CTX-M (27), SHV(12)	CIP, CHL, FUR, GEN, KAN, NAL, SUL, SXT, TET, TMP, TOB		(Blanc et al. 2006)

Table 1. Continued

Country	Animal host	Species (number)	blagene Type (number)	Non beta-lactam Resistance	Antibiotic usage	Reference
	Swine	E. coli (20)	CTX-M (8), SHV (12)	APR, CIP, GEN, NAL, STR, SUL, TET, TMP		(Escudero et al. 2010)
	Dog	E. coli (1)	SHV (1)	CHL, CIP, NAL, SUL, TET, TMP		(Teshager et al. 2000)
	Dog	E. coli (1)	CMY (1)			(Bogaerts et al. 2015)
		P. mirabilis (2)	CMY (2)	DOX, MIN		
	Dog	K. pneumoniae (2)	CTX-M (1), VIM (1), DHA (1)			(Gonzalez-Torralba et al. 2016)
		E. cloacae (1)	SHV (1)			
	Deer	E. coli (1)	CTX-M (1)	CIP, CHL, NAL, SXT, TET	Unknown	(Alonso et al. 2016)
	Rabbit	E. coli (1)	CMY (1)		Unknown	(Blanc et al. 2006)
		E. cloacae (3)	CTX-M (3)			

*[APR] refers to apramycin, [AMK] amikacin, [CIP] ciprofloxacin, [CHL] chloramphenicol, [CMX] co-trimoxazole, [DOX] doxycycline, [ENR] enrofloxacin, [FFC] florfenicole, [FLU] fluoroquinolones, [FOS] fosfomycin, [FUR] furazolidone, [GEN] gentamicin, [KAN] kanamycin, [LEV] levofloxacin, [MIN] minocycline, [MLS] Macrolides, [NAL] nalidixic acid, [NET] netilmicin, [NIT] nitrofurantoin, [NOR] norfloxacin, [OFX] oxofloxacin, [QUI] quinolones, [SPX] spectinomycin, [SXT] trimethoprim-sulfamethoxazole, [TEM] temocillin, [TET] tetracycline, [TMP] trimethoprim, [TOB] tobramycin.

1370 **Table 2.** ST/phylogroups, IS and plasmid types associated with beta-lactamase and mcr genes in the Mediterranean.
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Country	Animal Host	Species	Bla and/or mcr genes	ST and/or phylogroup	Plasmid type	Associated IS	Reference
Algeria	Poultry	E. coli	CTX-M 1	ST38, ST2179			(Belmahdi et al. 2016)
			SHV-12	ST1011, ST5086			
			CMY-2	ST744			
	Poultry	S. Heidelberg	CTX-M-1	ST15			(Djeffal et al. 2017)
	Cattle	A. baumanii	NDM-1	ST85			(Chaalal et al. 2016)
	Cattle	E. coli	NDM-5/ CMY-42/ CTX-M-15	ST1284	IncX3 (NDM-5)		(Yaici et al. 2016)
	Swine	K. pneumoniae	CTX-M-15	ST584			(Bachiri et al. 2017)
		E. coli	CTX-M 15	ST617, ST131, ST648, ST405, ST1431, ST1421, ST69, ST226			
	Dog	E. coli	CTX-M-15	A, B1, E			(Yousfi et al. 2016b)
			CTX-M-1/SHV-12	E			
			SHV-12	A, B1			
	Dog	E. coli	NDM-5	ST1284			(Yousfi et al. 2015)
	Dog	E. coli	OXA-48	A, D			(Yousfi et al. 2016a)
			NDM-5/ CTX-M-15/ CMY-42	A			
	Cat	E. coli	CTX-M-1	B1			(Yousfi et al. 2016b)
			CTX-M-15	A, U, E			
	Cat		OXA-48 / CMY-1	U			(Yousfi et al. 2016a)
			OXA-48	D			
	Barbary Macaques	K. pneumoniae	CTX-M-15	ST584			(Bachiri et al. 2017)
	Fish	A. baumanii	OXA-23	ST2			(Brahmi et al. 2016)
	Fish	E. coli	CTX-M-15	ST471, ST132, ST398, ST37,ST477, ST131, ST31			(Brahmi et al. 2015)
			CTX-M-9	ST8			
			TEM-24	ST31, ST471, ST66, ST21, ST74			

Table 2. Continued

Country	Animal Host	Species	blaand/or mcr genes	ST and/or phylogroup	Plasmid type	Associated IS	Reference
Tunisia	Poultry	E. coli	CTX-M-1	A, B1, D		ISEcp1	(Ben Sallem et al. 2012)
			CMY-2	B2		ISEcp1	
				D		ISEcp1D-IS10	
	Poultry		CTX-M-1			ISEcp1/IS26	(Jouini et al. 2007)
	Poultry	E. coli	CTX-M-1	B1, A			(Ben Slama et al. 2010)
			CMY-2	B1			
	Poultry	E. coli	CTX-M-1	A, B1, D, B2	IncI1		(Mnif et al. 2012)
			CTX-M-15	A, B1			
			CTX-M-1/CMY-2	B2	IncI1		
			CMY-2	A, D, B1	IncI1		
	Poultry	E. coli	CTX-M-1		IncI1		(Grami et al. 2013)
			CTX-M-9		IncI1		
	Poultry	E. coli	CTX-M-1	A0, A1, D2, B2			(Kilani et al. 2015)
	Poultry	E. coli	CMY-2	A, B1, D	IncI1, IncF, IncFIB, IncFIA		(Maamar et al. 2016)
			CTX-M-14	B1	IncF	ISEcp1-IS903	
			CTX-M-1	B1, D, A	IncI1, IncF, IncFIB, IncK, IncY, IncP, IncN		
			CTX-M-15	D		ISEcp1and ISEcp1-IS5	
	Poultry	E. coli	CTX-M-1/mcr-1	D, H, K	IncHI2/ST4		(Grami et al. 2016)
	Poultry	E. coli	CMY-2/mcr-1	A (ST2197)	IncP (mcr-1)	ISApl1	(Maamar et al. 2018)
					IncI1 (CMY-2)		
	Cattle	E. coli	CTX-M-1	A, B1			(Ben Slama et al. 2010)
			CTX-M-1/ TEM-20	B1			
	Cattle	E. coli	CTX-M-1			ISEcp1/IS26	(Jouini et al. 2007)
			CTX-M-14			ISEcp1 and IS903	
	Cattle	E. coli	CTX-M-15	ST10		ISEcp1	(Grami et al. 2014)
	Dog	E. coli	CTX-M-1		IncI1		(Grami et al. 2013)
			CTX-M-15		IncFII		

Table 2. Continued

Country	Animal Host	Species	blaand/or mcr genes	ST and/or phylogroup	Plasmid type	Associated IS	Reference
	Dog	E. coli	CMY-2	B1		ISEcp1	(Sallem et al. 2013)
			CTX-M-1	D, B1, A		ISEcp1	
	Cat	E. coli	CTX-M-1	B1, A, D		ISEcp1	(Sallem et al. 2013)
			CTX-M-1/ TEM-135	A		ISEcp1 (CTX-M-1)	
	Cat	E. coli	CTX-M-1		IncI1		(Grami et al. 2013)
	Dromedaries	E. coli	CTX-M-1	B1		ISEcp1	(Ben Sallem et al. 2012)
Egypt	Poultry	E. coli	CTX-M-15	clonal group O25b-ST131		ISEcp1	(Ahmed, et al. 2013)
	Poultry	E. coli	CTX-M	A, B1, B2, D			(Abdallah et al. 2015)
	Poultry	E. coli	CTX-M-14	D			(El-Shazly et al. 2017)
			SHV-12	D			
			CMY-2	A, B1, D			
	Poultry	E. coli	mcr-1	phylotype A, F, B1	IncFIB; IncI1; IncI2		(Lima Barbieri et al. 2017)
	Cattle	E. coli	mcr-1	ST10			(Khalifa et al. 2016)
Lebanon	Poultry	E. coli	CTX-M	ST156, ST5470, ST354, ST155, ST3224			(Dandachi et al. 2018a)
	Poultry	E. coli	mcr-1	ST515			(Dandachi et al. 2018b)
	Cattle	E. coli	CTX-M-15	A (ST1294, ST2325, ST1303, ST4623, ST5204) B1 (ST58, ST162, ST4252, ST155,ST196, ST540) D (ST69)			(Diab et al. 2016)
			CTX-M-14	D (ST457)			
			CTX-M-15/SHV-12	A (ST10, ST2450, ST5442)			
			CTX-M-14/SHV-12	D (ST457)			
			SHV-12	A (ST218, ST617, ST5204, ST1303,ST5728,ST1140, ST746)			
	Cattle	A. baumanii	OXA-23	ST2			(Al Bayssari et al. 2015a)
		P. aeroginosa	VIM-2	ST1762, ST1759			
	Swine	A. baumanii	OXA-23	ST491			(Al Bayssari et al. 2015a)

Table 2. Continued

Country	Animal Host	Species	Bla and/or mcr genes	ST and/or phylogroup	Plasmid type	Associated IS	Reference
	Fowl	A. baumanii	OXA-23	ST492, ST493			(Al Bayssari et al. 2015b)
			OXA-58/OXA-23	ST20			
		P. aeroginosa	VIM-2	ST1760, ST1761			
	Fowl	E. coli	OXA-48	ST38			(Al Bayssari et al. 2015b)
	Horse	A. baumanii	OXA-143	ST294			(Rafei et al. 2015)
	Rabbit	A. pitii	OXA-24	ST221			(Rafei et al. 2015)
Palestine	Poultry	E. coli	CTX-M	A, B, D			(Qabajah et al. 2014)
Turkey	Poultry	E. coli	CMY-2	A0, B2 D1, D2			(Pehlivanlar Onen et al. 2015)
			CTX-M-1/CMY-2	A0			
			CTX-M-1	A1, A0, D1, D2			
			CTX-M-1/SHV-5	D1			
			CTX-M-3	A0, D1			
			CTX-M-15	B1, D1, D2			
			SHV-12	D1			
			CTX-M-15/SHV-12	D2			
Italy	Poultry	E. coli	SHV-12		IncI1, IncFIB		(Bortolaia et al. 2010)
			CTX-M-1		IncI1, IncFIB, IncN		
			CTX-M-32		IncN		
	Poultry	E. coli	CTX-M-1		IncI1		(Accogli et al. 2013)
			CMY-2		IncI1		
	Poultry	E. coli	CTX-M	A, B1, B2, D			(Ghodousi et al. 2015)
			CIT like	B1, B2, D			
	Poultry	E. coli	CTX-M	B2, ST131			(Ghodousi et al. 2016)
	Swine	E. coli	OXA-181	B1 (ST359), A (ST641)	IncX3		(Pulss et al. 2017)
			mcr-1	A (ST641)	IncX4		
			CMY-2	A (ST641)	IncI1		
	Cat	E. coli	CMY	A		ISEcp1/IS26	(Bogaerts et al. 2015)

Table 2. Continued

Country	Animal Host	Species	Bla and/or mcr genes	ST and/or phylogroup	Plasmid type	Associated IS	Reference
	Dog	K. oxytoca	SHV-12, DHA-1	N.I	IncL/M		(Donati et al. 2014)
		K. pneumoniae	CTX-M-15,DHA-1	ST340	IncR (CTX-M-15)		
			CTX-M-15	ST101			
			SHV-28,	ST15			
			CTX-M-15,SHV-28,	ST15			
			CTX-M-1,SHV-28	ST15	CTX-M-1 in IncN and IncR		
			CTX-M-1	ST11			
	Cat	K. oxytoca	CTX-M-9	N.I	IncHI2		(Donati et al. 2014)
		K. pneumoniae	CTX-M-15, DHA-1	ST340	CTX-M-15/DHA-1 on IncR		
			SHV-28, CMY-2	ST15	CMY-2 on InCI1		
			CTX-M-15	ST101			
	Cat	E. coli	CTX-M-14/CMY-2	A (ST3848, ST3847)			(Nebbia et al. 2014)
			CTX-M-14	B2 (ST555, ST4181), B1 (ST602)			
			CTX-M-1	B2 (ST155)			
			CTX-M-15	B2 (ST131)			
Slovenia	Poultry	E. coli	CTX-M-1	D			(Zogg et al. 2016)
			SHV-12	B1 and D			
Spain	Poultry	E. coli	CTX-M-14	ST101, ST156,ST165,ST350, ST889, ST1137	IncK		(Sola-Gines et al. 2015)
			SHV-12	ST350, ST533	IncI1		
			CMY-2	ST429, ST131	IncK		
	Poultry	E. coli	CMY-2	A, D			(Cortes et al. 2010)
			CTX-M-14	A, B1, B2			
			CTX-M-32	A			
			CTX-M-9	B1			
			SHV-12				
			TEM-52	B1			

Table 2. Continued

Country	Animal Host	Species	Bla and/or mcr genes	ST and/or phylogroup	Plasmid type	Associated IS	Reference
	Poultry	E. coli	CTX-M-9	O25b:H4-B2-ST131.			(Mora et al. 2010)
	Poultry	E. coli	CTX-M, SHV	A, B1, D1			(Egea et al. 2012)
	Poultry, Swine, Cattle	E. coli	CTX-M, SHV	B2, D			(Doi et al. 2010)
	cattle	E. coli	mcr-1 /mcr-3/ CTX-M-55	ST533	non mobilizable IncHI2		(Hernandez et al. 2017)
	Swine	E. coli	CTX-M-1	A			(Cortes et al. 2010)
			SHV-5	A			
			SHV-12	B1			
	Dog	E. coli (1)	CMY (1)	ST2171	IncK	ISEcp1	(Bogaerts et al. 2015)
		P. mirabilis (2)	CMY (2)				
	Dog	K. pneumoniae	VIM-1	ST2090			(Gonzalez-Torralba et al. 2016)
	Deer	E. coli	CTX-M-1	ST224	IncN	IS26	(Alonso et al. 2016)
Croatia	Mussel	Aeromonas spp	CTX-M-15		IncFIB		(Maravic et al. 2013)
France	Poultry	E. coli	CTX-M-1			ISEcp1	(Meunier et al. 2006)
	Cattle	E. coli	CTX-M-1			ISEcp1	(Meunier et al. 2006)
			CTX-M-15			ISEcp1	
	Cattle	E. coli	CTX-M-15	B1		ISEcp1	(Valat et al. 2012)
	Cattle	E. coli	CTX-M-1	ST2497, ST2498			(Hartmann et al. 2012)
			TEM-71	ST178			
	Cattle	E. coli	CTX-M-15,	ST2212, ST2213, ST2210, ST2214,ST2215, ST88	F31:A4:B1/IncFII F2:A–:B–/IncFII and IncI1		(Madec et al. 2012)
	Cattle	K. pneumoniae	CTX-M-14	ST45	F2:A-:B-IncFII		(Dahmen et al. 2013b)
		E. coli	CTX-M-14	ST23, ST58, ST10, ST45	F2:A-:B-IncFII		
			CTX-M-1	ST23, ST58	IncI1/ST3		
	Sheep	K. pneumoniae	CTX-M-15, DHA	all ST274			(Poirel et al. 2013)
	Swine	E. coli	CTX-M-1			ISEcp1	(Meunier et al. 2006)

Table 2. Continued

Country	Animal Host	Species	Bla and/or mcr genes	ST and/or phylogroup	Plasmid type	Associated IS	Reference
	Dogs	E. coli	CTX-M-15	A (ST410, ST617)	IncFII		(Dahmen et al. 2013a)
			CTX-M-1	A (ST10), B1 (ST1303, ST1249)	IncFII		
					IncFII		
	Dog	A. baumanii	OXA-23	ST25			(Herivaux et al. 2016)
	Dogs	E. coli	CTX-M-1	ST345, ST1001, ST124	IncI1		(Poirel et al. 2013)
			CTX-M-15	NEW ST	N.T		
			TEM-52	ST359			
		K. pneumoniae	CTX-M-15, DHA-1	ST274			
			CTX-M-15,	ST15			
	Dogs	E. coli	CTX-M-1	A, B1,D	blaCTX-M-1/IncI1/ST3		(Haenni et al. 2014)
			CTX-M-grp9	B2			
			CMY-2	A, B1, B2, D	CMY-2/IncI1/ST2		
	Dog	E. cloacae	CTX-M-15	ST114,ST136,ST270,ST100	IncHI2		(Haenni et al. 2016)
			CTX-M-14	ST102	N.T		
			CTX-M-3	ST408	N.T		
			SHV-12	ST268	IncHI2		
	Dog	E. coli	CMY	ST55	N.T		(Melo et al. 2017)
			CMY	ST963	N.T		
			OXA-48	ST372	IncL		
	Cat	K. pneumoniae	CTX-M-15, DHA	ST274			(Poirel et al. 2013)
		E. coli	CTX-M-1	ST124, ST641			
			CTX-M-14	ST141			
	Cats	E. coli	CTX-M-15	A (ST617, ST410)			(Dahmen et al. 2013a)
			CTX-M-32	B1 (ST224)			
			CTX-M-3	B2 (ST493)			
			CTX-M-14	B1, (ST359), B2 (ST131)			
	Cat	E. cloacae	CTX-M-15	1 ST136, others ST114	IncHI2		(Haenni et al. 2016)
			SHV-12	N.T	IncA/C		

Table 2. Continued

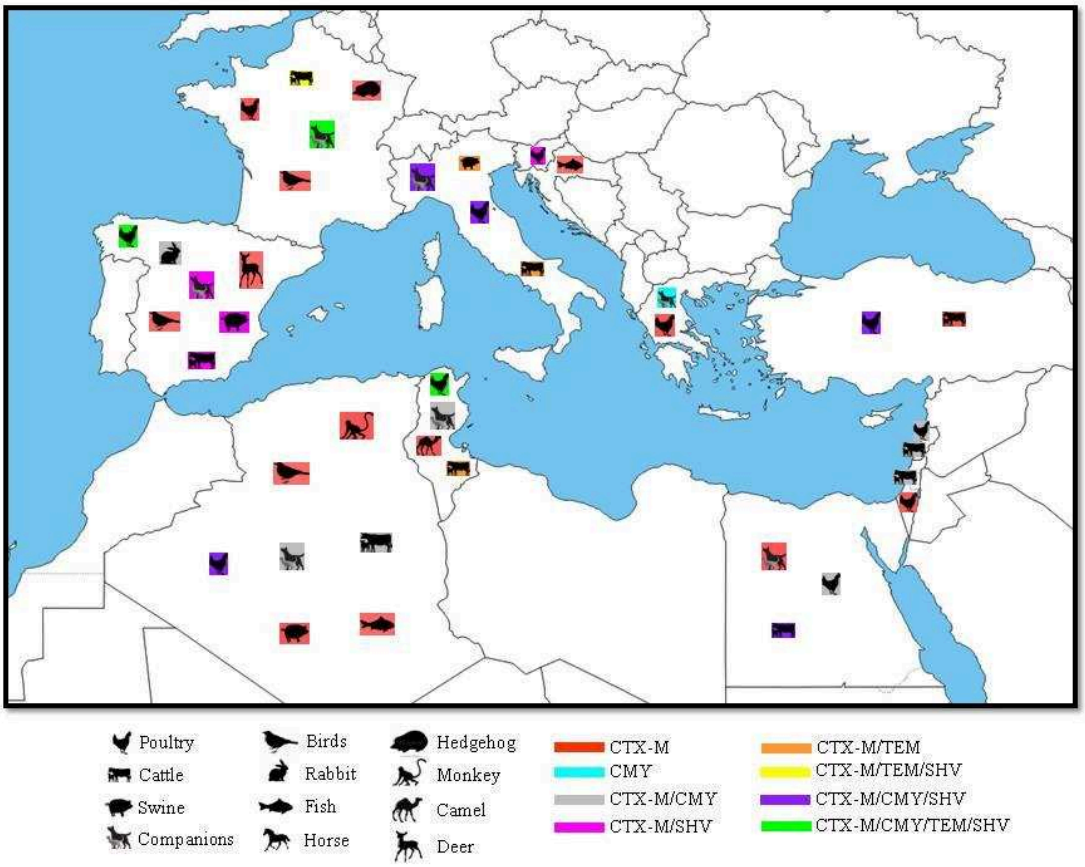
Country	Animal Host	Species	Bla and/or mcr genes	ST and/or phylogroup	Plasmid type	Associated IS	Reference
	Cat	E. coli	CTX-M-14	ST68	IncF		(Melo et al. 2017)
			CTX-M-1	ST673	IncFIB		
	Cat	A. baumanii	OXA-23	ST1/ST231			(Ewers et al. 2016)
	Hedgehog	K. pneumoniae	CTX-M-15, DHA	ST274			(Poirel et al. 2013)
	Tawny Owl	E. coli	CTX-M-1	ST93			(Poirel et al. 2013)
	Domestic goose	E. coli	CTX-M-15	ST10			(Poirel et al. 2013)
	Rock pigeon	E. coli	CTX-M-1	ST124			(Poirel et al. 2013)
	Horse	E. cloacae	CTX-M-15	ST127, ST372, ST145, ST114	IncHI2		(Haenni et al. 2016)
			SHV-12	ST135,ST145,ST118	IncHI2		
			CTX-M-1	ST268	N.T		
				ST107	IncP		
Greece	Dog	E. coli	CMY-2	ST212	IncI1/ST65		(Vingopoulou et al. 2014)

1372 Bla = beta-lactamase, ST = sequence type, IS = insertion sequence, N.T = non typeable.

Figure Legends:

Figure 1. Geographical distribution of ESBLs and their correspondent animal hosts in the Mediterranean Basin. N.B: only SHV and TEM genes confirmed by sequencing as ESBL were included.

Figure 2. Geographical distribution of carbapenemases and mcr colistin resistance gene with their hosts in the Mediterranean. N.B: only OXA genes confirmed by sequencing as carbapenemases were included.



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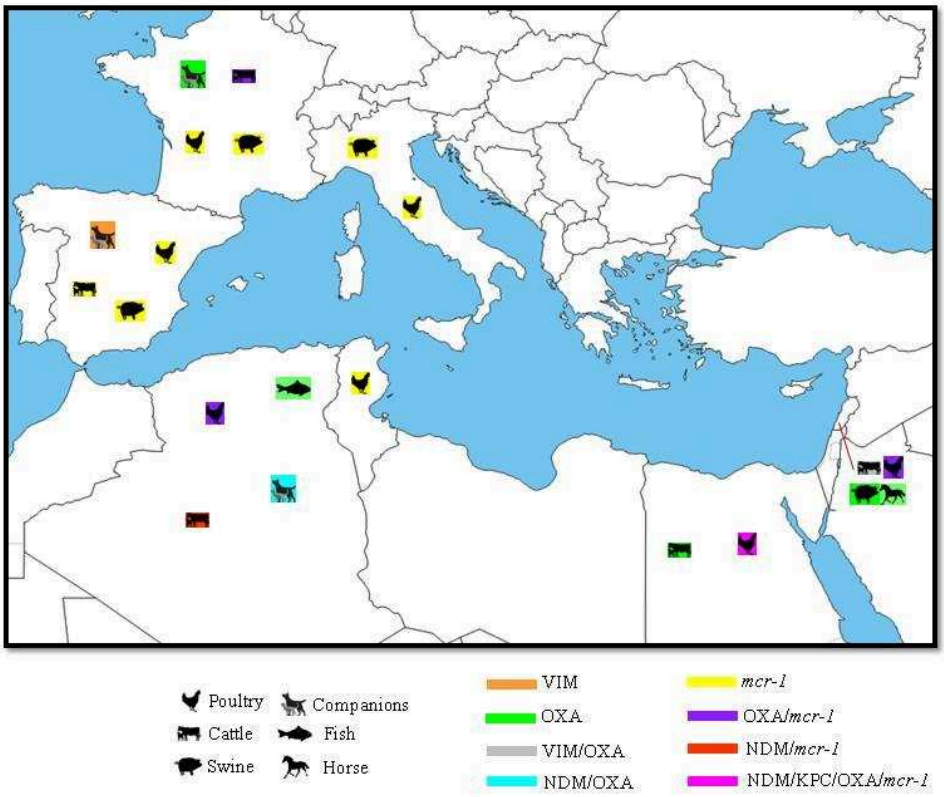

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Article 2

Colistin use in animals: a two side weapon against multi drug resistant organisms.

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Colistin Use in Animals: a Two-sided Weapon Against Multi-drug Resistant Organisms.

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Abstract

Colistin is widely used in animals for the treatment of infectious diseases but also for prophylaxis and as growth promoter. Colistin is over administered especially in poultry and pig production, to prevent E. coli and Salmonella infections, known to cause serious effects such as diarrhea and sepsis that cause huge economic losses. The excessive use of colistin, mainly in veterinary medicine, has led to the emergence of bacteria resistant to colistin that play an important role in the global emergence of resistance to this antibiotic. Colistin consumption should be monitored around the world, particularly in Africa and Asia, where there is no control over the level of consumption of colistin in animals added to the huge number of poultry and pigs breeding.

In November 2015, Liu et al have reported, for the first time in China, a new plasmid-mediated colistin resistance gene, namely mcr-1 gene that encodes a phospho-ethanolamine transferase. This resistance gene has been reported firstly in animals, then in human isolates and food from Enterobacteriaceae bacteria all over the world. The prevalence of mcr-1 gene in Gram-negative bacteria in food-producing animals, raises the question of the actual effectiveness of colistin administration in animals and their role in the transfer of colistin resistance in the public health. This review summarizes the potential impact of the use of colistin in veterinary medicine all over the world to eventually link this consumption to the prevalence of resistance to colistin, both in animals and humans. In addition, we discuss in this review the risk of the spread of bacteria resistant to colistin from farm animals and thus human food.

Introduction

The carbapenems and beta lactams have been widely used in the last decades to treat a variety of infectious disease (Olaitan et al., 2014a). Predominantly, to treat infections caused by Gram negative bacterial pathogens, such as P. aeruginosa, A. baumannii, K.pneumoniae, and E. coli (Olaitan et al., 2014a). However, simultaneously with this large use, resistance to a different class of antibiotics emerged among pathogens. The situation is further complicated by the reduced development of new antibiotics (Olaitan et al., 2014a). Unfortunately, only one new antibiotic (teixobactin) has been discovered in the last 30 years, compared to many antibiotics discovered in the 1940s to 1960s (Ling et al., 2015). Currently, the polymyxins are back in clinical practice, not because of an improved safety profile, but as antibiotics of last-line for the treatment of Gram-negative multidrug-resistant (MDR) causing bacterial infections (Biswas et al., 2012). Polymyxins including colistin and polymyxin B are polycationic antimicrobial peptides that are actually the last-resort antibiotic for the treatment of MDR, Gram-negative bacterial infections (Falagas et al., 2005). Colistin is a bactericidal which has an excellent activity against pathogens, such as A. baumannii, P. aeruginosa, K. pneumoniae, E. coli and Salmonella, including those currently resistant to antibiotics such as carbapenems (Falagas et al., 2005). However, colistin-resistant bacteria, which were initially sensitive to this drug, have emerged. Basically, Colistin has been used for the first time in the 1950s for the treatment of infections caused by Gram-negative bacteria (Justo and Bosso, 2015). In the 1970s, clinical use of polymyxin was significantly reduced due to nephrotoxicity concerns (Justo and Bosso, 2015). The return of polymyxin for antimicrobial therapy has been followed by the deficiency of new classes of antibiotics and the emergence of carbapenems resistance in Gram-negative bacteria (Olaitan et al., 2014a). Nowadays, increasing polymyxin resistance in clinical isolates is considered a serious problem due to the low number of currently effective antibiotics and the high consumption of colistin for the treatment of multidrug-resistant Gram negative bacteria not only in clinical treatment but also in animals (Al-Tawfiq et al., 2010; Kempf et al., 2016). The uncontrolled use of colistin in veterinary medicine has led to the worldwide emergence of colistin-resistant bacteria. Therefore, the World Health Organization (WHO) has recently included polymyxin as a critical antibiotic (Collignon et al., 2016). In a university hospital in Greece (Crete), authors have reported an increasing rate of infections caused by bacteria naturally resistant to polymyxin, namely Proteus, Providencia, Morganella and Serratia. Also, there have been reported resistance to polymyxin B bacteria which are normally susceptible to these drugs (Samonis et al., 2014).

Gram-negative bacteria harness various mechanisms to protect themselves from colistin in antibiotics, including a diversity of lipopolysaccharide (LPS) alterations, such as modifications of lipid A with phospho- ethanolamine and 4-amino-4-deoxy-L-arabinose. Many publications have summarized the mechanisms of resistance to polymyxin. These mechanisms underlying the polymyxin resistance have been well documented by Olaitan et al and Osei Sekyere et al (Olaitan et al., 2014b; Osei Sekyere et al., 2016).Colistin resistance is thought to be linked to lipopolysaccharide modification through changes in the mgrB gene and increased PhoP/PhoQ regulation (Baron et al., 2016). The worldwide prevalence of resistance to polymyxins is about 10% among Gram-negative bacteria and is highest in the Mediterranean countries and South east Asia (Al-Tawfiq et al., 2017). Colistin resistance has always been related to a chromosomal mechanism (Baron et al., 2016). The latest mechanism of polymyxin resistance has absolutely amended our view of colistin resistance as a worldwide problem. Recently, a Chinese team has demonstrated for the first time a novel new plasmid-mediated colistin resistance thought mcr-1 gene (Liu et al., 2016a), which was identified in Escherichia coli and Klebsiella pneumonia strains isolates from animals and humans. mcr-1 has an important implication because it can be acquired by pathogenic bacteria by horizontal transfer (Baron et al., 2016). To date, there are over 300 studies on mcr-1 mediated plasmid mediated colistin resistance worldwide.

In this review, we have focused on: (i) The worldwide spread of plasmid mediated colistin resistance in animals, (ii) MCR- variant (iii) Use of colistin in Veterinary Medicine and (iv) the risk of colistin resistance transmission from animals to humans.

The worldwide spread of plasmid mediated colistin resistance in animals

Prior to November 2015, extensive veterinary research demonstrated that different chromosomal mutations were often responsible for the development of colistin resistance. The first discovery in early November 2015 in China of a plasmid-mediated plasmid of colistin resistance encoding the mcr-1 gene of the enzyme phospho-ethanolamine transferase, was made mainly from E. coli strains of meat and pigs, during routine surveillance of food animals (Liu et al., 2016b).

Since the first detection of mcr-1 gene, there has been a great emergence of the presence of mcr-1 throughout the world. The mcr-1 gene has been found in human, animals and environmental isolates, in a number of countries (Schwarz and Johnson, 2016). Remarkably, the emergence of this plasmid in the animal world is more important.

Emergence of plasmid mediated colistin resistance in Asian countries

The plasmid mediated colistin resistance mcr-1 gene was first detected in china. Shortly after their discovery, an avalanche of epidemiological studies on mcr-1 in Chinese animals were conducted, focusing on the prevalence of the mcr-1 gene in different strain isolates, including E. coli, K. pneumoniae, Enterobacter cloacae and Salmonella strains (Bi et al., 2017; Cui et al., 2017; Kong et al., 2017; Lei et al., 2017; Lima Barbieri et al., 2017; Liu et al., 2017; Wang et al., 2017; Yang et al., 2017; Yi et al., 2017). This plasmid has been traced back to chicken isolates from the 1980s (Shen et al., 2016). In Malaysia, mcr-1 gene was first detected in E. coli from animals (chickens and pig) (Hu et al., 2016). In Vietnam, the mcr-1 gene was also detected in chicken and pig feces (Malhotra-Kumar et al., 2016; Nguyen et al., 2016). Recently, a study showed the zoonotic transmission of mcr-1 colistin resistance gene from small-scale poultry farms of Vietnam (Trung et al., 2017). Furthermore, the presence of mcr-1 genes in Laos was detected in E. coli isolates from humans and pig samples (Olaitan et al., 2016). In Lebanon, the first detection of mcr-1 colistin resistance gene occurred in 2015, where a mcr-1 E. coli strain harboring the TEM-135 like gene was isolated from chicken in southern Lebanon (Dandachi et al., 2018).

Likewise, mcr-1 from animals was detected in Cambodia (Stoesser et al., 2016), Japan (Kawanishi et al., 2017; Kusumoto et al., 2016) and Taiwan (Kuo et al., 2016; Lai et al., 2017)from E. coli and K. pneumoniae isolated from animals and human samples. Recently, Takahashi et al, found high prevalence of mcr-1, mcr-3 and mcr-5 in E. coli in diseased pigs in Japan (Fukuda et al., 2017). It has been observed that the highest percentage of the presence of mcr-1 in Asia is due to the uncontrollable use of colistin, especially in veterinary medicine.

Emergence of plasmid mediated colistin resistance in European countries

In Europe, the mcr-1 gene was detected in E. coli isolates from pigs, broilers, turkeys samples (Perrin-Guyomard et al., 2016) and veal calves (Haenni et al., 2016a, 2016b). Mcr-1 gene was identified in four Salmonella isolated from a sausage, a poultry feed and a boot swab from a broiler farm during a routine surveillance of the French agri- food sector (Webb et al., 2016).

The first appearance in Great Britain of E. coli carrying mcr-1 isolated from pigs dates from 2013 to 2015 (Duggett et al., 2016). Similarly, it was detected in two E. coli and one variant of Salmonella Typhimurium Copenhagen which were found to be MDR, including colistin,

with E. coli and Salmonella carrying the mcr-1 gene isolate from a pig (Anjum et al., 2016), as well as human excrement isolates and poultry meat samples (Doumith et al., 2016).
To date, plasmid has been detected in one E. coli isolate from a Danish patient with a bloodstream infection and in five E. coli isolates from imported chicken meat (Hasman et al., 2015a). As well, Spain is one of the European countries with the larger use of colistin in veterinary medicine (de Jong et al., 2013). This fact may correlate with the fact that Spain is the first country, in Southern Europe, that detected mcr-1 gene in nine strains from farm animals (poultry and swine) corresponding to five E. coli and four S. enterica (Quesada et al., 2016).In addition, Hernández et al. detected mcr-3 and mcr-1 colistin resistance genes in an E. coli isolate from cattle excrement in a Spanish slaughterhouse (Hernández et al., 2017). Mcr-2, another gene for colistin resistance mediated by a phospho-ethanolamine transferase plasmid was isolated from porcine and bovine E. coli in Belgium, with 76.7% nucleotide sequence homology to mcr-1 (Xavier et al., 2016a, 2016b). In Germany, E. coli plasmid-mediated colistin resistance occurs mainly in poultry production lines, while detection rates in cattle and pig isolates are considerably lower(Irrgang et al., 2016). In addition, it was detected in E. coli isolates from surrounding agricultural areas of three previously mcr-1-positive pig farms (Guenther et al., 2017).
Likewise, mcr-1 have also been reported in Italy (Cannatelli et al., 2016; Carnevali et al., 2016; Giufrè et al., 2016),Portugal (Campos et al., 2016; Figueiredo et al., 2016) and in Netherlands (Leverstein-van Hall et al., 2011; von Wintersdorff et al., 2016). Despite the wide number of European countries detecting mcr-1 in animal isolates, the spread of plasmid-mediated resistance in the European countries is considerably lower than in the Asian countries, especially in China.

Emergence of plasmid mediated colistin resistance in African countries

The first report of mcr-1 gene in Africa was detected in E. coli isolated from the Algerian chicken in 2015 (Olaitan et al., 2016) and in E. coli isolates from wild life in Bejaia (Bachiri et al., 2017). In Tunisia, Grami et al have reported a high prevalence of E. coli carrying mcr-1 in three chicken farms (Grami et al., 2016). Chickens were imported from France or derived from imported French chicks. The same IncHI2 plasmid has been reported to host these genes in cattle in France and in a dietary sample in Portugal (Tse and Yuen, 2016). This suggests a significant impact of food trade on the circulation of the mcr-1 gene(Grami et al., 2016).
This plasmid has also been reported in E. coli isolated from an animal in Egypt (Khalifa et al., 2016), a country with a high burden of infectious diseases and limited restrictions on

antimicrobial access. This plasmid has also been detected in E. coli isolates from human and chicken samples in South Africa (Coetzee et al., 2016; Perreten et al., 2016). It is now crucial to define the prevalence of the mcr-1 gene in poultry and other livestock in African countries in order to estimate the risk to human health.

Emergence of plasmid mediated colistin resistance in American countries

In Brazil, colistin-resistant E. coli isolates harboring mcr-1, and blaCTX-M or blaCMY-2 genes, were isolated from chicken meat. Moreover, it has also been demonstrated that most E. coli carried IncX4 plasmids already detected in human and animal isolates (do Monte et al., 2017). These results highlight a new reservoir of mcr-1 gene in South America (do Monte et al., 2017). In the United States, a colistin resistance gene carried by a transmissible plasmid was detected in two fecal samples of pigs carrying the mcr-1 gene (Meinersmann et al., 2017).

MCR- variants

Recently, other mcr variants, including mcr-2/3/3/4/5, have been added to the list of phospho-ethanolamine transferase genes causing colistin resistance in Enterobacteriaceae (Borowiak et al., 2017; Carattoli et al., 2017; Yin et al.). Three further plasmids mediated colistin resistance genes namely; mcr-3, mcr-4 and mcr-5, have been identified in Enterobacteriaceae, particularly from E. coli and Salmonella spp.

The mcr-2 gene that has 76,7% nucleotide sequence identity with mcr-1 gene was first reported in pigs and bovines in Belgium (Xavier et al., 2016b). The third mobile colistin resistance gene, mcr-3 (45% nucleotide identity with mcr-1) was reported in E. coli isolate from pigs in Malaysia (Yin et al.). Yin et al. also identified similar elements in a human K. pneumoniae isolate of Thailand and a human Salmonella enterica serovarTyphimurium isolate of the United States (Yin et al.). Subsequently, the coexistence of two plasmid-mediated colistin resistance genes, mcr-1 and mcr-3.2, was detected in the same strain isolated from cattle samples in Spain(Hernández et al., 2017). However, mcr-5 is different from mcr-1, mcr-2, mcr-3 and mcr-4, with only 34% to 36% amino acid sequence identity with the other proteins.

Use of colistin in veterinary medicine

Colistin (polymyxin E) is a cationic, multi-component, lipopeptide produced by Bacillus colistinus. It has been first isolated from the broth of Paenibacillus (Bacillus) polymyxa

(Falagas and Kasiakou, 2006). When first described in 1947, they were of great interest for their activity against pseudomonas aeruginosa. Colistin was introduced in the late 1950s because the bactericide was rapid and highly active against most species of Gram-negative bacteria, such as E. coli, salmonella and P. aeruginosa (Falagas et al., 2005). In the 1970s, colistin was replaced by new, more active and less toxic antimicrobial agents, such as aminoglycosides, quinolones and B-lactams, because they reported a higher frequency of neurotoxicity and nephrotoxicity (Poirel et al., 2017). In recent years, a recurrence of colistin use has been observed due to the emergence of infectious diseases caused by multi-resistant Gram-negative bacteria, particularly in human medicine. In veterinary medicine, colistin has been used regularly for decades for both curative treatment and disease prevention.

Over the last decade, colistin has been used in Europe for the treatment of intestinal infections caused by Enterobacteriaceae in pigs, poultry, cattle, sheep, goats and rabbits (Kempf et al., 2016). . It was also used in cattle, goats, sheep producing milk for human consumption and in laying hens (Catry et al., 2015), although in the UK, it has been recently used to treat infections in animals (Medicines Agency, 2016). In Brazilian livestock, colistin has been widely used in pigs, poultry and in animal feed as a growth promoter (Fernandes et al., 2016). The use of colistin potentially increases the selection pressure on bacteria to become resistant. Despite the significant potential consequences of colistin resistance, there has been no monitoring of global consumption of colistin in farm animals. In China, it has been used at over 8000 tones as a feed additive in animals (Walsh and Wu, 2016) and the annual use of colistin, ranging from 2470 to 2875 metric tons in food-producing animals in the past 5 years, might contribute to the rapid spread of mcr-1 (Shen et al., 2016). However, monitoring of data on the use of colistin in veterinary medicine in Africa remains limited. The wide distribution of the mcr-1 gene of plasmid colistin in animal isolates compared to human isolates, as well as the much greater use of colistin in animal compared to human, has been considered to suggest a flow from animals to humans.

The European Medicines Agency, which has reviewed the use of colistin in veterinary medicine in the EU and updated the use of colistin in animals, has recommended that these medicines should only be used as second-line treatment in animals and that their sales should be reduced and they should need drastic reductions in the use of colistin to meet their new recommendations (5 mg per population correction unit) (Medicines Agency, 2016). In the United Kingdom, the Veterinary Medicine Drug (VMD), in collaboration with other agencies, including Public Health England and the Food Standards Agency, assessed the relationship between the use of colistin in veterinary medicine and its implications for public

health. Following the detection of the mcr-1 gene, the Pig Veterinary Society re-categorized colistin in its prescribing principles for antimicrobials as an antibiotic of last resort, for which the use must be supported by laboratory sensitivity tests (VMD assesses the implications of colistin resistance in UK pigs., 2016)**.** Similarly, China's official Ministry of Agriculture has decided to ban the use of colistin as an additive in feed for animals (Walsh and Wu, 2016). They suggested that the use of colistin in medicine has probably accelerated the dissemination of mcr-1 in animals and, subsequently, in human beings.

Risk of transmission of colistin resistance from animals to humans

As mentioned earlier in this review, mcr colistin resistance gene is becoming prevalent in food producing animals worldwide. The spread of colistin resistance in animals is triggered by the concern to be transmitted to humans, where they can be causative agents of infections with limited therapeutic options when resistance to multiple drugs is encountered (Bettiol and Harbarth, 2015). The zoonotic transmission of bacteria can occur via direct/indirect contact or via consumption of under/uncooked animal products (Djeffal et al., 2017). Several studies have also highlighted the importance of the environmental routes in this transmission chain (Huijbers et al., 2014).

Unlike ESBL producers, the transfer of mcr-1 E. coli strains from animals to humans is not yet well established in the literature. The detection of mcr-1 in animals (Dandachi et al., 2018), environment (Yang et al., 2017; Zheng et al., 2016) but also in humans (Tada et al., 2017) is still new in several countries. However, studies on the possible transmission of positive strains of the mcr gene from animals to the general population are still rare. In their study, Olaitan et al revealed the transmission of a colistin resistant E. coli strain from a pig to its owner in Laos. This was demonstrated by both strains having the same sequence types and sharing the same virulence as well as same PFGE patterns (Olaitan et al., 2015). The transmission of mcr-1 was also suggested by Zhang et al when a mcr-1 E. coli strain was isolated from a patient with glomerulonephritis. The strain had ST354 and was clonally related four mcr-1 E. coli strains isolated from dogs in the pet shop where this patient was working (Zhang et al., 2016). More recently, mcr-1, mcr-2, mcr-3, mcr-4 and mcr-5 E. coli strains were isolated from vaginal swabs of women undertaking an infertility evaluation in China. Phylogenetic analysis of the isolated strains showed that the latter were identical or similar nucleotide sequences to those of animal origin in the same city, suggesting a possible transfer of mcr genes from animals to humans (Zhang et al., 2018). In Vietnam, mcr-1 fecal carriage in humans was significantly associated with exposure to mcr-1 positive chicken

(Trung et al., 2017). Other studies in the literature revealed no clonal relationship between mcr-1 in humans and those isolated from animals (Hasman et al., 2015b).

Conclusion

In summary, this study showed a spread of mcr colistin resistance genes in farmed and domestic animals worldwide. The spread of colistin resistance appears to be related to its overuse as therapeutic, prophylaxis and growth promoters. Although the zoonotic transmission of mcr positive strains is still not well documented and that the prevalence of these organisms in the clinical settings is still low compared to the one in animals, it is nevertheless possible that mcr isolates silently diffuse into the hospital settings without any notice. Indeed, in many countries, colistin resistance is monitored only when multi-drug resistant organisms are encountered. Therefore, the use of colistin in animals should be banned even in low prevalence countries, in order to preserve this antibiotic as a last resort therapeutic agent for infectious diseases caused by carbapenemase producing Gram-negative bacilli in the clinical settings.

Conflict of Interest Statement

No conflict of interest or financial disclosure for all authors.

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Authors' contributions

SC and ID wrote the review paper. JMR corrected the manuscript. All authors approved and revised the final version of the manuscript.

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645 **Table 1.** Distribution of mcr allele in animals versus antibiotic usage worldwide

Country	mcr allele	Animal Host	Species	Antibiotic used	Reference
			AFRICA		
Algeria	mcr-1	Poultry, Barbary macaques	E. coli	Colistin	(Olaitan et al. 2016) (Bachiri et al., 2017)
Egypt	mcr-1	Cow, poultry	E. coli	Colistin	(Khalifa et al. 2016) (Lima Barbieri et al. 2017)
Tunisia	mcr-1	Poultry	E. coli	-	(Grami et al. 2016)
South Africa	mcr-1	Poultry	E. coli	Colistin	(Perreten et al. 2016)
			EUROPE		
Belgium	mcr-1	Calves, swine, poultry	E. coli	Colistin, Banned in 2016 by MAPA	(Malhotra-Kumar et al. 2016a) (El Garch et al. 2017) (Monte et al. 2017)
	mcr-2	Calves, Swine	E. coli	-	(Xavier et al. 2016)
Great Britain	mcr-1	Swine, Poultry meat	E. coli, Salmonella spp	Colistin	(Duggett et al., 2016), (Doumith et al., 2016
Denmark	mcr-1	Poultry	E. coli	-	(Hasman et al., 2015)
Estonia	mcr-1	Swine	E. coli	-	(Brauer et al. 2016)
Italy	mcr-1	Swine, Reptiles Broilers	Salmonella spp, E. coli	Colistin with others.	(Carnevali et al. 2016) (El Garch et al. 2017) (Unger et al. 2017) (Nguyen et al. 2016)
Portugal	mcr-1	Swine	Salmonella spp	Colistin	(Campos et al. 2016)
Spain	mcr-1	Swine, turkey, cattle	E. coli, S. typhimurium, S. rissen	-	(de Jong et al., 2013) (Quesada et al., 2016) (Hernández et al., 2017).
Netherlands	mcr-1	Poultry	E.coli, K. pneumoniae	-	(Schrauwen et al. 2017)(Kluytmans-van den Bergh et al. 2016)
France	mcr-1	Swine, turkey, poultry cattle calves	E. coli, Salmonella spp	Colistin sulfonamides, tetracyclines	(Perrin-Guyomard et al. 2016) (Webb et al. 2016) (Brennan et al. 2016) (Haenni et al. 2016) (El Garch et al. 2017)

Germany	mcr-1	Swine, poultry, turkey, veal calves	Salmonella spp, E. coli	Colistin	(El Garch et al. 2017) (Falgenhauer et al. 2016) (Ewers et al. 2016a; Ewers et al. 2016b) (Pulss et al. 2017) (Roschanski et al. 2017) (Irrgang et al. 2016)
			ASIA		
Taiwan	mcr-1	Poultry, Swine	Salmonella spp	-	(Chiou et al. 2017)
Laos	mcr-1	Swine	E. coli	-	(Olaitan et al. 2016)
Malaysia	mcr-1	Poultry, Swine	E. coli	-	(Yu et al. 2016)
Japan	mcr-1	Retail chicken meat	E. coli	-	(Ohsaki et al. 2017)
Vietnam	mcr-1	Swine	E. coli	-	(Malhotra-Kumar et al. 2016b)
China	mcr-1	Swine, poultry, pets, Duck	E. coli, Cronobacter sakazakii, K. pneumoniae, Salmonella spp	Colistin florfenicol and olaquindox	(Shen et al. 2016) (Yi et al. 2017) (Li et al. 2017) (Liu et al. 2017a) (Kong et al. 2017) (Liu et al. 2017b) (Lima Barbieri et al. 2017) (Lei et al. 2017) (Wang et al. 2017) (Zhang et al. 2017) (Bai et al. 2016) (Li et al. 2016b) (Zhang et al. 2016) (Huang et al. 2017) (Yang et al. 2016) (Li et al. 2016a) (Liu et al. 2016)
			AMERICA		
Brazil	mcr-1	Poultry, swine	E. coli	Colistin Sulphate	(Fernandes et al. 2016)
Venezuela	mcr-1	Swine	E. coli	-	(Delgado-Blas et al. 2016)
United States	mcr-1	Swine	E. coli	-	(Meinersmann et al. 2017)

646 "-" = unknown

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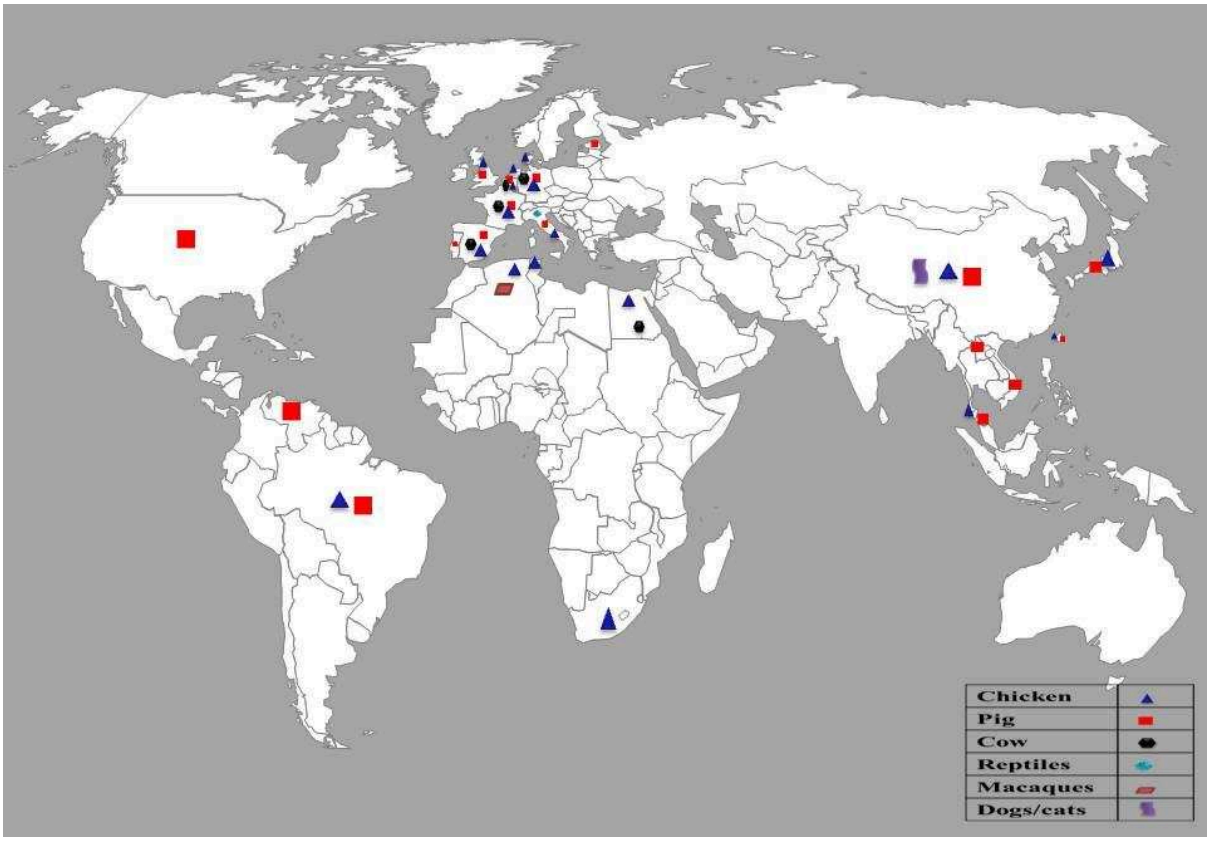
Figure Legends:

Figure 1. The Worldwide spread of plasmid mediated colistin resistance in animals.



691

692 **Figure 1.** The Worldwide spread of plasmid mediated colistin resistance in animals.

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Conclusion of Chapter I

In the Mediterranean region, ESBL, Ampc producers and to a lesser extent carbapenemase and mcr colistin resistant Gram-negative bacilli are highly prevalent in animals especially in chicken. The poultry production system is of particular importance since it can mediate the national as well as the global dissemination of multi-drug resistant organisms due to the frequent export/import of chicken between countries (1). The selection of beta lactamase producers appears to be mediated by the frequent use of non beta lactam antibiotics in the veterinary medicine. The control of antibiotic consumption is warranted in the Mediterranean region especially in Western Asia and North Africa were no accurate data are available neither at the level of the spread of multi-drug resistant organisms in animals nor at the level of antibiotic consumption.

Worldwide speaking, the continuous use of colistin in veterinary medicine appears to have promoted the dissemination of colistin resistant Gram-negative bacilli, notably the mcr mediated ones. The risk of transmission of resistant organisms from animals to humans is well documented for beta lactamase producers and to a lesser extent for colistin resistant isolates (2, 3). In view of the rapid dissemination of mcr-1 in Livestock and the rapid emergence of other plasmid mediated colistin resistance genes i.e. mcr-2, mcr-3, mcr-4 and mcr-5(4), the real efficacy of colistin use in food producing animals becomes questionable. A re-evaluation of colistin as well as non beta lactam prescription in livestock is therefore warranted especially in the Mediterranean area. Furthermore, the risk factors associated with the acquisition of colistin resistance from animals, in addition to its persistence in the human gut without colistin selective pressure should be also explored.

During our reviews, we found that Lebanon is one of the countries where little is known about the level of antibiotic consumption in animals as well as the level of multi-drug resistant organisms dissemination in the animal sector; hence the aim of the second chapter of this manuscript.

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Chapter II

Epidemiology of Multi-Drug Resistant organisms in Chicken, pigs and environment in Lebanon.

Introduction

Nowadays, the epidemiology of multi-drug resistant organisms has changed and is no more confined to the hospital settings (1). Studies have shown that the food producing animals are potent contributors to the dissemination of bacterial resistance (2). In livestock, resistant organisms can be transferred to humans via direct contact or indirectly via the consumption of under/uncooked animals products (3). Environmental routes play also a key role in the dissemination of multi-drug resistant organisms (4). These latter include air dust, fertilized soil with animal manures and contaminated wastewaters (5). In Lebanon, little is known about the prevalence of ESBL/ampC producers as well as colistin resistant Gram-negative bacilli in food producing animals and the surrounding environment.

Article 3 entitled **"Prevalence and characterization of multi-drug-resistant gram-negative bacilli isolated from Lebanese poultry: A nationwide study",** 981 fecal swab samples were collected from chicken farms distributed over the seven districts of Lebanon. The swabs were subcultured on a macconkey agar supplemented with cefotaxime for the screening of beta-lactamase producers. Double disk synergy test, ampC disk test were conducted for the phenotypic detection of ESBL and ampC producing Gram-negative bacilli. RT-PCR and standard PCR amplification were used for the molecular screening of ESBL and ampC beta lactamase genes, respectively. MLST typing of randomly chosen isolated multi-drug resistant E. coli strains, in addition to the MSP dendrogram for all isolated E. coli strains were performed in order to explore the relationship of isolated strains from all districts. The nationwide prevalence of ESBL/ampC producing Gram-negative bacilli in poultry was 20.7%. The main genes detected weer CMY, TEM and CTX-M beta lactamases. ESBL/ampC producing Gram-negative bacilli cross resistant to antibiotics commonly prescribed in the human medicine are highly prevalent over the Lebanese territory; in that more than 72% of isolated strains were co-resistant to at least two non-beta lactam antibiotics with gentamicin and trimethoprim-sulfamethoxazole being the most common.

Article 4 entitled "**First detection of mcr-1 plasmid mediated colistin resistant E. coli in Lebanese poultry"** describes the first detection of an mcr-1 positive E. coli strain from chicken in the south of Lebanon in 2015. The strain was an ESBL producer harboring the TEM-135 like gene.

In **Article 5** entitled **"Prevalence of multi drug resistance and colistin resistant Gram negative bacilli In Lebanese swine farms",** 114 fecal samples were collected from the main swine farms located in Lebanon. Three separate selective media supplemented with cefotaxime, ertapenem and colistin were used for the presumptive detection of ESBL/ampC,

carbapenemase producers as well as colistin resistant Gram-negative bacilli. RT-PCR was used for the screening of $bla_{SHV,}$ bla_{TEM} and bla_{CTX-M} genes. Standard PCR amplification and sequencing was done for the molecular detection of mcr colistin resistance genes. Furthermore, simplex PCRs were conducted for the detection of FOX, MOX, ACC, EBC, DHA and CMY ampC beta lactamase genes. Sixty seven percent of collected fecal samples were positive for an ESBL/ampC isolate. CTX-M and TEM were the most abundant beta lactamase genes detected. Furthermore, we report in this study the emergence of mcr-1 E. coli strains in Lebanese swine farms.

Article 6 entitled "**Dissemination of multi-drug resistant and mcr-1 Gram-negative bacilli in Broilers, farm workers and the surrounding environment in Lebanon"**, in this study we returned back to the same chicken farm where the first mcr-1 E. coli strain was isolated in 2015 from the south of Lebanon. Chicken fecal swabs, feed, litter and soil samples as well as fecal samples from the farm's workers were collected and screened for ESBL, ampC, carbapenemase producers and colistin resistant Gram-negative bacilli. Phenotypic tests including double disk synergy test, ampC disk test and carbe NP test were used for presumptive detection of ESBL, ampC and carbapenemase producers. RT-PCR was done for the screening of ESBL and mcr colistin resistance genes. The prevalence obtained in 2017 of ESBL/ampC producers as well as the one of resistance genes was compared to the prevalence of ESBL/ampC producing Gram-negative bacilli found in 2015. MSP dendrogram and MLST analysis of isolated strains in 2015 and 2017 were performed in order to explore the nature of multi-drug resistant organisms' evolution over the two years in this same farm in the south of Lebanon. Furthermore, in this farm, the types antibiotics as well as the cause of their administration was recorded via personal communication with the veterinarian of the farm. Conjugation experiments assessing the validity of the selective and co-selective pressure hypothesis of colistin and non beta-lactams use mediating the dissemination of multi-drug resistance in the chicken farm were also performed. Compared to 2015, the prevalence of ESBL/ampC production has significantly increased from 27% in 2015 to 59% in 2017. The rise was also observed at the level of CTX-M and TEM genes. On the other hand, mcr-1 positive strains were isolated from chicken, feed, litter and all workers' samples. MSP dendrogram and MLST analysis showed that the strains are multi-clonal.

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Article 3

Prevalence and characterization of multi-drug-resistant Gram-negative bacilli isolated from Lebanese poultry: A nationwide study.

Iman Dandachi, Elie S.Sokhn, Elias A.Dahdouh, Eid Azar, Bassel El-Bazzal, Jean-Marc Rolain, Ziad Daoud

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Prevalence and Characterization of Multi-Drug-Resistant Gram-Negative Bacilli Isolated From Lebanese Poultry: A Nationwide Study

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Currently, antimicrobial resistance is one of the most prominent public health issues. In fact, there is increasing evidence that animals constitute a reservoir of antimicrobial resistance. In collaboration with the Lebanese Ministry of Agriculture, the aim of this study was to determine the prevalence of intestinal carriage of multi-drug-resistant Gram-negative Bacilli in poultry farms at the national level. Between August and December 2015, 981 fecal swabs were obtained from 49 poultry farms distributed across Lebanon. The swabs were subcultured on MacConkey agar supplemented with cefotaxime (2 μg/ml). Isolated strains were identified using MALDI-TOF mass spectrometry. Multilocus sequence typing analysis was performed for *Escherichia coli*. Phenotypic detection of extended spectrum β-lactamases (ESBL) and AmpC production was performed using double disk synergy and the ampC disk test, respectively. β-lactamase encoding genes bla$_{CTX-M}$, bla$_{TEM}$, bla$_{SHV}$, bla$_{FOX}$, bla$_{MOX}$, bla$_{EBC}$, bla$_{ACC}$, bla$_{DHA}$, and bla$_{CMY}$ using PCR amplification. Out of 981 fecal swabs obtained, 203 (20.6%) showed bacterial growth on the selective medium. Of the 235 strains isolated, 217 were identified as *E. coli* (92%), eight as *Klebsiella pneumoniae* (3%), three as *Proteus mirabilis* (1%) and three as *Enterobacter cloacae* (1%). MLST analysis of *E. coli* isolates showed the presence of ST156, ST5470, ST354, ST155, and ST3224. The phenotypic tests revealed that 43.5, 28.5, and 20.5% of the strains were ampC, ESBL, and ampC/ESBL producers, respectively. The putative TEM gene was detected in 83% of the isolates, SHV in 20%, CTX-M in 53% and CMY ampC β-lactamase gene in 65%. Our study showed that chicken farms in Lebanon are reservoirs of ESBL and AmpC producing Gram-negative bacilli. The level of antibiotic consumption in the Lebanese veterinary medicine should be evaluated. Future studies should focus on the risk factors associated with the acquisition of multi-drug-resistant organisms in farm animals in Lebanon.

Keywords: ampC, ESBL, *E. coli*, poultry, carriage

INTRODUCTION

Antibiotic resistance is currently a major topic of interest for researchers and physicians. In particular, the rise of multi-drug resistance in Gram-negative bacteria is now a serious challenge encountered by healthcare professionals (Exner et al., 2017). Resistance in Gram-negative bacteria is mainly mediated via the production of extended spectrum β-lactamases (ESBL), ampC β-lactamases and carbapenemases (Schill et al., 2017). Genes encoding these enzymes are often located on plasmids carrying resistance genes to other commonly used antibiotics in clinical settings (Seiffert et al., 2013). Infections with these multi-drug-resistant organisms (MDROs) will thus pose therapeutic challenges; the antibiotic pipeline is drying up, and no new antimicrobial agents are anticipated in the near future to treat infections caused by MDROs (Bettiol and Harbarth, 2015).

In fact, it has been generally accepted that the main driver for the rapid evolution of bacterial resistance is the uncontrolled usage of antibiotics in human medicine. It is suggested that this theory is also applicable to the veterinary sector (Kempf et al., 2015). The European Centre for Disease Prevention and Control/European Food Safety Authority/European Medicines Agency (ECDC/EFSA/EMA) joint report stated that in 2014, the average antibiotic consumption in animals (152 mg/kg) was higher than in humans (124 mg/kg). Univariate analysis showed a signification correlation between fluoroquinolone consumption and resistance in *Escherichia coli* in the human and animal sectors, between polymyxins and tetracyclines and *E. coli* in animals, and for 3rd/4th generation cephalosporins and *E. coli* in humans (ECDC/EFSA/EMA, 2017). Antibiotics are heavily administered for therapeutic and prophylaxis purposes in veterinary medicine. As growth promoters, this practice is no longer adapted in the European Union, whereas it persists in North America and other countries (Economou and Gousia, 2015). In their study, Chantziaras et al. (2014) found a significant correlation between the use of antibiotics in livestock and the corresponding level of resistance toward these antimicrobials in *E. coli* strains isolated from pigs, poultry and cattle. During the last years, the prevalence of ESBLs, ampC, and carbapenemase producing Gram-negative bacteria has become extensively reported in food producing animals (Ghodousi et al., 2015; Gonzalez-Torralba et al., 2016; Haenni et al., 2016). In their review paper, Schwarz et al. (2016) showed that studies describing the epidemiology of resistant organisms in livestock targeted mainly swine, cattle and poultry. The prevalence of resistance varied from one country to another (Alonso et al., 2017). Although the extent to which food of animal origin contributes to the zoonotic transmission of multi-drug-resistant organisms, i.e., ESBL and carbapenemase producers, has not yet been well established (Madec et al., 2017), it suggests that sharing the same ESBL genes, plasmids and strains constitutes possible evidence of zoonotic transmission of MDROs from animals to humans (Leverstein-van Hall et al., 2011; Dahms et al., 2014). Furthermore, the increased risk of ESBL fecal carriage in individuals with a high degree of contact with broiler chickens is an indicator of transmission (Huijbers et al., 2014). Enteric-resistant strains in livestock can be easily transferred to humans through direct contact or through the handling/consumption of undercooked/uncooked animal products (Dahms et al., 2014).

In Lebanon, several studies addressing MDROs in hospital settings have been conducted. One study done at the American University of Beirut Medical Center between 2008 and 2011 reported that 1.07 and 2.45% of *E. coli* and *Klebsiella pneumoniae* clinical isolates, respectively, were ESBL producers and ertapenem-resistant (Baroud et al., 2013). Another study conducted in the north reported that over the period of 2009–2012, 9% and 28% of the bacteraemia episodes in febrile neutropenic patients were caused by carbapenem and third-generation cephalosporin-resistant Gram-negative bacilli, respectively (Moghnieh et al., 2015). However, very few studies have addressed this issue in the environment. One study showed that *Acinetobacter baumannii* was detected in 6.9% of water samples, 2.7% of milk samples, 8.0% of meat samples, 14.3% of cheese samples and 7.7% of animal samples (Rafei et al., 2015). Another study in which 115 stool samples were collected from livestock animals from different farms in north Lebanon reported the detection of four VIM-2 producing *Pseudomonas aeruginosa*, four OXA-23 producing *A. baumannii* and one OXA-23/OXA-58 co-producing *A. baumannii* (Al Bayssari et al., 2015a). Furthermore, Al Bayssari et al. (2015b) reported the isolation of an OXA-48 harboring *E. coli* isolate from fowl in Lebanon. More recently, Diab et al. (2016) detected a relatively high prevalence of CTX-M-15 producing *E. coli* in Lebanese cattle. In the above-mentioned studies in Lebanese livestock, MLST analysis revealed the presence of sequence types common to both humans and animals (Al Bayssari et al., 2015a; Rafei et al., 2015; Diab et al., 2016), which suggests that Lebanese farms are potent reservoirs of multi-drug-resistant organisms that could be transmitted to humans. In the present study and in collaboration with the Lebanese Ministry of Agriculture, our aim was to determine the national epidemiology of multi-drug-resistant Gram-negative bacilli in Lebanese chicken farms in terms of intestinal carriage.

MATERIALS AND METHODS

Ethics Statement

The Ministry of Agriculture in Lebanon granted approval to collect chicken samples from representative farms in the country as per the national norms for animal sampling and manipulation. This sampling was in conformity with the international regulations for animal safety. All of the involved farms officially received authorization from the Ministry of Agriculture, and this was considered, after undergoing an acceptance process, an official and legal document. Therefore, an Institutional Review Board (IRB) approval was obtained for the present study.

Samples Collection

Between August and December 2015, 981 rectal swabs were collected from 49 poultry farms distributed over the seven

districts of Lebanon. Six to seven farms were visited in each district. The average number of samples taken from each farm was 20 fecal swabs (**Table 1**). The 20 samples collected were randomly taken from each farm. Technical assistance, i.e., fecal swabs, gloves, costumes, and a portable refrigerator, were provided by the Ministry of Agriculture team. The collected swabs were directly placed in a portable refrigerator, and when they arrived at the University Laboratory, they were stored at −80°C until use. The farms visited were selected by considering their geographical location, presence or absence of a nearby community and the size of the farms (at least 3,000 chickens per breeding site). Eighty percent of the samples were gathered from broiler chickens, while 20% were taken from layers. The mean average age of the broilers and layers was 31 days and 14 months, respectively.

MALDI-TOF MS Identification

Rectal swabs were sub-cultured on a MacConkey agar supplemented with 2 μg/ml of cefotaxime for the preliminary screening of antibiotic-resistant Gram-negative bacilli. After overnight incubation at 37°C, colonies showing different morphologies were picked up from each selective plate and tested separately with MALDI-TOF MS for identification using the Microflex LT spectrometer (Bruker Daltonics, Bremen, Germany) (Seng et al., 2010; Singhal et al., 2015). The spectra obtained for each strain were stored and downloaded into a MALDI Biotyper 3.0 system to create a single main spectrum for each bacterial isolate. Thereafter, a dendrogram was constructed using MALDI Biotyper 3.0 software.

Antibiotic Susceptibility Testing

Using the Kirby–Bauer disk diffusion method, antibiotic susceptibility testing was performed. The results were interpreted according to EUCAST guidelines 2017 (European Committee on Antimicrobial Susceptibility Testing, 2017). Sixteen antimicrobial agents were used including ampicillin, aztreonam, cefotaxime, ceftazidime, cefoxitin, cefepime, amoxicillin-clavulanic acid, piperacillin-tazobactam, meropenem, imipenem, ertapenem, colistin, tigecycline, ciprofloxacin, gentamicin and trimethoprim-sulfamethoxazole (Bio-Rad, Marnes-la-Coquette, France). Phenotypic detection of ESBL was performed using the double-disk synergy test by placing an amoxicillin–clavulanic acid disk in the center between aztreonam, cefepime and ceftazidime. The observation of a "key hole effect" was considered a positive test. On the other hand, ampC β-lactamase detection was performed using the ampC disk test (Black et al., 2005). In brief, a lawn of cefoxitin-susceptible *E. coli* ATCC 25922 was inoculated on the surface of a Mueller Hinton agar plate. A 30-μg cefoxitin disk was placed on the inoculated surface. A sterile filter paper disk was moistened by adding 20 μl of a 1:1 mixture of saline and 100 × Tris-EDTA (catalog code T-9285; Sigma-Aldrich Corporation, St. Louis, MO, United States). Several colonies of the test isolate were then applied to the disk. The disk was then positioned with its inoculated face in contact with the agar surface. After overnight incubation, a flattening or indentation of the zone of inhibition around the cefoxitin disk was considered a positive result, while an absence of distortion was considered a negative one. Furthermore, for the presumptive detection of carbapenemases, the carba NP test was performed as previously described (Bakour et al., 2015). A bacterium was characterized as being multi-drug-resistant when resistance to at least three classes of antibiotics was observed (Magiorakos et al., 2012).

Molecular Characterization of β-Lactamase Encoding Genes

All of the isolates that showed a key hole effect or had cefoxitin resistance with non-susceptibility to cefepime were subjected to real-time PCR analysis for the detection of SHV, TEM and CTX-M encoding genes (Roschanski et al., 2014). Simplex PCRs for the genes encoding AmpC β-lactamases FOX, MOX, ACC, EBC, DHA, and CMY were conducted for all strains showing non-susceptibility to cefoxitin (Dallenne et al., 2010). Simplex PCR was also used to test the ADC ampC β-lactamase gene in *A. ba*umannii (Liu and Liu, 2015). DNA extraction was performed according to the manufacturer's instructions using EZ1 DNA extraction kits (Qiagen, Courtaboeuf, France) with the EZ1 Advanced XL biorobot.

Multilocus Sequence Typing

One *E. coli* strain from each cluster shown in the MSP dendrogram was chosen, and MLST typing was performed based on allelic profiles to determine their evolutionary relationship (Peng and Zong, 2011). Seven housekeeping genes were used: *adk, fumC, gyrB, icd, mdh, purA,* and *recA*. Analysis of the genes' allelic profiles was performed on the MLST[1] to determine the sequence type (ST) to which each isolate belongs.

Statistical Analysis

The prevalence, identification, and resistance profiles of isolated strains are all presented as the number (percentage).

RESULTS

Bacterial Identification

Out of 982 collected fecal swabs, 203 (20.6%) showed growth on selective medium. In total, 235 strains were isolated. All 235 isolated Gram-negative bacilli were identified by MALDI TOF mass spectrometry with a score value ≥1.9. The distribution at the species level was as follows: 217 were identified as *E. coli* (92%), eight as *K. pneumoniae* (3%), three as *Proteus mirabilis* (1%), three as *Enterobacter cloacae* (1%), two as *E. albertii*, one as *E. fergusonii* and one as *A. baumannii*. The MSP dendrogram of the 217 *E. coli* isolates revealed five clusters at a distance level of 500 (arbitrarily selected) (**Figure 1**). Cluster 1 was mainly formed by isolates from the Akkar District. Cluster 2 contained two isolates: one from Saida and the other from Baalbek. Cluster 3 was composed of three strains isolated from Jabal Lebnen District. Cluster 4 was mainly composed of isolates from the North Lebanon district, and Cluster 5 contained only one strain from Saida.

[1]http://mlst.warwick.ac.uk/mlst/dbs/Ecoli

TABLE 1 | Distribution of MDROs per farm and district.

		Collection date	Farm size	Age	Type	# of collected samples	# of positive samples	# of isolated strains
	Fl		18000	35 d	B	27	11	11
	F2		11300	35 d	B	27	5	6
	F3		20000	45 d	B	27	2	2
North Leb	F4	27-Aug	23000	4 m	L	20	9	18
	F5		4000	35 d	B	20	14	23
	F6		20000	25 d	B	20	13	14
	F7		15000	35 d	B	20	8	9
	F8		5000	25 d	B	20	5	5
Akkar	F9	31-Aug	4000	25 d	B	20	5	5
	F10		6000	25 d	B	20	9	11
	F11		4600	4 m	L	20	11	14
	F12		15000	40 d	B	20	11	14
	F13		6000	45 d	B	20	1	1
	F14		10700	36 d	B	20	4	4
Bekaa	F15	15-Sep	5000	45 d	B	20	6	7
	F16		3000	18 m	L	20	3	3
	F17		6000	36 d	B	20	1	1
	F18		6000	43 d	B	20	6	7
	F19		6000	43 d	B	20	3	3
Baalbek	F20	21-Sep	5000	14 m	L	20	3	3
	F21		6500	27 d	B	20	3	3
	F22		6700	12 m	L	21	1	1
	F23		11800	26 d	B	20	4	4
Nabatieh	F24	21-Oct	10000	27 d	B	20	2	2
	F25		10000	25 d	B	20	1	1
	F26		5000	25 d	B	20	1	1
	F27		10000	27 d	B	20	8	8
	F28		5000	28 d	B	20	4	4
Jabal Leb	F29	9-Nov	5000	25 d	B	20	7	6
	F30		10000	27 d	B	20	2	2
	F31		10000	28 d	B	20	4	5
	F32		18000	25 d	B	20	5	5
	F33		6000	25 d	B	20	3	3
	F34		6000	25 d	B	20	6	6
Saida	F35	7-Dec	3300	32 d	B	20	10	10
	F36		10000	25 d	B	20	5	6
	F37		10000	30 d	B	20	1	1
	F38		10000	28 d	B	20	6	6

F, farm; Aug, August; Sept, September; Oct, October; Nov, November; Dec, December; d, days; m, month; B, broiler; L, layer.

Phenotypic Profiles of Resistance

The disk diffusion susceptibility testing results are summarized in **Table 2**. All of the isolates were susceptible to tigecycline, colistin and carbapenems. Phenotypic identification using the double disk synergy test, ampC disk test and carba NP test revealed that 102 (43.5%) of the isolated strains were ampC β-lactamase producers, 67 (28.5%) were ESBL producers, and 48 (20.5%) were co-producers of ESBL and ampC β-lactamases. Both ESBL and ESBL/ampC production were detected in *E. coli*, *K. pneumoniae, E. fergusonii,* and *E. cloacae* (**Table 2**), whereas only AmpC production was detected in *E. coli, K. pneumoniae, P. mirabilis, E. albertii,* and *A. baumannii*. In addition, 18 *E. coli* strains (7.5%) did not show a key hole effect and were resistant to cefoxitin but tested negative with the ampC disk test. Moreover, 32% of the isolated strains were co-resistant to gentamicin, ciprofloxacin, and trimethoprim-sulfamethoxazole, whereas 40% were resistant to at least two non-β-lactam antibiotics, 19.5% were resistant to only one non-β-lactam, and 8% were susceptible to all of the non-β-lactam antibiotics tested.

Prevalence of MDR-GNB

The distribution of samples showing positive growth on the selective medium was as follows: 54 samples in the North District, 38 in the Akkar District, 37 in Saida, 26 in Bekaa, 24 in Jabal Lebnen, 16 in Baalbek and eight in Nabatieh.

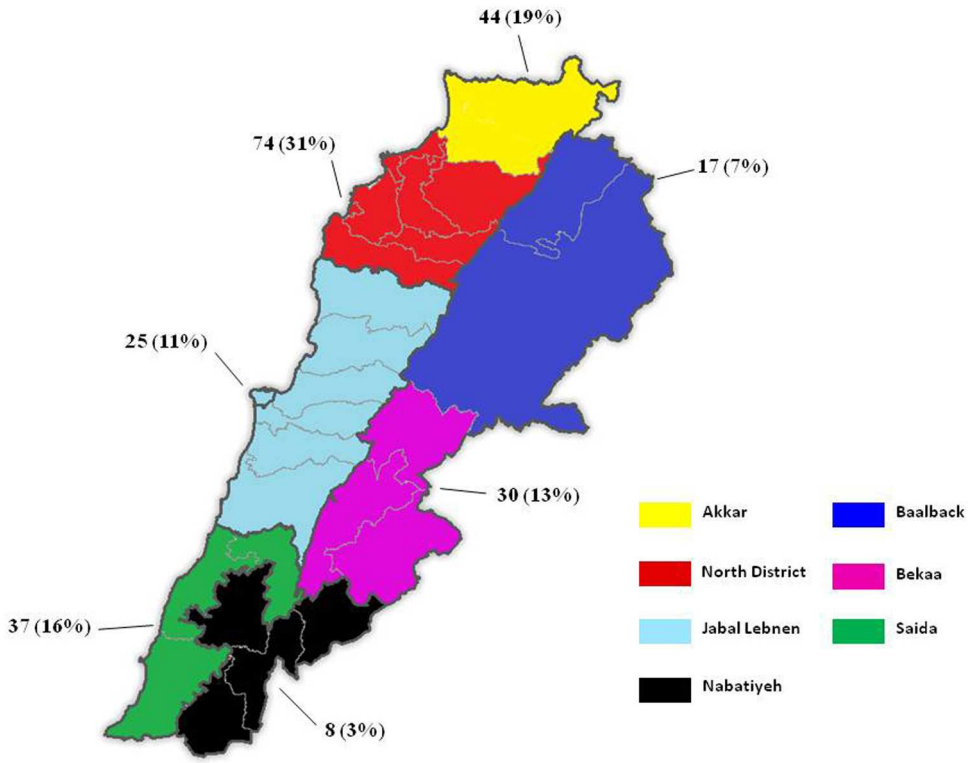

FIGURE 1 | MSP dendrogram of *Escherichia coli* isolates.

The number of positive samples from broilers exceeded the one obtained from Layers (176 vs. 27, respectively). Isolated strains (235) originated from 38 out of the 49 visited farms, i.e., 77.5% of the farms were positive for at least one multi-drug-resistant Gram-negative bacilli. As shown in **Figure 2**, the highest prevalence was detected in the north-west of the country, with 74 and 44 isolated strains for the North and Akkar Districts, respectively, whereas the lowest prevalence was detected in the north–east and south–east of Lebanon.

PCR Screening of CTX-M, SHV, TEM, and AmpC β-Lactamase Genes

One hundred and twelve isolates suspected to be ESBL producers were subjected to a real-time PCR assay for the detection of SHV, TEM, and CTX-M encoding genes. Of the 112 strains selected, 93 (83%) harbored the TEM gene, 59 (53%) the CTX-M gene and 22 (20%) the SHV gene. Overall, 49% (55) of the ESBL suspected isolates harbored only one gene, 46% (52) harbored at least two genes with the highest concordance being between the TEM and CTX-M genes, and 4% (five) showed the co-existence of all three genes together (**Table 3**). In parallel, 152 strains including 4 *K. pneumoniae*, 3 *P. mirabilis*, 2 *E. albertii,* and 143 *E. coli* were positive for bla_{CMY}; whereas fifteen *E. coli* strains were negative fall ampC β-lactamase genes tested. Furthermore, in *A. baumannii* the ADC gene was detected.

MLST Typing

The MLST typing of the strains, each chosen from the major district-related isolates grouped in each cluster, revealed that they belong to five different STs: ST156 for Cluster 1, ST5470 for Cluster 2, ST354 for Cluster 3, ST155 for Cluster 4 and ST3224 for Cluster 5.

DISCUSSION

Many years ago, hospitals and health care settings were regarded as the sole source of antimicrobial resistance. However, recent evidence has shown that food producing animals constitute a potent reservoir of multi-drug-resistant organisms (Belmahdi et al., 2016; Bachiri et al., 2017). This was mainly linked to the over-use of antimicrobial agents in veterinary medicine for treatment, growth promotion and prophylaxis (Economou and Gousia, 2015). Although the zoonotic transmission of multi-drug-resistant organisms from animals to humans remains controversial (Olsen et al., 2014), several studies have shown a direct link between direct contact with farm animals and the acquisition of bacterial resistance (Huijbers et al., 2014). One study conducted by Olaitan et al. (2015) demonstrated the zoonotic transmission of a colistin-resistant *E. coli* strain from a pig to its owner. This owner usually fed his pig without wearing any protective equipment. The two colistin-resistant isolates (in the pig and its owner) belonged to the same sequence type and

TABLE 2 | Resistance profiles and phenotypes of multi-drug-resistant organisms isolated in this study.

Species	AMP	AZT	CTX	CAZ	FOX	FEP	AMC	TZP	SXT	CIP	GENT	% of ESBL producers	% of AmpC producers	% of ESBL/AmpC co-producers
Escherichia coli (*n* = 217)	217 (100)	49 (23)	195 (90)	120 (55)	104 (48)	31 (14)	77 (35)	28 (13)	150 (59)	134 (62)	152 (70)	27	44	21
Klebsiella pneumonia (*n* = 8)	8 (100)	2 (25)	8 (100)	3 (38)	2 (25)	2 (25)	2 (25)	2 (25)	6 (75)	7 (88)	7 (88)	50	37.5	12.5
Proteus mirabilis (*n* = 3)	3 (100)	0 (0)	2 (67)	0 (0)	3 (100)	0 (0)	3 (100)	0 (0)	3 (100)	3 (100)	1 (33)		100	
Enterobacter cloacae (*n* = 3)	3 (100)	1 (33)	3 (100)	2 (67)	0 (0)	0 (0)	0 (0)	0 (0)	1 (33)	1 (33)	3 (100)	100		
Escherichia albertii (*n* = 2)	2 (100)	0 (0)	1 (50)	1 (50)	2 (100)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)		100	
Escherichia fergusonii (*n* = I)	1 (100)	0 (0)	1 (100)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (100)	0 (0)	100		
Acinetobacter baumannii (*n* = I)	1 (100)	0 (0)	1 (100)	1 (100)	1 (100)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)		100	

Resistance profiles are presented as a number (percentage). N, number; %, percentage; AMP, ampicillin; AZT, aztreonam; CTX, cefotaxime; CAZ, ceftazidime; FOX, cefoxitin; FEP, cefepime; AMC, amoxicillin-clavulanic acid; TZP, piperacillin-tazobactam; SXT, trimethoprim-sulfamethoxazole; CIP, ciprofloxacin; GENT, gentamicin.

presented with the same virulence and PFGE pattern (Olaitan et al., 2015).

In Lebanon, very few studies have looked at the prevalence of MDROs in farm animals (Al Bayssari et al., 2015a). Our study is the first epidemiological study in Lebanon quantifying the prevalence of multi-drug-resistant Gram-negative bacilli in chicken farms in terms of intestinal carriage at the national level. The prevalence is similar to the one previously reported from cattle (84%) in Lebanon (Diab et al., 2016). The flock's size did not influence the prevalence of resistance in each farm (**Table 1**). On a global level, the prevalence found in our study is approximate to the one reported in Romania (69%) (Maciuca et al., 2015) and Ecuador (60%) (Ortega-Paredes et al., 2016) but is higher than the ones described in Germany (44%) (Kola et al., 2012), Japan (23%) (Kawamura et al., 2014), and Vietnam (3.2%) (Nguyen et al., 2015). Differences in the screening methodologies, sample size used and the level of antibiotic consumption in each country could explain these variations (Rhouma et al., 2016).

Escherichia coli was the most common multi-drug-resistant organism isolated; MALDI-TOF MSP dendrogram and MLST analysis revealed the presence of five clusters from which the representative strains belonged to different STs. Within each cluster, strains isolated from farms of the same district were grouped together; this is especially true for the Akkar and North Lebanon strains. This observation reveals that strains of the same region are closely related. Although PFGE is the standard method for the detection of clones, due to the large number of strains isolated in this study, PFGE typing was not performed; rather, we referred to the MSP dendrogram as a possible rapid tool for strain differentiation according to their geographical and/or phenotypic distribution in epidemiological studies as certain previous studies have suggested (Berrazeg et al., 2013; Khennouchi et al., 2015). With the exception of ST155, none of the sequence types identified in this study were among those frequently reported in chicken such as ST10, ST23, ST48, ST58, ST115, ST117, ST350, and ST648 (Olsen et al., 2014). However, looking at the Warwick *E. coli* MLST database, we found that the STs detected in our study were previously reported from livestock, cats and dogs, and humans. ST155 has been commonly reported in poultry (Pires-dos-Santos et al., 2013), and it appears to be associated with a zoonotic risk, which has been suggested by some studies (Lazarus et al., 2015). This emphasizes the hypothesis that MDROs in food-producing animals can be transmitted to humans and may be causative agents of infections with therapeutic challenges when high resistance is encountered. It should also be mentioned that clones in animals and humans are not always shared; some studies have shown that *E. coli* strains in food-producing animals differ from those reported in humans (Randall et al., 2012; Wu et al., 2013). This suggests that only some bacterial clones might be transmitted to the human population.

As our study showed, ESBL producers dominate the Lebanese poultry sector. The prevalence of ampC producers is also elevated (43.5%). ESBL and ampC-producing Gram-negative bacilli were previously reported in clinical and community settings in Lebanon (Dandachi et al., 2016). Molecular characterization revealed that 50% of isolated strains co-harbored at least two

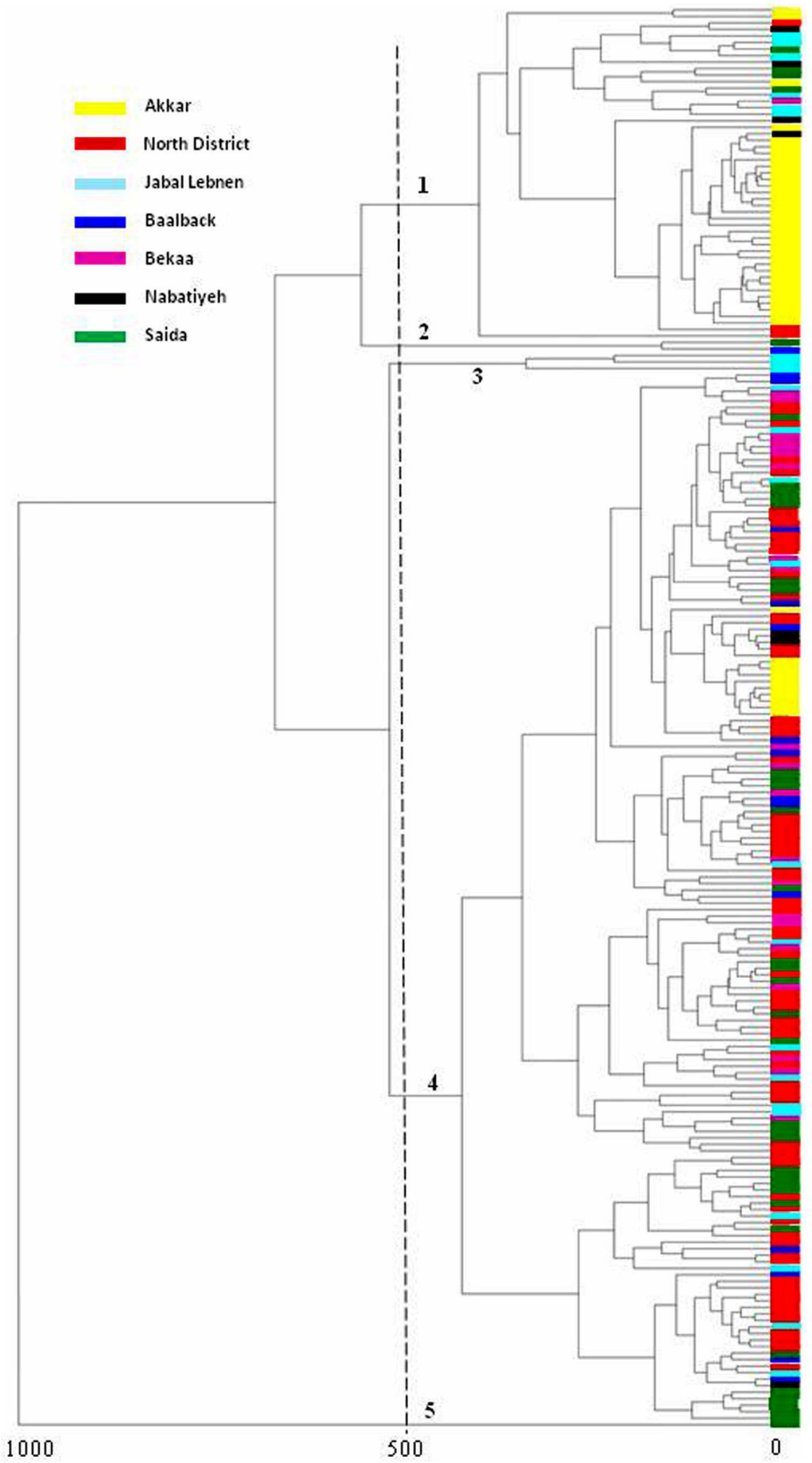

FIGURE 2 | Prevalence of MDROs in Lebanese poultry farms. Prevalence is expressed as the "number of isolates (percentage)."

TABLE 3 | Characteristics of the different phenotypes/genotypes of ESBL and ESBL/AmpC producers found in this study.

Species	Phenotype	β -lactamase genes			Co-resistance to non β -lactams
Escherichia coli	ESBL		bla TEM	bla CTX-M	SXT-CIP-GNT
			bla TEM	bla CTX-M	SXT-CIP
			bla TEM	bla CTX-M	CIP-GNT
			bla TEM	bla CTX-M	SXT-GNT
		bla SHV	bla TEM		SXT-CIP-GNT
		bla SHV	bla TEM		CIP
		bla SHV	bla TEM		SXT-GNT
		bla SHV	bla TEM		SXT-CIP
		bla SHV	bla TEM		SXT
				bla CTX-M	SXT-CIP-GNT
				bla CTX-M	SXT-CIP
				bla CTX-M	N.R
			bla TEM		SXT-CIP-GNT
			bla TEM		SXT-GNT
			bla TEM		SXT-CIP
			bla TEM		CIP-GNT
			bla TEM		GNT
			bla TEM		N.R
		bla SHV	bla TEM	bla CTX-M	SXT-CIP-GNT
		bla SHV			GNT
	AmpC/ESBL		bla TEM		SXT-CIP-GNT
			bla TEM		SXT-GNT
			bla TEM		CIP-GNT
			bla TEM		SXT
			bla TEM		N.R
			bla TEM	bla CTX-M	SXT-CIP-GNT
			bla TEM	bla CTX-M	SXT
			bla TEM	bla CTX-M	CIP-GNT
			bla TEM	bla CTX-M	SXT-CIP
			bla TEM	bla CTX-M	SXT-GNT
			bla TEM	bla CTX-M	N.R
		bla SHV	bla TEM		GNT
		bla SHV	bla TEM		CIP-GNT
				bla CTX-M	SXT-CIP-GNT
				bla CTX-M	N.R
		bla SHV		bla CTX-M	CIP-GNT
		bla SHV	bla TEM	bla CTX-M	SXT-CIP-GNT
Klebsiella pneumoniae	ESBL	bla SHV	bla TEM		SXT-CIP-GNT
		bla SHV	bla TEM	bla CTX-M	SXT-CIP-GNT
			bla TEM	bla CTX-M	CIP-GNT
	AmpC/ESBL	bla SHV	bla TEM	bla CTX-M	SXT-CIP-GNT
			bla TEM	bla CTX-M	SXT-CIP-GNT
		bla SHV	bla TEM		SXT-GNT
Escherichia fergusonii	ESBL		bla TEM	bla CTX-M	CIP
Enterobacter cloacae	ESBL			bla CTX-M	GNT

SXT, trimethoprim-sulfamethoxazole; GNT, gentamicin; CIP, ciprofloxacin; N.R, no resistance.

β-lactamase genes with the most common being CTX-M and TEM. Moreover, the only AmpC β-lactamase encoding gene was the CMY ampC β-lactamase. This gene was previously reported in poultry (Dierikx et al., 2013; El-Shazly et al., 2017) as well as in food producing animals (Sato et al., 2014; Aguilar-Montes de Oca et al., 2015) and healthy pets (Donati et al., 2014; Liu et al., 2016). As per the phenotypic and genotypic detection of AmpC production, these showed that there are some strains that were negative with the ampC disk test but positive for an ampC β-lactamase gene and vice-versa. Phenotypically false negatives shows the importance of the molecular testing in the detection of AmpC production. On the other hand, in the 15 *E. coli* strains that were negative for plasmidic ampC β-lactamase genes; one explanation for this might be due to an overexpression

of the chromosomal ampC gene mediated by a mutation in the promoter/attenuator region as described in previous studies (Escudero et al., 2010; Haenni et al., 2014). Regarding non-β-lactam co-resistance in ESBL and/or ampC producers, antimicrobial resistance toward gentamicin was relatively high in this study. In fact, 66% of ESBL and/or ampC producing Gram-negative bacilli were gentamicin resistant. This could possibly be linked to the frequent use of this antibiotic in Lebanese farms as several studies have reported (El-Rami et al., 2012; Diab et al., 2016). One study conducted by Abdelnoor et al. (2013) found a significant association between gentamicin resistance in *E. coli* isolates and the use of this antimicrobial agent as a food additive in poultry in Lebanon. Another study launched a questionnaire-based survey on the most common antibiotics used in Lebanese livestock and found that gentamicin and streptomycin are the most common and heavily used antimicrobial agents (Kassaify et al., 2013). Another thing to mention is that in this study, no carbapenemase producers were detected. There might be two possible explanations for this: the first one is that carbapenemase producers are really scarce in Lebanese chicken farms; the second one is that these isolates were missed due to the medium used for the screening of multi-drug-resistant organisms. As has been reported, OXA-48 carbapenemase producers are frequently found in hospitals and nursing homes and in fowls in Lebanon (Al Bayssari et al., 2015b). OXA-48 carbapenemases do not always confer resistance to third-generation cephalosporins unless there is another mechanism of resistance that co-exists in the same bacterial cell (Poirel et al., 2012). Therefore, Oxacillinase producers could have been missed or under-estimated in our study.

Our study has two main limitations. The first one is that the primers used for blaTEM and blaSHV screening were universal, and thus, the possibility of having non-ESBL variants cannot be ruled out. However, as the strains presented with a typical ESBL phenotype, i.e., the key hole effect and resistance to penicillin, monobactams and third-generation cephalosporins with susceptibility to carbapenems, the TEM-positive strains were considered as ESBL producers and were included in the description of the MDR-GNB prevalence in this study. The second limitation is the low number of isolates subjected to MLST typing. MLST and PFGE analysis remain the gold standard for clone/cluster detection in epidemiological studies regardless of the number of strains (McGregor and Spratt, 2005; Zou et al., 2010).

CONCLUSION

Our study illustrates the current epidemiology of multi-drug-resistant Gram-negative bacilli in Lebanese chicken farms. ESBL and ampC producers cross-resistant to antibiotics used in human medicine are highly prevalent across the territory. Our study suggests that poultry farms are potent reservoirs of antimicrobial resistance in Lebanon. Although very few studies have reported the detection of carbapenemase producers in Lebanese Livestock (Al Bayssari et al., 2015a,b), it will likely only be a matter of time before these organisms become prevalent in Lebanese animal farms. This is especially true if no strict rules are implemented to control the overuse and misuse of antibiotics for treatment, growth promotion and prophylaxis in Lebanese agriculture. We believe that the prescription of antibiotics often used in human medicine should be reduced or even banned in the veterinary sector.

AUTHOR CONTRIBUTIONS

ID, ES, and ED conducted the phenotypic and molecular work. BE-B was responsible for the collection of the samples. EA, J-MR, and ZD reviewed and edited the manuscript.

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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.

Article 4

First detection of mcr-1 plasmid mediated colistin resistant E. coli in Lebanese poultry.

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Letter to the Editor

First detection of *mcr-1* plasmid-mediated colistin-resistant *Escherichia coli* in Lebanese poultry

Sir,

The wide dissemination of multidrug-resistant Gram-negative bacteria (MDR-GNB), especially carbapenem-resistant bacteria, as common causative agents of human infections has necessitated the re-use of old antibiotics, namely colistin, which was abandoned in the past owing to its undesired nephrotoxicity in the human body [1]. Colistin belongs to the polymyxin group of polypeptide antibiotics that attack the lipopolysaccharide (LPS) and phospholipids in the outer cell membrane of GNB, leading to cellular leakage and subsequent bacterial death [1]. Resistance to colistin is mainly due to modifications to LPS and lipid A by the addition of aminoarabinose or phosphoethanolamine [1]. Prior to the end of 2015, such modifications were only due to chromosomal mutations of target genes involved in those pathways. Recently, the plasmid-mediated colistin resistance gene *mcr-1*, a member of the phosphoethanolamine transferase enzyme family in *Escherichia coli*, was reported in *E. coli* in China from pigs and meat [2]. Subsequently, *mcr-1* plasmid-mediated colistin-resistant bacteria have been detected in animals and humans across Asia, Africa, the Americas and Europe [3].

Here we report the first detection of a single *mcr-1*-positive colistin-resistant *E. coli* strain isolated from poultry in Lebanon. This isolate was recovered in Sidon on 14 August 2015 from a rectal swab obtained during a surveillance study aimed at determining the epidemiology of MDR-GNB in Lebanese poultry (unpublished data). In that study, 982 faecal swabs were collected from 49 chicken farms located in the seven districts of Lebanon. Swabs were cultured on MacConkey agar plates supplemented with cefotaxime (2 μg/mL) for the screening of MDR organisms. Identification of the isolated strain was performed using matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF/MS). Antibiotic susceptibility testing was performed by the disk diffusion method (Sanofi-Diagnostic Pasteur, Marnes-la-Coquette, France). The *E. coli* isolate showed an extend-spectrum β-lactamase (ESBL) phenotype and was resistant to penicillins, ceftazidime, cefotaxime, aztreonam, ciprofloxacin, gentamicin and trimethoprim/sulfamethoxazole and, surprisingly, was also resistant to colistin with an inhibition zone diameter of 10 mm. To confirm colistin resistance, colistin Etest strips (bioMérieux, Marcy-l'Étoile, France) and broth microdilution were used. Etest and broth microdilution revealed minimum inhibitory concentrations (MICs) of 2 μg/mL and 4 μg/mL, respectively, thus confirming colistin resistance in this isolate. Using standard PCR amplification and sequencing as described previously [3], the *mcr-1* gene was confirmed in this *E. coli* isolate. The obtained sequence was deposited in GenBank with the accession no. **MF197562**. A conjugation experiment using *E. coli* J53 as recipient was also conducted but was unsuccessful, suggesting that either *mcr-1* is located on a non-conjugative plasmid or it is chromosomally located. PCR amplification and sequencing revealed that the isolate harboured a $bla_{\text{TEM-135-like}}$ ESBL gene with a difference of six base pairs only at the extremities. Multilocus sequence typing (MLST) was performed based on seven housekeeping genes and revealed that the isolate belongs to ST515. This ST differs from those previously reported in *E. coli* isolates harbouring the *mcr-1* gene in food-producing animals. However, ST515 *mcr-1*-harbouring *E. coli* has been isolated from the blood of a male patient at an emergency department in Canada [2]. We thus suppose that this isolate could be a candidate for human infections with possible therapeutic challenges if ever transmitted and introduced into hospital and community settings.

In Lebanon, although insignificant, colistin resistance is not new in that it has been reported in clinical settings since the early 2000s. However, the mechanism of colistin resistance was not previously investigated. To the best of our knowledge, the first and only determination of colistin resistance mechanism in Lebanon was recently performed by Okdah et al., where three colistin resistant *Klebsiella pneumoniae* strains were isolated from Sahel Hospital in Beirut [4]. Colistin resistance in these isolates was mediated by inactivation of *mgrB*, *phoQ*, *pmrA* and *pmrB* genes involved in the modification of LPS in the outer cell membrane, the primary target of colistin in GNB [4]. Here we report the first detection of the *mcr-1* plasmid-mediated colistin resistance gene in Lebanon. As demonstrated by Olaitan et al., *mcr-1*-harbouring strains can be readily spread from animals to the human gut [5] and thus our finding sparks concerns over the transmission of *mcr-1* strains to the Lebanese community. Nowadays, carbapenem-resistant isolates are disseminated in clinical and community settings in Lebanon. This dissemination has necessitated the frequent use of colistin and non-β-lactam antibiotics in Lebanese hospitals [4]. Therefore, it is expected that *mcr-1*-positive strains, when transmitted from animals to humans in Lebanon, will be easily selected and further diffused into the country by the selective pressure applied by the use of colistin and other antibiotics in clinical settings. Surveillance studies addressing the current epidemiology of colistin resistance are thus warranted in Lebanon. In addition, the usage of colistin in veterinary medicine should be re-evaluated, as unpublished data have revealed its heavy use in animals in Lebanon.

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Competing interests

None declared.

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Article 5

Prevalence of multi drug resistance and colistin resistant Gram-negative bacilli In Lebanese swine farms.

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Prevalence of ESBL Producing Gram-Negative Bacilli and Emergence of mcr-1 Colistin Resistance Gene in Lebanese Swine Farms

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Abstract

Livestock are considered reservoirs of multi-drug resistant organisms that can be transferred to humans via direct/indirect routes. Once transmitted, these organisms can be responsible for infections with therapeutic challenges. The aim of this study was to determine the prevalence of extended spectrum cephalosporin and colistin resistant Gram-negative bacilli in Lebanese swine farms. In May 2017, 114 fecal samples were collected from swine farms in south Lebanon. Separate media supplemented with cefotaxime, ertapenem and colistin were used for the screening of resistant organisms. Double disk synergy test and ampC disk test were performed to detect ESBL and ampC producers respectively. Detection of beta-lactamase and mcr genes was done using RT-PCR. Of 114 fecal samples, 76 showed growth on the medium with cefotaxime. In total, 111 strains were isolated with 94.5% being E. coli. Phenotypic tests showed that 98, 6 and 7 strains were ESBL, ampC and ESBL/ampC producers, respectively. CTX-M and CMY were the main beta-lactamase genes detected. On the medium with colistin, 19 samples showed growth. In total, 23 colistin resistant E. coli strains harboring the mcr-1 gene were isolated. This is the first study in Lebanon determining multi-drug resistance epidemiology in pigs. The prevalence of ESBLs is high and the emergence of colistin resistance is alarming.

Introduction

Resistance in Gram-negative bacilli toward the most common antibiotics administered in the human medicine i.e. beta-lactams has significantly increased in the last decade.[1] Resistance to beta lactams and carbapenems in Gram-negative bacteria is mainly mediated via the production of extended spectrum beta lactamases (ESBLs), ampC beta lactamases and carbapenemases.[1] Genes encoding these enzymes are often co-localized on plasmids harboring resistance genes to other commonly prescribed antibiotics in human medicine such as aminoglycosides and quinolones. [1] Resistant organisms' dissemination often results in reducing beta lactam antibiotics efficacy limiting thus treatment options of infectious diseases.[2] This is currently emphasized with the recent emergence of colistin resistance in Gram negative bacilli. Colistin belongs to the polymyxin antibiotics family that acts on the lipopolysaccharide chain of the bacteria and leads to increased permeability of the outer membrane and subsequent cellular leakage followed by cell death.[3] In human medicine history, colistin was abandoned because of its nephrotoxicity and neurotoxicity inside human body.[4] However, due to the wide spread of multi drug resistant organisms, mainly carbapenem resistant ones; colistin was re-introduced in clinical settings.[5] This antibiotic revival had to face the emergence of colistin resistance in bacteria of human as well as of animal origin.[6] Prior to 2015, colistin resistance was thought to be only mediated via chromosomal mutations that leads to the alteration of the lipid A subunit of the LPS chain via the addition of 4-amino-4-deoxy-L-arabinose(L-Ara4N) and/or phosphoethanolamine (PEtN) [6] thus resulting in a reduced binding to colistin and subsequently bacterial resistance.[6] However, in 2015, Liu et al reported the first detection of a transferable phosphoenolamine transferase named mcr-1 gene in E. coli strains isolated from pigs and meat.[7] In this context, mcr-1 was reported from clinical and animal isolates across all continents. Furthermore, mcr variants i.e mcr-2,[8] mcr-3,[9] mcr-4 [10] and mcr-5 [11] have also emerged.

Nowadays, farm animals are considered as reservoirs of antimicrobial resistance. [12] The unregulated use of antibiotics is considered among the most common drivers for the emergence of resistance in livestock. [13] Indeed, antibiotics are not only given for treatment but are also prescribed for prophylaxis and administered as growth promoters.[13] The major public health concern about multi-drug resistance spread in animals is the potential transmission to human via direct contact or indirectly through the consumption of under/uncooked animal origin food.[14] Once transmitted, these organisms can cause infections with limited therapeutic options, especially the ones cross resistant to antibiotics frequently used in the human medicine.[15]

In Lebanon, the dissemination of multi-drug resistant organisms in the clinical settings is well documented;[16, 17,18,19, 20] however, studies addressing multi-drug resistance in animals remain scarce. One study carried by Diab el al. showed a relatively high prevalence of the CTX-M-15 ESBL type in E. coli of cattle origin in Lebanon.[21] More recently, a nationwide study conducted in Lebanese chicken farms reported an elevated level of ESBL/ ampC producing Gram-negative bacilli intestinal carriage.[22] Recently, our group reported the first detection of an E. coli isolated from poultry in south Lebanon harboring the mcr-1 colistin resistance gene in addition to the TEM-135 like ESBL gene.[23] In pigs, only one study reported the detection of an OXA-23 producing Acinetobacter baumannii in northern Lebanon.[24] The prevalence of multi drug resistant organisms in the Lebanese swine farms remains unknown. In collaboration with the ministry of agriculture, the aim of this study was to determine the prevalence of extended spectrum cephalosporin and colistin resistant Gram negative bacilli in Lebanese swine farms.

Materials and Methods

Ethics statement and collection of samples

The Ministry of Agriculture in Lebanon approved the collection of fecal samples from swine farms. The sampling was realized in compliance with the national guidelines for animal safety. On the 30th of May 2017, one hundred eleven fecal samples were randomly collected from three different swine farms located in south Lebanon. In addition, 3 fecal samples were taken from 3 wild pigs living in the same region. The number of samples collected was relatively proportional to the farms size which ranged from 20 to 120 pigs per farm (table 1). The fecal samples were collected using sterile urine cups and directly placed in a portable refrigerator; then when arrived at the University laboratory, they were stored at -80 °C until being used.

Screening of resistant organisms and identification

Each fecal sample was mixed in a sterile container and then a swab was used to subculture a considerable amount on MacConkey agars supplemented separately with 2µg/ml of cefotaxime, ertapenem (1µg/ml) and colistin (4mg/l) for the screening of resistant Gram-negative bacilli. Following overnight incubation at 37°C, isolated colonies with different morphologies were separately taken from each plate and identified by MALDI-TOF MS with a score value ≥1.9 using the Microflex LT spectrometer (Bruker Daltonics, Bremen,

Germany).[25] Thereafter, the strains were conserved in 40% glycerol aliquots at -80 °C for further tests.

Antibiotic susceptibility testing

Antibiotic susceptibility testing was performed using the Kirby-Bauer disk diffusion method and interpreted according to the European Committee on Antimicrobial Susceptibility testing (EUCAST) guidelines 2017.[26] A total of sixteen antibiotics were tested involving eleven beta-lactams (ampicillin, amoxicillin-clavulanic acid, aztreonam, cefotaxime, ceftazidime, cefoxitin, cefepime, piperacillin-tazobactam, ertapenem, meropenem, imipenem) and five non beta-lactams (colistin, gentamicin, ciprofloxacin, trimethoprim-sulfamethoxazole and tigecycline) (Bio-Rad, Marnes-la-Coquette, France). The phenotypic detection of ESBL was done using the double disk synergy test by placing an amoxicillin-clavulanic acid disk between cefepime, ceftazidime and aztreonam. Formation of a keyhole effect was considered as a phenotypic indication of ESBL production. Regarding screening of ampC beta lactamase and carbapenemase production, ampC disk test and carba np test were performed respectively as previously described.[27,28] Furthermore, all isolates having a narrow diameter zone of inhibition around the colistin disk were subjected to colistin broth micro-dilution test as previously described.[26] An isolate is termed as multi-drug resistant if this latter was resistant to three different classes of antibiotics at least.[29]

PCR identification of beta lactamase genes

All isolates showing a keyhole effect or having resistance to both cefoxitin and cefepime were subjected to real time PCR analysis for blaCTX-M, blaSHV and blaTEM genes screening.[30] Furthermore, all strains found positive to the ampC disk test were also tested for genes encoding AmpC beta lactamases FOX, MOX, ACC, EBC, DHA and CMY using simplex PCRs.[31] DNA extraction was performed using EZ1 DNA extraction kit (Qiagen, Courtaboeuf, France), following manufacturer instructions with an EZ1 Advanced XL biorobot.

Molecular characterization of mcr-1 colistin resistance gene

All strains having a colistin MIC $\geq$ 2µg/ml were subjected to standard PCR amplification and sequencing for the detection of mcr-1 colistin resistance gene. DNA extraction was done

using an EZ1 DNA extraction kit (Qiagen, Courtaboeuf, France) with an EZ1 Advanced XL biorobot. Primers used in molecular analysis were previously described in other studies.[32]

Results

Prevalence of beta lactamase producers and colistin resistant Gram-negative bacilli

Out of 114 fecal samples collected, 76 (66.5%) showed positive growth on the selective medium supplemented with cefotaxime. In total, 111 multi drug resistant strains were isolated according the following distribution: 65 strains in farm 1, 9 in farm 2, 35 in farm 3 and 2 isolates from the wild pigs. MALDI TOF MS identification revealed that Escherichia coli made up to 94.5% of isolated MDR strains, Escherichia fergusonii 3.5% and Klebsiella pneumoniae 2% (table 1). Besides, 23 colistin resistant E. coli strains isolated from 19 fecal samples were obtained. No carbapenemase producers were detected in this study.

Phenotypic profiles of beta lactamase producers

The resistance profiles of isolated ESBL and/or ampC producing Gram-negative bacilli are summarized in table 2. All ESBL/ampC producing strains were susceptible to colistin and carbapenems. Carba np test, double disk synergy test and ampC disk test, revealed the absence of carbapenemase producers, 98 isolates (88.5%) were categorized as ESBL producers, 7 (6%) as ESBL/ampC co-producers and 6 strains (5.5%) as solely ampC producers. K. pneumoniae isolates were only ESBL producers whereas 3 E. fergusonii were categorized as ampC producers and 1 as an ESBL producer. Co-production of ESBL and ampC was only detected in E. coli isolates. Regarding non beta lactam antibiotics resistance in the afore-mentioned strains, one isolate was co-resistant to all non beta lactams tested: tigecycline, gentamicin, ciprofloxacin and trimethoprim-sulfamethoxazole, 32 (29%) were co-resistant to 3 non beta lactams, 59 (53%) to 2 non beta lactams, 16 (14%) to one non beta lactam and three strains were susceptible to all non beta lactam antibiotics. Overall, 83% of beta lactamase producing Gram-negative bacilli in this study were co-resistant to at least two non beta lactams.

Molecular characterization of beta lactamase genes

One hundred five Gram negative bacilli having ESBL phenotypes were subjected to real time PCR analysis for the screening of CTX-M, TEM and SHV encoding genes. CTX-M was detected in 83 (79%) ESBL isolates, TEM in 57 (54%) and SHV in 9 (8.5%). In total, 12 strains (11%) showed the co-existence of the three bla genes together, 43 (41%) showed the

co-existence of two bla genes and 57 (54%) harbored only one beta lactamase gene. In addition, CMY was the only ampC encoding gene detected in ampC and ESBL/ampC co-producers.

Colistin resistant isolates: resistance profiles and genotype

The detailed profile of the resistance of E. coli colistin resistant strains isolated in this study is depicted in Figure 1. To summarize, four of the twenty three strains were colistin resistant and also ESBL producers whereas the remaining strains (19 isolates) were susceptible to all beta lactams tested, except for ampicillin. Resistance rates towards non beta lactam antibiotics varied: 8 strains were co-resistant to gentamicin, ciprofloxacin and trimethoprim-sulfamethoxazole, 7 strains were resistant to two non beta lactams, 2 were resistant to only one non beta lactam antibiotic and 6 strains were susceptible to all non beta lactams tested. Colistin MICs of the 23 E. coli isolates ranged between 4 and 16 µg/ml except one strain having a MIC of 256 µg/ml. Standard PCR and sequencing revealed that all the strains were mcr-1 positive. In the four ESBL mcr-1 positive resistant isolates, CTX-M was detected in 2 strains while SHV and TEM were detected in all four (figure 1).

Discussion

Antimicrobial resistance is rapidly evolving and disseminating worldwide. In the context of antimicrobial resistance in the one health concept, livestock (i.e. pigs, poultry and cattle) is now considered as a major reservoir of multidrug-resistant organisms and antibiotic resistance genes. [12] In Lebanon, few studies have been conducted to determine the prevalence of multi-drug resistant organisms in Lebanese Livestock; [21] however in pork, only one study reported the detection of a carbapenemase producing A. baumannii isolate from a pig in northern Lebanon.[24] To the best of our knowledge, our study is the first in Lebanon to describe the epidemiology of beta lactamase producing Gram-negative bacilli in Lebanese swine farms. It is worth mentioning that the number of samples collected was not relatively high since only few swine farms are accessible in Lebanon. The role of the Ministry of Agriculture was essential to carry out this study since it provided the legal permission to access and sample the different sites. In our investigation, ESBL/ampC producing Gram negative bacilli were detected in 66.5% of the collected fecal samples (table1). Compared to other epidemiological studies investigating pigs worldwide, the prevalence in Lebanon is not far from what is reported in Belgium (75 %) [33] and Germany (88 %) [34] but is still much higher

than the ones reported in China (32 %),[35] UK (23%),[36] Denmark (18.5%),[37] Switzerland (15%) [38] and Thailand (2.4%).[39] Differences in the number of samples and screening methodologies, in addition to the level and type of antibiotics prescribed in the farms of each country could explain these differences.[3] The aforementioned concept applies also to prevalence of mcr-1 positive E. coli strains detected in our previous study (17%) compared to other international studies: Portugal (98%),[40] Vietnam (37.5%),[41] China (20.6%),[7] Japan (1%),[42] France (0.5%) [43] and USA (0.35%).[44]

In this study, 83% percent of ESBL/ampC producers were co-resistant to at least two non-beta lactam antibiotics with the highest level of resistance being observed against trimethoprim-sulfamethoxazole and ciprofloxacin. During our samples collection, we tried hard to collect correct data on the types and quantities of antibiotics used in the different farms; a mission nearly impossible. Indeed, despite the official presence of the Ministry of Agriculture, the cooperation of the farm owners was not easy to get; and there was no clear distinction between different uses of antibiotic in farms investigated (treatment of infections, prevention on infection, and growth enhancement). Unofficially, we were informed that enrofloxacin is frequently administered to pork in Lebanon. In fact, it has been reported that in pigs, penicillins are used to treat necrotic enteritis whereas as cephalosporins such as cefquinome and ceftiofur are prescribed for polyarthritis, septicemia, polyserositis and respiratory infections.[2] Use of non-beta lactams such as gentamicin, fluoroquinolones, aminoglycosides and colistin was also reported.[45,46] On the other hand, it is not clear to us to which extent international guidelines and recommendations for hygiene and waste management in pig farms are applied in our country. Questionable hygiene, poor feed quality and bad waste management imply another important drive in the emergence of multi-drug resistance in pigs in addition to the over-use of antibiotics that facilitates the transmission of resistant organisms from pigs to their surrounding environment and vice versa. At the molecular level, the most commonly detected beta lactamase gene was the CTX-M. This gene was highly reported in Lebanon in the clinical settings [16, 47] as well as in cattle [21] and poultry.[22] CTX-M is also the main ESBL type reported globally in farm animals.[36, 37,39, 48] As for ampC producers, this study showed that CMY was the only ampC beta lactamase gene detected in swine farms of Lebanon. The same observation was also made in chicken farms (data not shown). It has been worldwide shown that this gene is the most common ampC beta lactamase gene detected in poultry, [49,50] food producing animals [51,52] as well as in healthy pets.[53,54] In this study, it has not escaped our notice that no carbapenemase producers were

detected. This is in accordance with another study performed by our group in poultry farms [22] reflecting that carbapenemase producers are really scarce in Lebanese livestock. Furthermore, in this study we report for the first time the detection of mcr-1 in pork of Lebanon. In this country, mcr-1 gene was first reported in chicken during an epidemiological study aiming at determining the prevalence of multi-drug resistant organisms in Lebanese chicken farms.[23] The MIC values of colistin in mcr-1 producing E. coli isolates in this study range between 4 and 16 µg/ml. These results are in accordance with other studies showing that mcr-1 harboring isolates do not usually have elevated colistin MICs.[55,56] Some reports showed that mcr-1 positive E. coli isolate could have a colistin MIC as low as 2µg/ml.[57] In our collection of mcr-1 strains, only one ESBL producing E. coli had a colistin MIC of 256 µg/ml. This elevated MIC might be attributed to additional chromosomal mutations in the phoP/Q, pmrA/B and mgrB genes as reported previously in the literature.[5] However, further genomic analysis is needed to explore this possibility. Delannoy et a1. reported the isolation of E. coli strains harboring mcr-1 and having amino acids mutations in the phoP/Q, pmrA/B and mgrB genes from diseased pigs in France.[57] Furthermore, it is worth mentioning that, as shown in figure 1, none of the colistin resistant isolates was Pan-Drug resistant, but rather remained susceptible to the majority of the tested antibiotics, except four strains that were ESBL producers. The co-existence of mcr-1 and ESBL/carbapenemase encoding genes was previously reported in several studies in the literature.[58,59] Resistance profiles of mcr-1 strains in this study possibly illustrate an over-estimated fear of colistin resistance. E. coli colistin resistant isolates will pose therapeutic challenges only if transmission of MDR strains to humans occurs.

In conclusion, this study describes the epidemiology of ESBL/ampC producing Gram-negative bacilli in Lebanese swine farms. The emergence of mcr-1 in pigs is alarming. The level of antibiotic consumption in Lebanese swine farms remains unknown; a more transparent policy should be adopted in this context. Therefore, the surveillance and control programs addressing antibiotic consumption in Lebanese farms, especially in pigs, are urgently needed. Future studies should not only focus on antimicrobials usage but also on the risk factors associated with the carriage of multi-drug resistant organisms in pigs.

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552 **Table 1** Distribution of ESBL/ampC producing and colistin resistant Gram-negative bacilli
553 per farm

	AB used	n collected samples	n of ESBLs/ampcs samples	n of ESBLs/ampcs isolates	Species	n of Col/R samples	n of Col/R isolates	Specie
Farm 1 (n = 120)	Enrofloxacin	60	42	65	60 E.coli 4 E.fergusonii 1 K.pneumoniae	8	8	E.coli
Farm 2 (n = 20)	unknown	15	8	9	8 E.coli 1 K.pneumoniae	4	5	E.coli
Farm 3 (n = 100)	unknown	36	24	35	E.coli	7	10	E.coli
W.P (n = 3)	unknown	3	2	2	E.coli	0	0	

554
555 W.P = wild pigs, AB = antibiotic, n = number, Col/R = colistin resistant.

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558 **Table 2** Resistance profiles of ESBL/ampC producing Gram negative bacilli

	Antibiotic susceptibility testing												Phenotype		
Species	AMP	CTX	AZT	FOX	CAZ	AUG	FEP	PTZ	TGC	SXT	CIP	GNT	% of ESBL	% of ampC	% of ESBL/ampC
E.coli (n = 105)	103 (98)	70 (67)	45 (43)	25 (24)	44 (42)	48 (46)	57 (54)	1 (1)	1 (1)	97 (92)	82 (78)	44 (42)	90	3	7
E.fergusonii (n = 4)	4 (100)	2 (50)	3 (80)	4 (100)	4 (100)	4 (100)	0 (0)	0 (0)	0 (0)	1 (20)	4 (100)	3 (80)	20	80	
K.pneumoniae (n = 2)	2 (100)	2 (100)	1 (50)	0 (0)	1 (50)	1 (50)	2 (100)	0 (0)	0 (0)	1 (50)	1 (50)	0 (0)	100		

559

560 Resistance profiles are presented as number (percentage).
561 n = number, % = percentage, AMP = ampicillin, CTX = cefotaxime, AZT = aztreonam, FOX = cefoxitin, CAZ
562 = ceftazidime, AUG = amoxicillin-clavulanic acid, FEP = cefepime, TZP = piperacillin-tazobactam, TGC =
563 tigecycline, SXT = trimethoprim-sulfamethoxazole, CIP = ciprofloxacin, GNT = gentamicin.

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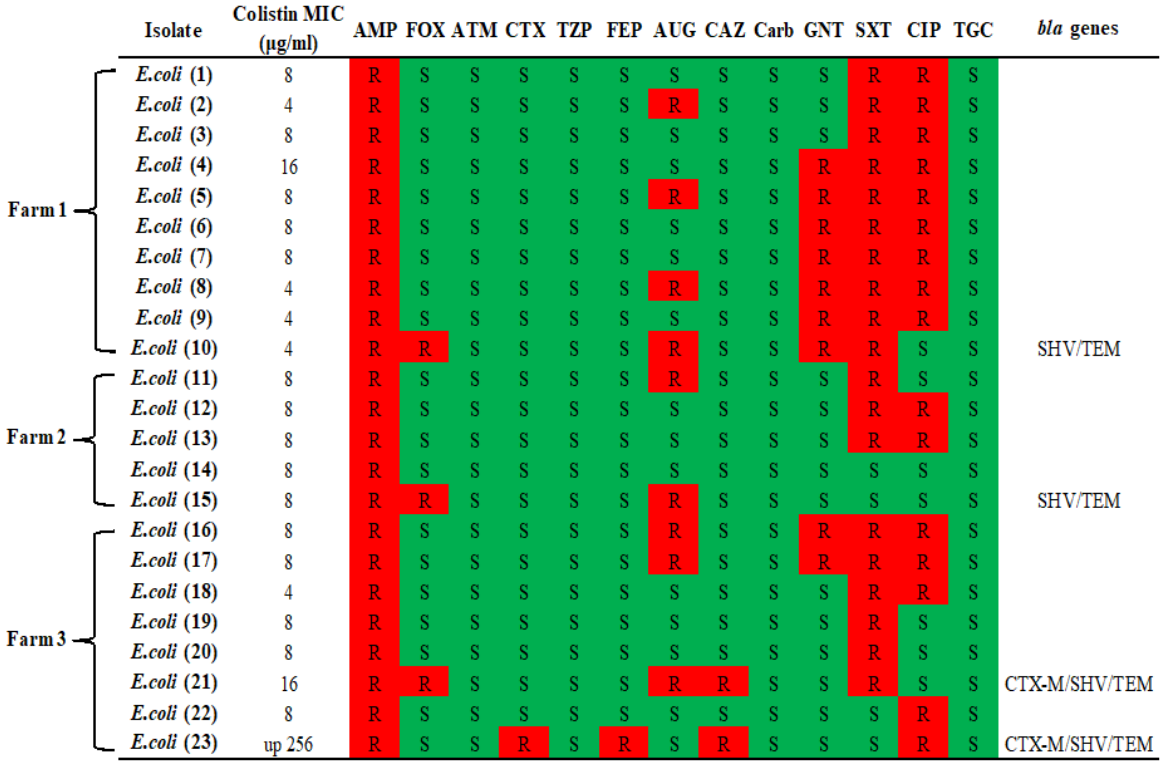
Figure Legends

FIG1. Resistance profiles of mcr-1 colistin resistant E. coli isolates. R= resistant, S = sensitive, bla = beta lactamase, AMP = ampicillin, CTX = cefotaxime, AZT = aztreonam, FOX = cefoxitin, CAZ = ceftazidime, AUG = amoxicillin-clavulanic acid, FEP = cefepime, TZP = piperacillin-tazobactam, Carb = carbapenems, TGC = tigecycline, SXT = trimethoprim-sulfamethoxazole, CIP = ciprofloxacin, GNT = gentamicin.



	Isolate	Colistin MIC (µg/ml)	AMP	FOX	ATM	CTX	TZP	FEP	AUG	CAZ	Carb	GNT	SXT	CIP	TGC	*bla* genes
Farm 1	*E.coli* (1)	8	R	S	S	S	S	S	S	S	S	S	R	R	S	
	E.coli (2)	4	R	S	S	S	S	S	R	S	S	S	R	R	S	
	E.coli (3)	8	R	S	S	S	S	S	S	S	S	S	R	R	S	
	E.coli (4)	16	R	S	S	S	S	S	S	S	S	R	R	R	S	
	E.coli (5)	8	R	S	S	S	S	S	R	S	S	R	R	R	S	
	E.coli (6)	8	R	S	S	S	S	S	S	S	S	R	R	R	S	
	E.coli (7)	8	R	S	S	S	S	S	S	S	S	R	R	R	S	
	E.coli (8)	4	R	S	S	S	S	S	R	S	S	R	R	R	S	
	E.coli (9)	4	R	S	S	S	S	S	S	S	S	R	R	R	S	
	E.coli (10)	4	R	R	S	S	S	S	R	S	S	R	R	S	S	SHV/TEM
Farm 2	*E.coli* (11)	8	R	S	S	S	S	S	R	S	S	S	R	S	S	
	E.coli (12)	8	R	S	S	S	S	S	S	S	S	S	R	R	S	
	E.coli (13)	8	R	S	S	S	S	S	S	S	S	S	R	R	S	
	E.coli (14)	8	R	S	S	S	S	S	S	S	S	S	S	S	S	
	E.coli (15)	8	R	R	S	S	S	S	R	S	S	S	S	S	S	SHV/TEM
Farm 3	*E.coli* (16)	8	R	S	S	S	S	S	R	S	S	R	R	R	S	
	E.coli (17)	8	R	S	S	S	S	S	R	S	S	R	R	R	S	
	E.coli (18)	4	R	S	S	S	S	S	S	S	S	S	R	R	S	
	E.coli (19)	8	R	S	S	S	S	S	S	S	S	S	R	S	S	
	E.coli (20)	8	R	S	S	S	S	S	S	S	S	S	R	S	S	
	E.coli (21)	16	R	R	S	S	S	S	R	R	S	S	R	S	S	CTX-M/SHV/TEM
	E.coli (22)	8	R	S	S	S	S	S	S	S	S	S	S	R	S	
	E.coli (23)	up 256	R	S	S	R	S	R	S	R	S	S	S	R	S	CTX-M/SHV/TEM

FIG1. Resistance profiles of mcr-1 colistin resistant E. coli isolates. R= resistant, S = sensitive, bla = beta lactamase, AMP = ampicillin, CTX = cefotaxime, AZT = aztreonam, FOX = cefoxitin, CAZ = ceftazidime, AUG = amoxicillin-clavulanic acid, FEP = cefepime, TZP = piperacillin-tazobactam, Carb = carbapenems, TGC = tigecycline, SXT = trimethoprim-sulfamethoxazole, CIP = ciprofloxacin, GNT = gentamicin.

Article 6

Dissemination of multi-drug resistant and mcr-1 Gram-negative bacilli in Broilers, farm workers and the surrounding environment in Lebanon.

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Dissemination of multi-drug resistant and mcr-1 Gram-negative bacilli in Broilers, farm workers and the surrounding environment in Lebanon

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Abstract

Objectives

Poultry are nowadays regarded as reservoirs from which multi-drug resistant organisms can be readily transferred to the surrounding ecosystem. The aim of this study was to explore the prevalence of ESBL/ampC and mcr-1 Gram-negative bacilli in chicken, farmers and the surrounding environment in Lebanon.

Methods

In May-2017, we went to the same farm where the first mcr-1 E. coli was detected in 2015 in Lebanon. 200 chicken fecal swabs, 6 farmers' fecal samples and 41 environmental samples were collected. RT-PCR was performed to screen for beta-lactamase and mcr genes using newly designed primers and probes. MLST typing and statistical analysis comparing the prevalence of resistant organisms and genes in 2015 and 2017 was performed.

Results

ESBL/ampC beta lactamases were detected in chicken (59%), workers (67%), litter (100%), feed (100%) and soil (100%). mcr-1 was detected in 73% and 100% of chicken and farmers samples, respectively. Three mcr-1 positive E. coli strains were isolated from litter and feed. Compared to 2015, the prevalence of ESBL/ampC producers as well as TEM and CTX-M genes increased significantly in 2017. MSP dendrogram of isolated strains in 2015 and 2017, in addition to MLST, shows the presence of different clones as well as different sequence types.

Conclusions

This study showed a massive dissemination of mcr-1 strains from 2015 to 2017. The evolution of resistance appears to be multi-clonal and related to the diffusion of plasmids carrying ESBL and mcr-1 genes. Colistin use should be banned in the Lebanese veterinary medicine.

Introduction

Gram-negative bacilli (GNB) are among the most common causative agents of hospital and community acquired infections (1). Among other organisms, resistance in Gram-negative bacteria has taken major concern in the last decade (2). This is due to their rapidly evolving and disseminating mechanisms of resistance against commonly prescribed antibiotics in the human medicine i.e. cephalosporins and carbapenems (3). Extended spectrum beta lactamases (ESBLs), ampC beta lactamases and carbapenemases are the main mediators of resistance encountered nowadays in Gram-negative bacteria (4). Recently, the emergence of colistin resistance worsened the situation. Colistin is a polymyxin antibiotic that has previously been discontinued in clinical settings, but has recently been reintroduced due to the wide dissemination of multi-drug resistant Gram-negative bacteria, notably the carbapenem resistant ones (5). Colistin resistance is mediated either through chromosomal mutations that mediates the modification of the lipid A moiety of the LPS chain (6), or via plasmidic acquisition of a phosphoenolamine transferase gene i.e. mcr-1(7), mcr-2 (8), mcr-3 (9), mcr-4 (10) and mcr-5 (11).

Many years ago, the epidemiology of resistant GNB was thought to be restricted to the hospital settings. However, nowadays, evidence has shown the presence of an external reservoir of resistance in "livestock" (12). Many studies reported a high prevalence of ESBLs as well as colistin-resistant Gram-negative bacilli in farm animals (13) (14). The main driven for this abundance is the uncontrolled usage of antibiotics in veterinary medicine (15). The European Centre for Disease Prevention and Control/European Food Safety Authority/European Medicines Agency (ECDC/EFSA/EMA) report showed that in 2014, the average antibiotic consumption in animals (152 mg/kg) out passed the one in humans (124 mg/kg) (16). Univariate analysis showed a significant correlation between tetracycline and polymyxin consumption and resistance in Escherichia coli in animals and between fluoroquinolones and E. coli in both human and animal sectors (16). Furthermore, a recent publication of the WHO guidelines on use of medically important antimicrobials in food producing animals recommended an overall reduction but also a complete restriction use of all medically important antimicrobial classes for growth promotion and disease prevention in food producing animals (17). According to the WHO CIA report, these antimicrobials include 3rd, 4th and 5th generation cephalosporins, glycopeptides, macrolides, ketolides and polymyxins (18). The main concern about the spread of resistant organisms in animals is their potential transmission to humans where they could be causative agents of infections with limited therapeutic options when multi-drug resistance is encountered(19).

The increased carriage of ESBLs in humans with frequent contact with broilers (20), sharing the same plasmids/ESBL genes (21), sequence types, virulence and PFGE patterns (22) between humans and animals have all been considered evidence of resistance transmission between these two compartments. Although, direct contact with animals has been suggested to be the main player in this transmission, environmental routes in farm animals are increasingly being considered (20). These latter include transmission via air (23), dust (24), soil fertilized with animal manures (25) and contaminated wastewaters (26). The epidemiology of multi-drug resistant Gram-negative bacteria is thus complex at the human-animal-environment interface (27).

In Lebanon, our group reported a considerable nationwide prevalence of ESBL/ampC producing Gram-negative bacilli (20.6%) in poultry farms in 2015 (28). Similarly, a study conducted in cattle revealed high abundance of CTX-M-15 producing E. coli over the Lebanese territory (29). Scattered other reports described the detection of OXA-23/OXA-58 producing Acinetobacter baumannii, VIM-2 producing Pseudomonas aeruginosa and OXA-48 E. coli strains in livestock and fowl respectively (30, 31). In addition, our group reported the first detection of an isolated positive E. coli mcr-1 strain of chicken in Lebanon in 2015 on a farm in southern Lebanon (32). In this context, the purpose of this study was to return to the same farm where we found mcr-1 two years ago and to do further investigations on the prevalence of ESBL and mcr-1 positive Gram-negative bacilli, not only in chickens, but also in farm workers and the surrounding environment.

Materials and Methods

Ethics statement

The Ministry of Agriculture of Lebanon has agreed to the collection of fecal swabs from broilers in the south in accordance with national animal handling and sampling standards. Sampling was in accordance with international animal safety guidelines. The farm workers provided us with fecal samples with their complete satisfaction and without any obligation.

Collection of samples from broilers, environment and workers

On the 15th of May 2017, 200 fecal swabs were collected from broilers in the farm where the first mcr-1 positive E. coli strain was first isolated in 2015 in Saida - southern Lebanon (32). Ten chicken feed samples, 10 poultry litter samples as well as 21 soil samples surrounding the farm were also collected using sterile cups. Six fecal samples were also provided by the workers in this farm using sterile urine cups. All collected samples were put in a portable

refrigerator and transported directly to the University laboratory where they were stored at -80 °C for later use. In addition, the list of antibiotics used in this farm was recorded.

Screening of beta lactamase and colistin-resistant Gram-negative bacteria

MacConkey agars supplemented with cefotaxime (2mg/l), ertapenem (1mg/l) and colistin (4mg/l) were used for the screening of ESBL, carbapenemase producers and colistin-resistant Gram-negatives respectively. The chicken fecal swabs were simply subcultured on the different media. This also applies to the workers fecal samples; however for these, each fecal sample was first mixed using a swab and then this swab was used for subculture. On the other hand, each soil, feed and litter sample was incubated in a 400 ml of sterile distilled water for 2 hours at room temperature. Thereafter, an initial vacuum pump filtration using filter papers (pores size 10-15µm) to remove sediments was performed and the 400 ml filtered from each sample was divided into 3 sterile cups containing each 100 ml. Then, using mixed ester cellulose filter papers with 0.45 µm pores size, each 100 ml was filtered again and put on a separate selective media and incubated overnight at 37 °C. Following incubation, well isolated colonies growing on the selective media were taken separately and identified using MALDI-TOF MS with a score value ≥1.9 using the Microflex LT spectrometer (Bruker Daltonics, Bremen, Germany) for correct identification (33),(34). For each strain, the spectra obtained were stored and downloaded into a MALDI Biotyper 3.0 system for the construction of an MSP dendrogram. Following identification, the strains were conserved in 40% glycerol aliquots and preserved at -80 °C for later testing.

Antibiotic susceptibility testing

Antibiotic susceptibility testing was performed using the Kirby-Bauer disk diffusion method. Sixteen antibiotics were used: ampicillin, amoxicillin-clavulanic acid, aztreonam, ceftazidime, cefotaxime, cefepime, cefoxitin, piperacillin-tazobactam, colistin, meropenem, ertapenem, imipenem, tigecycline, ciprofloxacin, gentamicin and trimethoprim-sulfamethoxazole (Bio-Rad, Marnes-la-Coquette, France). The diameters zones of inhibition were interpreted according to EUCAST guidelines 2017(35). Furthermore, colistin broth micro-dilution test was performed as previously described. An isolate showing resistance to at least three different classes of antibiotics was termed as being multi-drug resistant (36). Phenotypic detection of ESBL, ampC beta lactamases and carbapenemases was performed using the double disk synergy test, ampC disk test and Carba NP test respectively(37)(38).

Real time PCR screening of beta lactamase and mcr genes

All strains having a colistin MIC of > 2 mg/l were subjected to RT-PCR for the screening of mcr colistin-resistant gene (39) (40). For a more rapid screening, new primers and probes were designed for the detection of mcr-3, mcr-4 and mcr-5 plasmid mediated colistin resistance gene by real time PCR (table 1). Furthermore, isolates showing a keyhole effect or having non susceptibility to third generations cephalosporins were screened for the presence of bla_{CTX-M}, bla_{SHV} and bla_{TEM} genes(41). Bacterial DNA was extracted using an EZ1 DNA extraction kits (Qiagen, Courtaboeuf, France) with an EZ1 Advanced XL biorobot.

Multilocus sequence typing

Colistin-resistant E. coli strains isolated from workers and environmental samples as well as fifteen selected ones from chicken were subjected to MLST typing based on their allelic profiles using seven housekeeping genes: adk, fumC, gyrB, icd, mdh, purA and recA (42). ESBL producers in workers were also subjected to MLST typing. The sequence type (ST) of each strain was determined using the allelic profiles analyzed based on the Warwick MLST database (http://mlst.Warwick.ac.uk/mlst/dbs/Ecoli).

Statistical analysis

The prevalence of multi-drug resistant Gram-negative bacilli, resistance genes as well as resistance patterns, were compared between the years 2015 and 2017 via Fisher Exact test using Epi Info™ version 7.2 (43). A P value ≤ 0.05 was considered statistically significant.

Results

Identification of Isolated strains

Of the 200 rectal swabs collected from chicken, 181 E. coli strains were isolated on the medium supplemented with cefotaxime. In farm workers and poultry litter, four and seventeen E. coli strains were detected, respectively. In feed samples, 3 Acinetobacter baumannii, 3 Pseudomonas aeruginosa, one Achromobacter xylosoxidans and one Serratia rubideae were isolated from 8 samples. Similarly, in soil samples, non-fermenters were the most common organisms found in addition to enterobacteriaceae: 4 Pseudomonas putida, 2 Pseudomonas monteilii, 4 Acinetobacter genomospecies, 4 Stenotrophomonas maltophilia, 4 Enterobacter cloacae, 5 E. coli and one Ochrobactrum haematophilium. On the other hand, on the medium supplemented with colistin, 121 colistin-resistant E. coli strains, 30 Klebsiella pneumoniae and 1 Enterobacter asburiae were isolated from chicken. All 6 workers carried

colistin-resistant isolates: 6 E. coli and 1 K.pneumoniae. From feed samples, two colistin-resistant E. coli strains and one A. baumanii were detected. In poultry litter, a single colistin-resistant E. coli strain was isolated while in soil, no colistin-resistant bacteria were found.

Resistance Phenotypes of isolated strains

The detailed antibiotic susceptibility testing of Gram-negative bacilli isolated in this study is summarized in Table 2. Overall, ESBL was the main mechanism of resistance found in all sources followed by ESBL/ampC and ampC production. High resistance rates were found against non beta-lactam antibiotics, notably gentamicin, trimethoprim-sulfamethoxazole and ciprofloxacin. In chicken, 163 (90%) ESBL/ampC strains were co-resistant to colistin, one strain was resistant to all non beta-lactams tested, 119 (66%) were resistant to three non beta-lactams, 54 (30%) to two and 7 (4%) to only one non beta-lactam. Same pattern of co-resistance was also observed in strains isolated from poultry litter, soil and feed samples, where (17) 100%, 20 (89%), 4 (50%) were at least resistant to two non beta-lactams respectively; the most common of these being resistance to both ciprofloxacin and trimethoprim-sulfamethoxazole. Conversely, farm workers isolates were mainly susceptible to non beta-lactams with only one being co-resistant to ciprofloxacin and trimethoprim-sulfamethoxazole. Compared to 2015, the prevalence of antibiotic resistance has increased significantly for all beta-lactam and non beta-lactams except cefepime, ciprofloxacin and tigecycline (figure 3 A).

As for colistin-resistant isolates grown on the media supplemented with colistin, broth micro-dilution testing revealed colistin MICs ranging from 4 to 16 mg/l in E. coli strains isolated from chicken except for four isolates having colistin MICs ranging from 64 mg/l to 256 mg/l. K. pneumoniae isolates from chicken displayed colistin MICs reaching 256 mg/l for 26 of them whereas four strains had an MIC of 8 mg/l. Furthermore, one Enterobacter asburiae with a colistin MIC of 256 mg/l was also detected. In workers, feed and litter strains, colistin MICs ranged from 4 and 8 mg/l. From all sources, phenotypic test revealed that all strains were sensitive to the majority of the beta-lactams, tested with only 5 and one in chicken and workers, respectively, being ESBL producers. Different rates of resistance were also detected against non beta-lactams; overall only 7 strains were resistant to one non-beta lactam antibiotic whereas the other strains (156) were co-resistant to at least two non beta-lactams. As depicted in figure 3 B, gentamicin and colistin resistance were significantly more prevalent in ESBL producers compared to ESBL negative mcr-1 positive strains.

Prevalence of ESBL/ampC and colistin-resistant isolates in all sources

As shown in Figure 1, ESBL/ampC producing Gram-negative bacilli were detected in all feed, soil and litter samples whereas in chicken and farm workers, these latter were detected in 59% and 67% of collected fecal samples, respectively. The abundance of colistin resistance was higher in chicken (73%) and farmers (100%) as compared to the environmental samples (litter (6%), feed (20%)). In fact, the prevalence of ESBL/ampC producers detected in poultry in Saida region has significantly increased, from 27% in 2015 to 59% in 2017.

Personal communication with the veterinarian of the visited farm revealed that colistin and gentamicin are often prescribed for gastrointestinal infections and doxycycline for respiratory infections of poultry in this farm.

Detection of beta lactamase and mcr genes

In chicken CTX-M, TEM and SHV genes were detected in 70, 116 and 23 ESBL/ampC positive E. coli strains, respectively. As shown in figure 3 A, the prevalence of CTX-M and TEM beta lactamase genes has significantly increased in 2017 compared to 2015. All Farmers' and feed's isolates harbored CTX-M with two and four of them co-harboring also the TEM gene respectively. In poultry litter, CTX-M and TEM genes were detected in 16 strains. TEM encoding gene was found in 15 strains isolated from soil samples, CTX-M in 14 and SHV in 3 isolates. Furthermore, of the 181 ESBL and/or ampC producers detected, 125 were also positive for the mcr-1 colistin resistance gene. In parallel, all colistin-resistant E. coli and K. pneumoniae strains isolated from chicken, farm workers, poultry litter and feed were positive for mcr-1. No other mcr variants were detected. One A. baumannii and one E. asburiae isolates from feed and chicken were negative for the mcr-1 gene, respectively.

MSP dendrogram analysis and MLST typing

As shown in figure 2, no cluster formations were formed in the MSP dendrogram neither at the level of the geographical location in 2015 neither at the level of the resistance phenotype. This also applies to the E. coli strains isolated in 2017 where the ESBL and mcr-1 ones were dispersed randomly in the dendrogram. Combining the spectra of ESBL E. coli strains isolated from Saida in 2015 with those isolated in 2017, shows that these latter do not form independent clusters.

MLST typing of chicken strains surrounding farmers' and environmental strains in the MSP dendrogram revealed the presence of: ST101, ST746, ST1196, ST359, ST1140, ST2220,

ST5687 and ST2481 in addition to unknown sequence types. The colistin-resistant E. coli strain isolated from litter had ST746, whereas the two E. coli isolates detected in feed samples were of ST101 and ST3941. ST101 was shared by chicken and feed strains whereas ST746 was shared between litter and chicken isolates. Farm workers' isolates displayed with ST1011 for colistin-resistant E. coli and ST10, ST59 for ESBL producers; unknown sequence types were also detected in both ESBL and colistin-resistant E. coli strains isolated from workers.

Discussion

It is now becoming clear that the epidemiology of multidrug-resistant organisms has changed and is no longer confined to the hospital setting(12). ESBL, carbapenemase producers and colistin resistant Gram-negative bacilli are frequently detected in livestock, pets and wild type animals (4). The poultry production system is of special interest since it forms a complex and vulnerable ecosystem that can be easily hacked by resistant organisms. Indeed, once introduced, these latter can disseminate nationally but also globally due to the frequent import/export of broilers worldwide (44). Moreover, it has been shown that resistant organisms in food producing animals can be readily transmitted to humans via direct or indirect contact (20) and via environmental routes (24). In their study, Laube et.al reported the detection of ESBL/ampC producing E. coli strains from broilers fecal samples (100%), dust samples (71%), litter (95%), farmers' boot swabs (90%) in addition to 54% of different environmental swabs such as scales water and feeding troughs (4).

Following our first detection of the mcr-1 positive strain of E. coli in poultry in southern Lebanon in 2015(4) and in addition to the high abundance of ESBL/ampC producers detected at the national level in chicken farms during the same year (4); we found it crucial to return to the same farm in southern Lebanon where we found the mcr-1 strain and explore the evolution of bacterial resistance in chicken. It is important to mention that from 2015 to 2017 no infection control measures were taken in the chicken farm. In addition, gentamicin and colistin were often prescribed as treatment for gastrointestinal infections and doxycycline for respiratory infections. Moreover, it should be mentioned that although the veterinarian of the visited farm stated that antibiotics are only administered for therapeutic purposes, he also admitted that once an infection occurs, the antibiotic is provided for the entire herd and not only for the sick animal. In other words, the antibiotic is theoretically prescribed only for therapeutic purposes but technically is also administered as prophylaxis. Our study shows that from 2015 to 2017, the prevalence of ESBL/ampC producers has significantly increased

from 27% to 59% in Saida – south of Lebanon. In addition, mcr-1 positive strains are highly prevalent in the chicken feces but also in feed, litter and workers. The presence of multi-drug resistance in feed samples is questionable and can have two plausible explanations: first that these resistant organisms are contamination from the farm housing environment as some studies have suggested (45); or it can be due to the hidden use of antibiotics as growth promoters in this farm.

In our investigation, we found that ESBL producers were more resistant to gentamicin compared to the mcr-1 positive isolates. Conversely, mcr-1 strains were more resistant to colistin. This suggests that ESBL producing Gram-negative bacilli are co-selected with the frequent use of gentamicin, while mcr-1 strains are selected with colistin use in the chicken farm. Gentamicin was previously described as being among the most common antibiotic administered to livestock in Lebanon (29). Rami et.a1 demonstrated a significant correlation between the use of gentamicin and tetracycline as growth promoters and the corresponding number of resistant E. coli strains in poultry farms (46). Similarly, another study showed an association between the use of gentamicin as food additive and the number of gentamicin resistant E. coli isolates (47).

Constructed MSP dendrogram (figure 2) reveals no cluster formation, at either the geographical location or at the phenotypic level. MLST analysis of mcr-1 E. coli strains also revealed the presence of different sequence types in chicken, workers and environment, except the detection of two mcr-1 colistin-resistant E. coli strains sharing the same sequence type “ST101” and phenotype from chicken and feed. ST101 is an international ST described in broilers (48), pigs (49) and clinical settings (50). Many studies even associated ST101 to clinical E. coli strains harboring NDM-1 in Canada, Germany, UK, Australia and Pakistan (50). ST101 is thus a potent candidate for the zoonotic transmission to humans. Furthermore, ST746 was shared between mcr-1 E. coli strains detected in chicken and poultry litter. Again, this ST has been reported in animals (24) as well as in OXA-48 producing E. coli strains isolated in clinical settings (51). The variety of sequence types detected together with the MSP dendrogram patterns observed suggest that the dissemination of bacterial resistance from 2015 to 2017 is multi-clonal and is related to the diffusion of plasmids carrying ESBL and mcr-1 genes.

To summarize, this study reported the dissemination of mcr-1 E. coli strains in broilers, farmers and the surrounding environment in Lebanon. The overuse of antibiotics seems to have played a key role in the massive spread of colistin resistance since the first detection of mcr-1 in 2015 (32). Colistin use in animals should be banned in the Lebanese veterinary

medicine. Moreover, the relatively high abundance of multi-drug resistance in all sources emphasizes the hypothesis that when aiming to control the dissemination of resistant organisms; besides controlling antibiotic use, environmental routes should be also targeted. Moreover, it is worth mentioning that this study is the first in Lebanon to report the isolation of mcr-1 positive E. coli strains from humans. Okdah et. al previously reported the detection of colistin-resistant K. pneumoniae strains isolated from patients in Beirut (52). However, in these latter, the mechanism of colistin resistance was due to mutations in the phoP/Q, pmrA/B and mgrB genes (52). Therefore, our study points out that mcr-1 is present in the Lebanese farmers and might be introduced to the community and hospital settings if no strict infection control measures in animals and their surrounding environment are implemented. Further works are warranted to quantify the magnitude of this emerging problem in Lebanon.

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Transparency Declarations

No conflict of interest or financial disclosure for all authors.

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531 **Table 1.** Primers and Probes used for the detection of mcr genes via RT-PCR in this study.

Target gene	Primer/Probe Name	Sequence (5' - 3')	Amplicon size (base pair)	Reference
mcr-1	PE_F1	GCAGCATACTTCTGTGTGGTAC	145	chabou
	PE-R1	ACAAAGCCGAGATTGTCCGCG		
	PE-Probe1	6 FAM –GACCGCGACCGCCAATCTTACC-TAMRA		
mcr-2	mcr-1.2-RT-F	CTGTGCCGTGTATGTTCAGC	151	
	mcr-1.2-RT-R	TTATCCATCACGCCTTTTGAG		
	mcr-2-VIC1	VIC-TGACCGCTTGGGTGTGGGTA-TAMRA		
mcr-3	mcr-3-RT-F1	TGAATCACTGGGAGCATTAGGGC	144	This study
	mcr-3-RT-R1	TGCTGCAAACACGCCATATCAAC		
	mcr-3-PE1	6 FAM-TGCACCGGATGATCAGACCCGT-TAMRA		
mcr-4	mcr-4-RT-F1	GCCAACCAATGCTCATACCCAAAA	112	This study
	mcr-4-RT-R1	CCGCCCCATTCGTGAAAACATAC		
	mcr-4-PE1	6 FAM-GCCACGGCGGTGTCTCTACCC-TAMRA		
mcr-5	mcr-5-RT-F1	TATCCCGCAAGCTACCGACGC	126	This study
	mcr-5-RT-R1	ACGGGCAAGCACATGATCGGT		
	mcr-5-PE1	6 FAM-TGCGACACCACCGATCTGGCCA-TAMRA		

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Table 2. Resistance Profiles of Gram-negative bacilli isolated in this study.

		Antibiotic Susceptibility testing														Phenotype				Genotype		
Source	Species	AMP	FOX	ATM	CTX	TZP	FEP	AMC	CAZ	CARB	COL**	GNT	SXT	CIP	TGC	% of ESBL producers	% of ampC producers	% of ESBL/ampC producers	% of colistin-resistant mcr-1 positives	% of CTX-M positives	% of TEM positives	% of SHV positives
Chicken	E. coli (n = 302)	298 (99)	175 (58)	177 (59)	144 (48)	35 (12)	101 (33)	158 (52)	165 (55)	0	284 (94)	237 (78)	250 (83)	285 (94)	12 (4)	33	18	9	81	55	91	18
	K.pneumoniae (n = 30)	30 (100)	12 (40)	1 (3)	1 (3)	4 (13)	1 (3)	18 (60)	1 (3)	0	30 (100)	29 (97)	30 (100)	30 (100)	0	3			100			
Workers	E. coli (n = 10)	9 (90)	1 (10)	5 (50)	5 (50)	0	5 (50)	2 (20)	4 (40)	0	6 (60)	6 (60)	5 (50)	7 (70)	0	50	10		60	100	20	
	K.pneumoniae (n = 1)	1 (100)	0	0	0	0	0	0	0	0	1 (100)	1 (100)	1 (100)	1 (100)	0				100			
Litter	E. coli (n = 18)	17 (94)	13 (72)	17 (94)	15 (83)	0	11 (61)	11 (61)	13 (72)	0	1 (6)	15 (83)	17 (94)	17 (94)	0	61		33	6	94	94	
Feed	A. baumanii* (n = 3)	3 (100)	3 (100)	3 (100)	3 (100)	0	3 (100)	3 (100)	3 (100)	0	1 (33)	0	0	1 (33)	0	67		33		100	67	
	P. aeruginosa* (n = 3)	3 (100)	2 (67)	1 (33)	1 (33)	0	1 (33)	2 (67)	0	0	0	1 (33)	3 (100)	1 (33)	1 (33)	100				100	67	
	S. rubideae (n = 1)	1 (100)	1 (100)	0	1 (100)	0	0	1 (100)	0	0	0	0	1 (100)	0	1 (100)	100				100		
	A. xylosoxidans (n = 1)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	0	0	1 (100)	0	1 (100)	0	100				100		
	E. coli (n = 2)	2 (100)	0	0	0	0	0	0	0	0	2 (100)	2 (100)	2 (100)	2 (100)	0				100			
Soil	P. putida* (n = 4)	4 (100)	4 (100)	4 (100)	4 (100)	0	4 (100)	4 (100)	4 (100)	0	0	0	4 (100)	0	4 (100)	50	50			50	50	
	P. monteilii* (n = 2)	2 (100)	2 (100)	2 (100)	2 (100)	0	2 (100)	2 (100)	1 (50)	0	0	0	2 (100)	2 (100)	2 (100)	100				100	50	
	A. baumannii* (n = 3)	3 (100)	3 (100)	3 (100)	3 (100)	2 (67)	3 (100)	3 (100)	3 (100)	0	0	2 (67)	2 (67)	2 (67)	2 (67)		33	67		67	33	
	S. maltophilia (n = 4)	4 (100)	4 (100)	4 (100)	4 (100)	1 (25)	2 (50)	4 (100)	1 (25)	0	0	0	4 (100)	1 (25)	3 (75)	50				50	50	25
	E. cloacae (n = 4)	4 (100)	4 (100)	4 (100)	4 (100)	2 (50)	4 (100)	4 (100)	3 (75)	0	0	3 (75)	3 (75)	3 (75)	0	50		50		25	75	25
	E. coli (n = 5)	5 (100)	4 (80)	5 (100)	4 (80)	0	3 (60)	4 (80)	5 (100)	0	0	2 (40)	5 (100)	4 (80)	3 (60)	100				80	100	20
	O. haematophilium (n = 1)	1 (100)	1 (100)	1 (100)	1 (100)	0	1 (100)	1 (100)	1 (100)	0	0	0	1 (100)	0	1 (100)	100						

Resistance profiles are presented as number (percentage).

*: susceptibility to carbapenem was based on imipenem and meropenem.

**: colistin resistance was determined by colistin broth micro-dilution test.

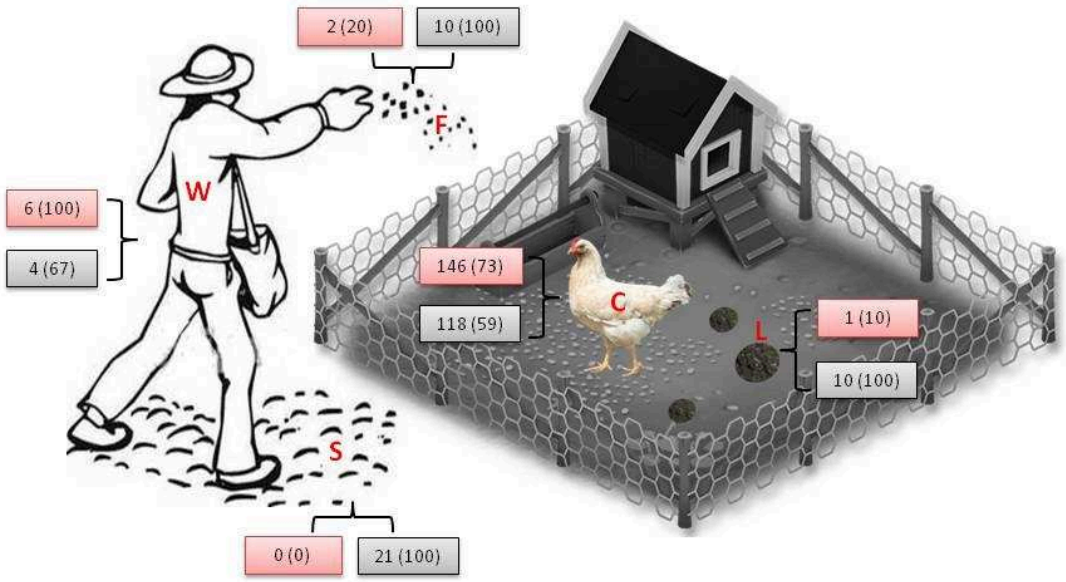
n = number, % = percentage, AMP = ampicillin, CTX = cefotaxime, AZT = aztreonam, FOX = cefoxitin, CAZ = ceftazidime, AMC = amoxicillin-clavulanic acid, FEP = cefepime, TZP = piperacillin-tazobactam, CARB= carbapenems i.e. imipenem, meropenem and ertapenem, COL = colistin, TGC = tigecycline, SXT = trimethoprim-sulfamethoxazole, CIP = ciprofloxacin, GNT = gentamicin

Figure Legends

Figure 1. Prevalence of colistin-resistant and ESBL/ampC producing Gram-negative bacilli in chicken, farmers and environment. Prevalence is expressed as "number of positive samples (percentage)" C = chicken, W = worker, S = soil, L = litter, F = feed. Red highlight = colistin resistance, Black highlight = ESBL/ampC.

Figure 2. MSP Dendrogram of A) E. coli strains isolated from Chicken in 2015, B) negative ESBL positive mcr-1 isolates and ESBL E. coli strains isolated from chicken in 2017 and C) E. coli strains isolated from chicken in Saida region in 2015 along with the ESBL and negative ESBL positive mcr-1 E. coli strains isolated from chicken in 2017.

Figure 3. A) Comparison of the antibiotic and resistance genes prevalence in ESBL E. coli strains isolated from Saida region in 2015 and the ESBL strains E. coli strains isolated from chicken in 2017 B) Comparison of gentamicin and colistin resistance prevalence in ESBL and non ESBL mcr-1 positive E. coli strains isolated in 2017. ǂ = P value ≤ 0.05



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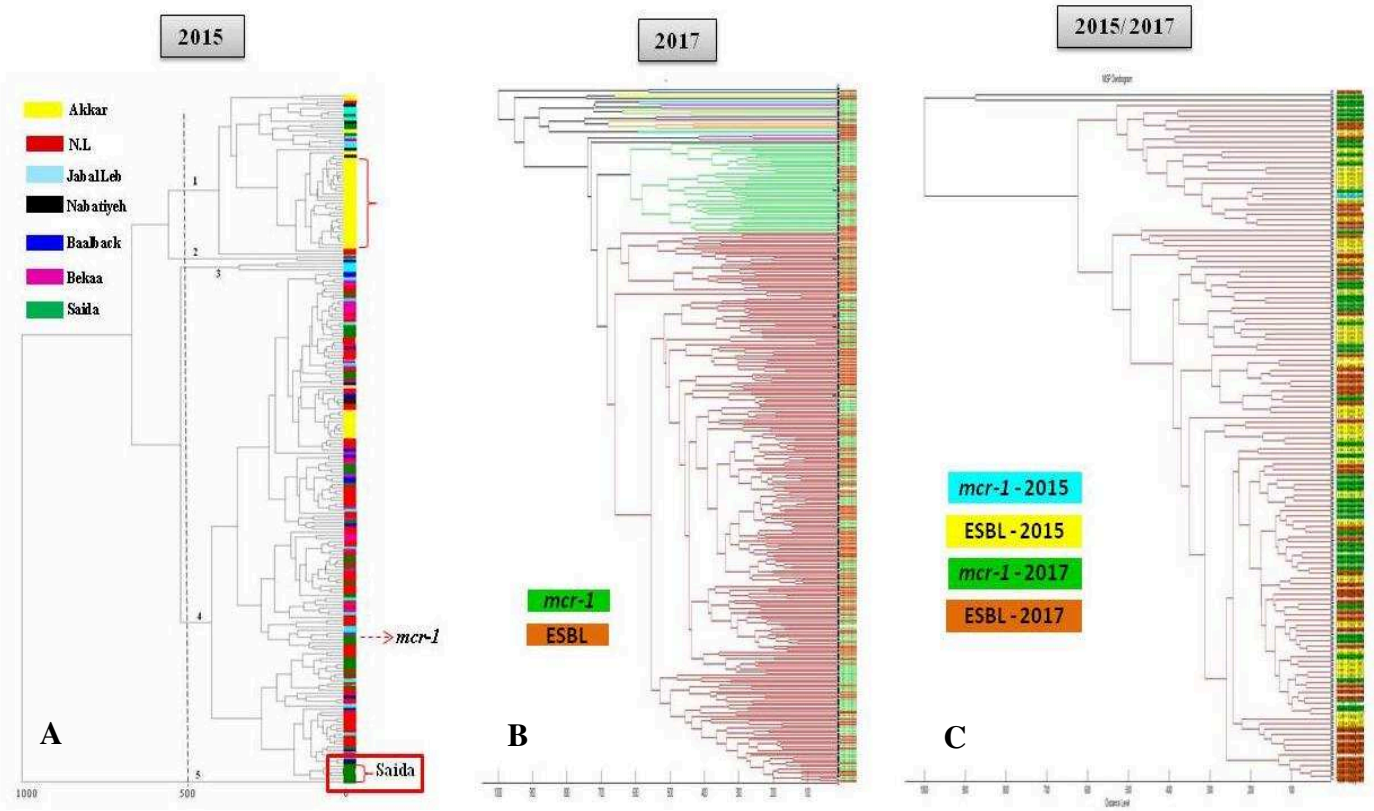

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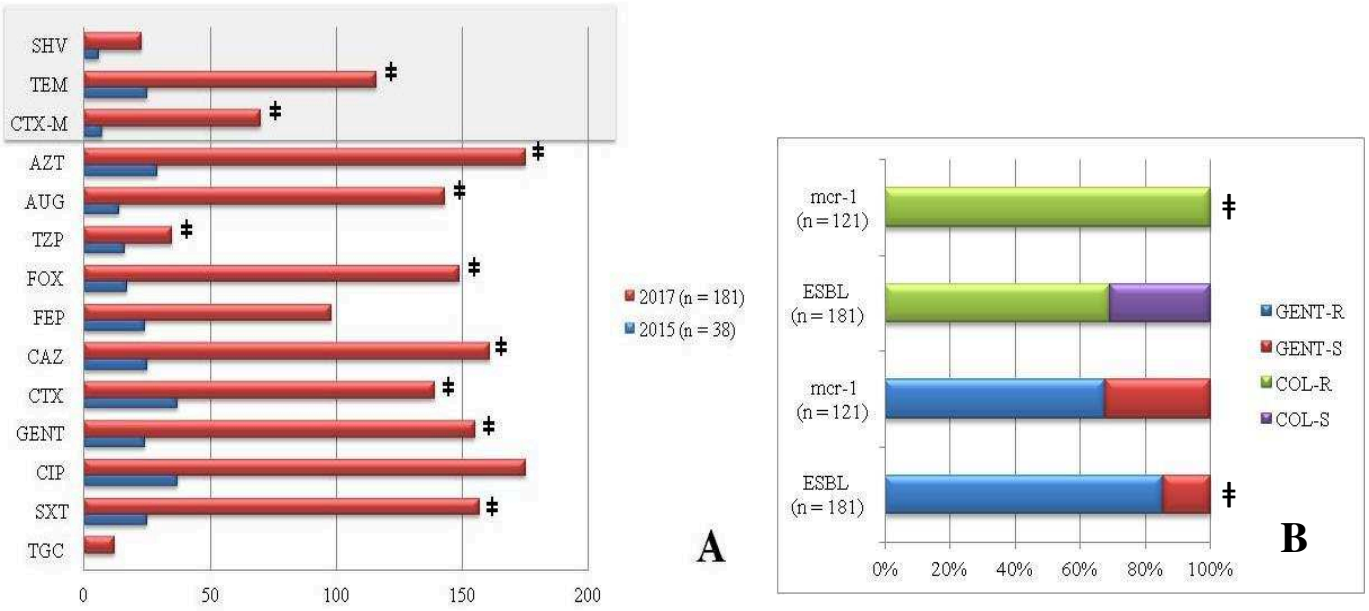

Figure 3. A) Comparison of the antibiotic and resistance genes prevalence in ESBL E. coli strains isolated from Saida region in 2015 and the ESBL strains E. coli strains isolated from chicken in 2017 B) Comparison of gentamicin and colistin resistance prevalence in ESBL and non ESBL mcr-1 positive E. coli strains isolated in 2017. ǂ = P value ≤0.05

Conclusion of Chapter II

In Lebanon, ESBL and ampC producing Gram-negative bacilli are highly prevalent in chicken and swine farms. The main genes promoting beta lactam resistance were CMY, TEM and CTX-M beta lactamases (1). Like the studies conducted in cattle in Lebanon (2) and in several countries worldwide (3) (4), we have found that chicken and pigs are hidden reservoirs of mcr-1 colistin resistant Gram-negative bacilli. The dissemination of mcr-1 is huge in pigs, chicken and surprisingly in the farm's workers. The dissemination of resistance in poultry in Lebanon appears to be multi-clonal and mediated by the diffusion of plasmids carrying resistance genes. Questionable sanitary conditions, food quality, waste management and antibiotic consumption are all potent contributors to the emergence and spread of ESBL/ampC and colistin resistant Gram-negative bacilli in farm animals of Lebanon. Besides banning colistin use in the veterinary section in Lebanon; future work should rely on the possible infection control measures that can be taken at the national level in order to limit the dissemination of colistin resistance in livestock. In addition, surveillance studies targeting the spread of mcr-1 colistin resistant Gram-negative bacilli are warranted in the clinical and community settings of Lebanon in order to quantify the magnitude of this emerging problem.

During our surveillance study in 2015 we have isolated a colistin hetero-resistant Enterobacter cloacae strain from a chicken farm in the south of Lebanon. The strain was mcr negative and the mechanism of colistin resistance was unknown; hence the aim of the third chapter of this manuscript.

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Chapitre III

Genomic Analysis of a colistin Hetero-resistant Enterobacter cloacae isolate.

Introduction

Colistin belongs to the polymyxin family of antibiotics (1). Previously abandoned due to its nephrotoxicity and neurotoxicity inside the human body; colistin was re-introduced into the clinical settings in view of the dissemination of carbapenem resistant Gram-negative bacilli (2). The use of colistin was thereafter faced with the emergence of colistin resistance. In Gram-negative bacilli, this latter is mediated either through the acquisition of a mcr colistin resistance gene or via chromosomal mutations that promotes the modification of the lipid A moiety of the lipopolysaccharide chain (3). More recent studies highlighted the contribution of the resistance nodulation division (RND) family of efflux pumps in resistance to colistin in Gram-negative bacilli (4).

Article 7 entitled **"Colistin Hetero-resistance in Enterobacter cloacae from Lebanon mediated by over-expression of acrAB-tolC efflux pump through inactivation of acrR local repressor gene"**, we investigated the mechanism of colistin hetero-resistance in an Enterobacter cloacae strain isolated in 2015 from a chicken farm located in the south of Lebanon. The strain was mcr negative and presented with an elevated colistin MIC up to 1024µg/ml. New primers were designed in order to explore any mutations in the pmrA, pmrB, phoP, phoQ and mgrB genes. Carbonyl Cyanide m-Chlorophenylhydrazine test, quantitative RT-PCR to determine any over-expression of acrAB/tolC efflux pump as well as whole genome sequencing were used to decipher the mechanism of colistin hetero-resistance in this isolate. The strain presented with an elevated colistin MIC up to 1024µg/ml and had no mutations in the genes commonly known to mediate colistin resistance in Gram-negative bacilli. qRT-PCR showed an over-expression of the acrAB-tolC efflux pumps. Using whole genome sequencing, it appears that this over-expression was mediated by a deletion of three amino acids in the local repressor gene "acrR" of the acrAB-tolC efflux pump.

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Article 7

Colistin Hetero-resistance in Enterobacter cloacae from Lebanon mediated by over-expression of acrAB-tolC efflux pump through inactivation of acrR local repressor gene.

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Colistin hetero-resistance in Enterobacter cloacae from Lebanon mediated by over-expression of acrAB-tolC efflux pump through inactivation of the acrR local repressor gene.

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Abstract

Objectives

Nowadays, the dissemination of colistin resistance has raised major concerns. Indeed, colistin is currently considered the last resort therapeutic agent against multi-drug resistant organisms. During a surveillance conducted in chicken farms in Lebanon in 2015, we isolated a colistin hetero-resistant Enterobacter cloacae strain. The aim of this study was to explore the mechanism of colistin hetero-resistance in this atypical E. cloacae isolate.

Methods

Carbonyl Cyanide m-Chlorophenylhydrazine test, mRNA quantification and whole genome sequencing were used to decipher the mechanism of colistin hetero-resistance in the isolated E. cloacae strain from chicken.

Results

The strain E. cloacae isolated from in southern Lebanon in 2015 was an ampC producer harboring the MIR-20 gene and was hetero-resistant to colistin with an MIC of 1024 μg/ml. The strain was positive with the CCCP test and showed an over-expression of the acrAB-tolC efflux pump. Whole genome sequencing revealed a deletion of three amino acids in the acrAB-tolC local repressor gene "acrR"; this mutation was annotated as deleterious with PROVEAN.

Conclusion

We have recently reported that colistin hetero-resistance in E. cloacae could be mediated by the over-expression of the acrAB-tolC efflux pump. This study highlighted the importance of efflux pumps repressors in controlling the susceptibility of Gram-negative bacilli toward colistin.

Introduction

Enterobacter species including Enterobacter cloacae are ubiquitous opportunistic pathogens that are widely encountered in nature and in human/ animals' intestinal microbiota (1). Multi-drug resistance in these species occurs via the production of ampC beta lactamases, ESBL and carbapenemases (2). Recently, colistin resistance has also emerged in Enterobacter spp. This latter is provoked via the acquisition of mcr colistin resistance gene or via chromosomal mutations that lead to the modification of the lipid A moiety of the lipopolysaccharide chain (3). Other mechanisms of colistin resistance include capsule formation and efflux pump utilization (3). In fact, it has been shown that efflux pumps are key players in the intrinsic resistance of bacterial species against a variety of substances including detergents, dyes and antimicrobial agents (4). Among others, the resistance nodulation division family of efflux pumps has been described in the literature as a potent contributor to the multi-drug resistance phenotype observed in clinically relevant bacterial species (5)(6). This is mainly owing to their broad spectrum of substrate specificity and their tripartite structure that allows the exclusion of molecules outside the bacterial cell directly from the cytosol and the cytoplasmic space (5).

During surveillance study conducted in Lebanon in 2015, one colistin hetero-resistant E. cloacae strain was isolated from poultry in the south. Colistin hetero-resistance is defined as colistin susceptible isolates with MIC below 2 µg/mL from which a subpopulation growing in the presence of >2µg/mL of colistin are detected (6). The mechanisms of colistin resistance are not well understood in Enterobacter species. The aim of this study was therefore to explore the mechanism of the colistin hetero-resistance phenotype observed.

Materials and methods

Samples collection and strain isolation

In August 2015, we conducted a nationwide surveillance study in Lebanon, aiming at determining the prevalence of ESBL/ampC producers in poultry (2). In brief, 981 fecal swabs were collected from chicken farms distributed over the seven districts in Lebanon. The swabs were subcultured on a selective medium for the screening of beta lactamase producers. MALDI-TOF MS spectrometry was used for bacterial identification (2).

Phenotypic testing

Antibiotic susceptibility testing was performed as previously described (2). Double disk synergy test, ampC disk test and carba NP test, were used for the detection of different beta

lactamases (2). Broth micro-dilution test was performed for colistin MIC determination (7). Furthermore, Carbonyl Cyanide m-Chlorophenylhydrazine (CCCP) test was done to assess the possible contribution of efflux pumps to colistin resistance (8).

Molecular characterization of colistin resistant and beta lactamase genes

Colistin resistance genes phoP, phoQ, pmrA, pmrB and mgrB were amplified and sequenced using newly designed primers (supplementary table 1). RT-PCR analysis was used for the detection of CTX-M, SHV, TEM and mcr-1/2 genes (2)(7). Furthermore, simplex PCR assays and sequencing were conducted for the screening of FOX, MOX, ACC, EBC, DHA, CMY ampC beta lactamase genes (2).

Quantitative RT-PCR of acrAB-tolC efflux pump

Total bacterial RNA was extracted using the TRI REAGENT® - RNA /DNA /PROTEIN ISOLATION REAGENT kit (Thermofisher) and quantified with the Nano-Drop ND-1000-UV-Vis Spectrophotometer (Applied Biosystems, Carlsbad, CA, USA). Using Super Script Platinum One-Step Quantitative RT-PCR system with ROX kit (Thermo Fisher Scientific Inc.) the transcriptional levels of acrA, acrB and tolC genes were quantified (6). The rpoB housekeeping gene was used as internal control. The fold change in gene expression was calculated by the comparative threshold cycle (CT) method (6). Colistin susceptible E. cloacae NH141 (6) was used for the comparative analysis of acrAB-tolC efflux pump expression.

Whole genome sequencing and annotation

Total genomic DNA of the isolated colistin hetero-resistant E. cloacae was sequenced on the MiSeq sequencer (Illumina, San Diego, CA, USA) with the Mate Pair strategy (6). Genomic assembly was done using CLC genomics WB4 version 4.9 and A5-miseq pipeline (6). Multiple genomic sequence alignment was performed with Mauve alignment tool (6). Genome annotation was done by Rapid Annotation using Subsystem Technology (RAST) (9). The nucleotide and protein sequences obtained were blasted against GenBank database (10). The Sequence type of the isolated strain was identified using the center for genomic epidemiology MLST1.8 (11). ARG-ANNOT was used for the detection of antibiotic resistance genes in Silico(6). Protein Variation Effect Analyzer (PROVEAN) was used to predict the functional effect of amino acids mutations within protein sequences (12).

Results

Phenotypic analysis

The isolated E. cloacae strain was susceptible to carbapenems, resistant to cefotaxime, cefoxitin, ceftazidime, ciprofloxacin, gentamicin and trimethoprim-sulfamethoxazole and was surprisingly hetero-resistant to colistin (supplementary figure 1 A). AmpC disk test was positive. Broth micro-dilution test revealed that this isolate had a colistin MIC of 1024µg/ml. As shown in supplementary Figure 1, the E. cloacae was hetero-resistant to colistin and upon the addition of CCCP, the resistant subpopulation have disappeared.

Genotypic and transcriptional analysis

PCR amplification and sequencing showed that the strain harbored the MIR-20 ampC beta lactamase gene. No mcr colistin resistance genes were detected. Furthermore, no mutations were found in the pmrA/B, phoP/Q and mgrB genes. Quantitative RT-PCR revealed an over-expression of the acrAB-tolC efflux pump in the colistin hetero-resistant E. cloacae strain compared to the susceptible one (supplementary table 2). In order to investigate the mechanism of efflux pump over-expression, whole genome sequencing was thus performed.

Genome analysis

The colistin hetero-resistant E. cloacae genome was 5 444 571 bp long with 55% GC content. Three plasmids were identified: IncHI2, IncHI2A and IncA/C2. The genome is composed of 5107 protein coding sequences and 77 RNAs. In silico analysis revealed the presence of resistance genes against aminoglycosides “AadA1 and AadA2”, trimethoprim-sulfamethoxazole “SULI”, fluoroquinolones “FlqOqxBgb and FlqOqxA”, florfenicol “FloR” and macrolides “MphE” (table 1). MLST 1.8 showed that the strain belonged to ST523. Analysis of the nucleotide and protein sequence showed the presence of truncated ompF and pmrC genes. Furthermore, a deletion of three amino acids “DLE” at position 72-74 in the acrAB-tolC local repressor gene “acrR” gene was detected. This latter mutation was annotated by PROVEAN as being deleterious with a score of “-16.544” (figure 1).

Discussion

Recently, evidence has shown that livestock are contributors to the dissemination of multi-drug resistance in humans (2). Colistin hetero-resistance is of particular interest in the clinical settings; since this latter cannot be easily discriminated based on routine diagnostic testing. As a consequence, upon exposure to colistin, the undetected resistant subpopulation might

proliferate and lead to therapeutic failures in addition to inducing cross resistance to the host antimicrobial lyzosyme(6). In GNB, colistin hetero-resistance was previously described in Acinetobacter baumannii, Klebsiella pneumoniae and in Enterobacter spp (6). In their study, Guerin et al. attributed colistin hetero-resistance in clinical isolates of E. cloacae to the expression of the arn operon and the phoP/Q two component system (13). More recently, we have shown that over-expression of the acrAB-tolC efflux pumps mediated by naturally produced level of soxRS induces colistin hetero-resistance in E. cloacae clinical isolates (6). In our isolated colistin hetero-resistant E. cloacae strain, no mutations were detected in the genes commonly known to promote colistin resistance in GNB. Based on the aforementioned study conducted by Telke et al., the over-production of efflux pump was thus suggested. The colistin hetero-resistant E. cloacae strain was positive with the cccp test revealing a possible contribution of efflux pumps to colistin resistance. CCCP is an efflux pump inhibitor that increases the bacterial membrane permeability by interfering with the proton motive force and electrochemical gradient (14) . Transcriptional analysis revealed an over-expression of the acrAB-tolC efflux pump in the colistin hetero-resistant E. cloacae. Indeed, in the literature, the over-expression of efflux pumps was mainly attributed to mutations in the local repressor genes, global regulatory gene, promoter region of the transporter gene or insertion elements upstream the transporter gene (5). Our findings are consistent with the previous studies, in that a deleterious deletion of three amino acids was found in the local repressor gene of the acrAB-tolC efflux pump “acrR”. The acrAB-tolC efflux pump system in E. cloacae is similar to the one described in E. coli (15). The expression of this efflux pump is tightly regulated by the local repressor acrR and global activators marA, Rob and soxRS (16). acrR mutation mediating over-expression of acrAB-tolC was previously reported as being responsible for tigecycline and ciprofloxacin resistance in E. coli strains (17)(18). Warner et al. found that deletion of the acrR gene affected polymyxin B susceptibility of E. coli strains when grown in Luria broth (16). As for E. cloacae, our study is the first to associate the mutation of the acrR gene with colistin resistance by acrAB-tolC over-expression. The truncated ompF porin in the isolated colistin hetero-resistant E. cloacae might have contributed to the resistance pattern observed towards fluoroquinolones and beta-lactam antibiotics (19). As for the truncated pmrC gene, more genomic work is warranted in order to decipher the effect of pmrC inactivation on colistin susceptibility in GNB. pmrC was reported in the literature as a mediator of colistin resistance via its over-expression and subsequent addition of a pEtN group to the LPS chain (20), however the effect of its inactivation on antibiotic resistance remains unknown.

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Table 1. Phenotypic versus genotypic characteristics of the colistin hetero-resistant Enterobacter cloacae

Strain	Sequence Type	Isolation date	Sample origin	Sample type	Colistin MIC	Resistance profile	Resistance genes	Chromosomal mutations
E. cloacae	ST523	Aug-15	Chicken	Fecal swab	1024 µg/ml	CTX, CAZ, FOX, GENT SXT CIP	MIR-20, AadA1/AadA2, SULI, FlqOqxBgb/FlqOqxA, FloR, MphE	acrR: D72_E74del S75G pmrC: Codon Stop71C ompF: Codon Stop136V

Aug-15 = August 2015, CTX = cefotaxime, CAZ = ceftazidime, FOX = cefoxitin, GENT = gentamicin, CIP = ciprofloxacin, SXT = trimethoprim-sulfamethoxazole

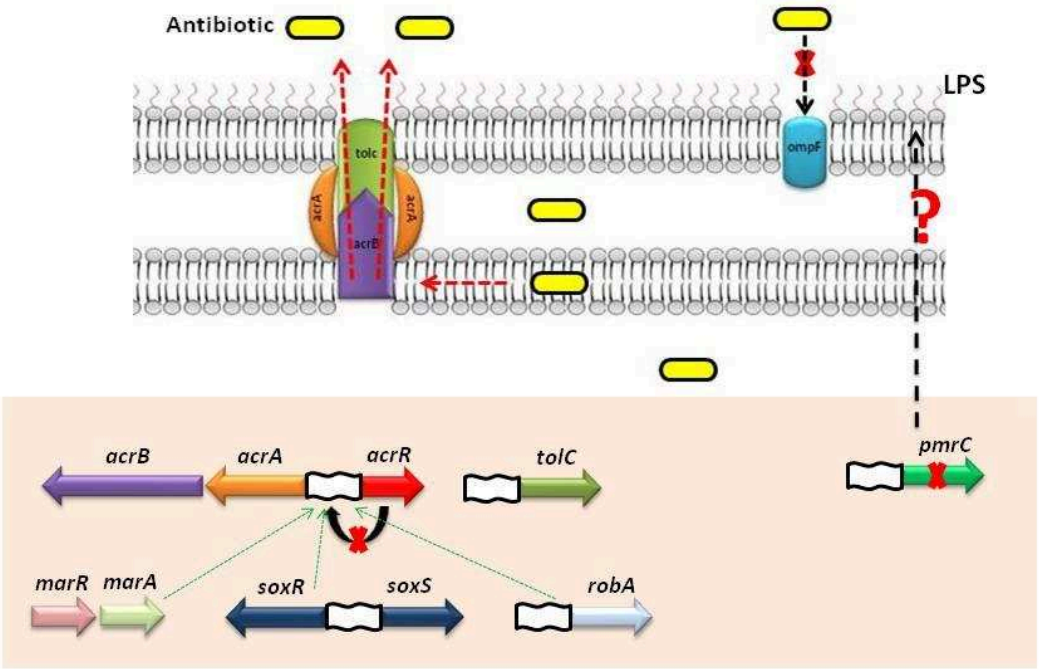

Figure 1. Suggested mechanism of colistin hetero-resistance in the isolated E. cloacae. Green arrows: activators, red arrow: repressor.

Supplementary Material

Table 1. Primers designed for colistin resistance genes amplification in this study

Target Gene	Primer name	Sequence (5' - 3')	Length (base pair)	Amplicon size (base pair)	Annealing Temperature
mgrB	mgrB-F	CCATTTCACCACCTCAATAAAAA	23	296	55°C
	mgrB-R	TGACAGTACAGTTAGCCCCTGTT	23		
phoP	phoP-E-F	CCACAACAACATAATCAGCGTTA	23	980	55°C
	phoP-E-R	GCCAGGGTATAAAACAGATTGCT	23		
	phoP-I-F	GAAGACGGTCTGTCGCTAATTC	22	360	55°C
	phoP-I-R	GAGCATTAAGGAATCTTTGCTCA	23		
phoQ	phoQ-E-F	CAGGCTCTTACTGACTCGGATTA	23	1630	55°C
	phoQ-E-R	GACGTTTGCGTAAGAAAATTCAG	23		
	phoQ-I-F	GTTCACCCCTTTACGCTGATAC	22	668	55°C
	phoQ-I-R	AGCAAAAGCTGAACGAGATCC	21		
pmrA	pmrA-E-F	CAGCCAGATGACGCTTATCA	20	865	55°C
	pmrA-E-R	CGTATGGCATTCGTGCAGTA	20		
	pmrA-I-F	GGTTTGCAGATTCAGCGTAATA	22	394	55°C
	pmrA-I-R	AGACGATCTGTTATTGCAGGAAG	23		
pmrB	pmrB-E-F	AGGCGTCGAGTTCATCTACAAG	22	1198	55°C
	pmrB-E-R	CTTGAAGTCCACATTCACAACCT	23		
	pmrB-I-F	GTCACTGTAATCGTTGTCCCTGT	23	480	55°C
	pmrB-I-R	TCCTATGTATTCCGACATGGAAG	23		

F = forward, R = reverse, E = external fragment, I = internal fragment.

Table 2. Relative expression of acrAB/tolC in the colistin hetero-resistant E. cloacae

Gene	Col S- E.cloacae	Col R- E.cloacae	P value
tolC	1.0 ± 0.03	2.06 ± 0.04	<0.0001
acrA	1.0 ± 0.02	1.91 ± 0.07	0.0002
acrB	1.0 ± 0.05	1.93 ± 0.05	0.0003

Col S = colistin susceptible, Col R = colistin resistant.

Figure 1: CCCP test of the colistin hetero-resistant E. cloacae

Conclusion of Chapter III

Colistin is a polymixin B antibiotic that attacks the lipopolysaccharide and phospholipids in the outer cell membrane of Gram-negative bacilli, leading to cellular leakage and subsequent bacterial death(1). The mechanisms of resistance toward colistin in Gram-negative bacilli are diverse and are still not well understood in some species such as in Enterobacter spp (2). During the genomic analysis of the colistin hetero-resistant Enterobacter cloacae isolated from poultry in the south of Lebanon, we found that colistin hetero-resistance was mediated by an over-expression of acrAB/tolC efflux pumps promoted by a deletion of three amino acids in the local repressor gene acrR. The regulation of efflux pumps in Gram-negatives is complex and involves several local and global activators as well as repressor genes (3). The knowledge behind colistin resistance is still young and will absolutely in the future uncover more mechanisms that are nowadays unknown. These latter might include outer membrane proteins or even genes having different functions in different bacterial species and thus contributing differently to colistin resistance.

The surveillance of colistin resistance is not limited to livestock but is also warranted in the clinical settings especially in countries were no sufficient data are available such as in Algeria; hence the aim of the fourth chapter of this manuscript.

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Chapter IV

Collaborative Studies

Surveillance of colistin and carbapenem resistance in patients in Algeria.

Introduction

In this chapter, we present the collaborative study that I performed during my PhD studies in France. The prevalence of ESBL and carbapenemase producing Gram-negative bacilli is in a constant rise in the clinical settings (1). This increase necessitated the re-introduction of colistin into the human medicine as a last resort therapeutic agent against carbapenem resistant organisms (2). Recently, resistance to colistin has emerged and became prevalent in hospitals as well as in other ecosystems (3).

Article 8 entitled **"Colistin- and carbapenem-resistant Klebsiella pneumoniae clinical isolates, Algeria"** describes the detection of three Klebsiella pneumoniae strains isolated from patients at three different periods. The three isolates were of ST101 and carried the SHV-106, TEM-183 and CTX-M-15 ESBL genes. In addition two of them were carbapenem and colistin resistant via the production OXA-48 carbapenemase and mutated pmrA/B and mgrB gene, respectively.

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Article 8

Colistin- and carbapenem-resistant Klebsiella pneumoniae clinical isolates, Algeria.

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Colistin- and carbapenem-resistant Klebsiella pneumoniae clinical isolates, Algeria

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Abstract

This study investigates the molecular mechanisms of colistin and carbapenem resistance in Klebsiella pneumoniae ST101 strains. The three Klebsiella pneumoniae carried $bla_{CTX\text{-}M\text{-}15}$, $bla_{TEM\text{-}183}$ and $bla_{SHV\text{-}106}$ genes and two co-harbored $bla_{OXA\text{-}48}$. As for colistin resistance, the isolates had amino acid substitutions in pmrA/B and a truncated mgrB gene in one isolate.

Dear Sir,

The prevalence of extended spectrum β-lactamase (ESBL) and carbapenemases-producing Klebsiella pneumoniae isolates is constantly rising in clinical settings (1).Consequently, colistin, a previously abandoned antimicrobial agent due to its nephrotoxicity and neurotoxicity in humans, was re-introduced in clinical settings for the treatment of infections caused by multidrug-resistant (MDR) organisms (2). Recently, resistance to last resort antibiotics, namely colistin and carbapenems, has emerged and other resistant Gram- negative bacilli have been isolated in clinical settings worldwide (3,4).

In Algeria, the first report of a colistin-resistant isolate was published in 2015 and described an Acinetobacter baumannii ST 2 isolated from patients in the university Hospital center of Béni-Messous in Algiers. This isolate presented with a deleterious insertion of an amino acid named "Alanine" in the pmrB gene at position 163 (5). Thereafter, the mcr-1 plasmid-mediated colistin resistance gene was described in Escherichia coli after its isolation from animals as well as in clinical settings (6). Here we report the first detection of a colistin- resistant K. pneumoniae co-harboring OXA-48 carbapenemase which was isolated from a hospital in Algeria.

In 2016, three colistin-resistant K. pneumoniae isolates were recovered in Annaba University hospital, in Algeria, from three different patients who have in common an urogical surgery antecedent (Table). The patients were admitted to the infectious diseases unit for recurrent urinary tract infection, where urine cytobacteriology and antibiotic susceptibility testing were performed. Of note, two of the aforementioned patients had previously received colistin for treatment of their recurrent urinary tract infection.

Identification of the isolates was done using matrix-assisted laser desorption an ionization time-of-flight mass spectrometry (MALDI-TOF MS) (Microflex;Bruker Daltonics) (7). Antibiotic susceptibility testing was performed by disk diffusion method. Interpretation of results was done according to the European Committee following the Antimicrobial Susceptibility Testing (EUCAST) guidelines. The three isolates were resistant to ceftazidime, cefotaxime, ceftriaxone, cefoxitin, aztreonam, fosfomycin, gentamicin, ciprofloxacin, nalidixic acid, nitrofurantoin and colistin; however they remained sensitive to amikacin, trimethoprim/sulfamethoxazole and imipenem. In addition, two of the three K. pneumoniae strains were resistant to ertapenem. The minimum inhibitory concentration (MIC) of colistin, imipenem and ertapenem for isolates was determined by broth micro-dilution, which revealed that all isolates were resistant to colistin (MIC≥ 16µg/ml) with only two of them being also resistant to ertapenem (MIC≥ 4 µg/ml) (Table). It is to mention that sensitivity to imipenem was further tested using E-test. The latter revealed the presence of imipenem MICS of 0.25, 1.5, 1 mg/l in M5, M6 and M7 strains, respectively. The carbapenemase activity of the two carbapenem-resistant isolates (M6, M7) was

thereafter confirmed by a positive modified Carba-NP test performed as previously described (5) (Table).

MLST analysis, according to the Pasteur schemes available at the Institute Pasteur's MLST Web site (www.pasteur.fr/mlst/), revealed that all of them belonged to the same sequence type "ST101".

RT-PCR amplification of carbapenemases-encoding genes bla_{OXA-48}, bla_{NDM}, bla_{VIM}, bla_{KPC} and beta lactamase genes bla_{CTX-M}, bla_{TEM}, and bla_{SHV} showed that all isolates were positive for $bla_{CTX-M-15}$, $bla_{TEM-183}$ and $bla_{SHV-106}$,with only two co-harboring bla_{OXA-48}. None of the isolates expressed bla_{NDM} , bla_{VIM} or bla_{KPC}.

The molecular mechanism of colistin resistance was investigated by PCR amplification and sequencing of the pmrA, pmrB, phoP, phoQ, mgrB, mcr1 and mcr2 genes. The plasmid-mediated colistin resistance genes mcr-1 and mcr-2 were absent in the three K. pneumoniae strains. Sequence analysis revealed no mutations in phoP and phoQ genes but showed an inactivating insertion in the mgrB gene in one isolate (M5) on nucleotide 94 with 95% identity at the nucleotide level with IS 903B insertion sequence (IS5 family of insertion sequences). The A217V pmrA substitution was observed in two strains (M5, M6) with a mutation in the pmrB gene for the three isolates (Table).

Colistin is the last-line antibiotic for treatment of infections by Gram-negative bacteria such as K. pneumoniae and the ongoing emergence of colistin and carbapenem resistance represents a serious problem for the management of infections caused by these bacteria (8). This study is in accordance with recent studies that highlighted the emergence of colistin resistance in MDR K. pneumoniae arising from loss-of-function by inactivation of the mgrB gene and activation of the Pmr system inducing modification of the lipopolysaccharide (8–10). The A217V pmrA mutation showed in this study was reported in another case of K. pneumoniae colistin resistance in Serbia (3), also in a colistin-resistant clone of K. pneumoniae ST101 harboring bla_{oxa-48}. In this study, authors concluded that this mutation in pmrA could have played a role in the development of colistin resistance.

These data would strengthen the presumption that this mutation was responsible for colistin resistance. The T246A pmrB mutation was also showed in polymyxin B-resistant K. pneumoniae isolated from rectal swabs in Brazil {Formatting Citation}. In this study, the authors suggest that the specific pmrB (T246A) mutation found was not capable of producing polymyxin resistance alone, since this mutation was also found in polymyxin-susceptible isolates and was considered not deleterious by PROVEAN software. To our knowledge, all other pmrB mutations (V212G, T256A) have never been described (11).

There are only three reports of genomic investigation on colistin-resistant and carbapenemase-producing K. pneumoniae ST101 (Figure). Two of these strains (from Serbia and Turkey) were OXA-48-producing with amino acid changes in the pmrB gene (3,12) and the third one (from Tunisia) was also OXA-48 producing with mgrB truncated by the same 2- kb sequence insertion between nucleotides 123 and 124 of the mgrB coding sequence (13).

Thus, this is the first description of colistin-and carbapenem-resistant Klebsiella pneumoniae ST101 in Algeria. The analysis results of M5 colistin-resistant strains with mgrB truncation collected from individuals not treated with colistin shows that the clinical use of colistin may not be the only reason for the emergence of colistin resistance. Another possibility is the horizontal transmission between patients, who have in common a stay in the urological unit of the same hospital. Thus, a possible spread of nosocomial infections to a larger number of patients and healthy individuals should be prevented. It is urgent to establish a powerful monitoring system in each hospital with perfect coordination between all Algerian hospitals to detect as soon as possible an infectious epidemic and prevent the spread of such multidrug-resistant bacteria inducing infections that are difficult to treat (14).

Funding

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Conflict of interest

We have no conflict of interest to declare.

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Table 1. Description of colistin-and carbapenem-resistant K. pneumoniae isolates from Algeria

	Isolation date	Clinical sample	Colistin prescription	CT MIC (µg/ml)	IMP MIC (µg/ml)	ERT MIC (µg/ml)	*Bla* genes	*mgrB* mutations	*pmrA* mutations	*pmrB* mutations	*phoP/Q* mutations
M5	23/05/2016	Urine	NO	64	0.25	<1	$bla_{CTX-M-15}$, $bla_{SHV-106}$, $bla_{TEM-183}$	IS903B	A217V	V212G, T256A	WT
M6	20/10/2016	Urine	YES	16	2	8	$bla_{CTX-M-15}$, $bla_{SHV-106}$, $bla_{TEM-183}$, bla_{OXA-48}	WT	A217V	T246A	WT
M7	30/11/2016	Urine	YES	64	1	8	$bla_{CTX-M15}$,$bla_{SHV-106}$ $bla_{TEM-183}$, bla_{OXA-48}	WT	WT	T246A	WT

CT – colistin ; IMP – imipenem ; ERT – ertapenem; MIC – minimum inhibitory concentration ; WT – Wilde Type

Figure Legends

Figure 1. Geographical distribution of the various Klebsiella pneumoniae ST101 phenotypes by country origin (carbapenemase and colistin resistance). Other carbapenemases include KPC, NDM and OXA-181.

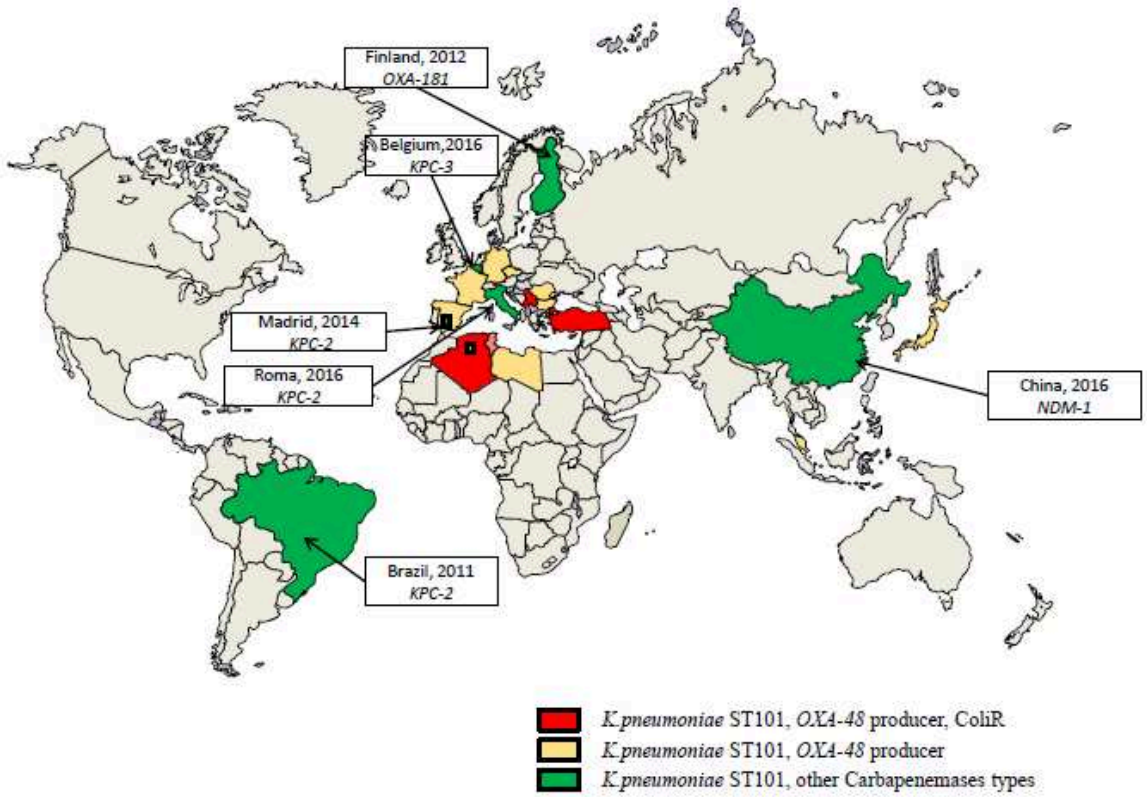

Figure 1. Geographical distribution of the various Klebsiella pneumoniae ST101 phenotypes by country origin (carbapenemase and colistin resistance). Other carbapenemases include KPC, NDM and OXA-181.

Conclusion of Chapter IV

In Algeria, the dissemination of multi-drug resistant Gram-negative bacilli has been previously well documented in the livestock (1) and clinical settings (2). In this study, the detection of similar Klebsiella pneumoniae strains in three different periods during the same year in the same hospital suggests an epidemic situation of colistin carbapenem co-resistance in the Algerian hospitals. Surveillance studies quantifying the magnitude of this issue in the clinical settings in Algeria are thus needed. Furthermore, the implementation of strict infection control measures including hand sanitization, isolation of infected patients in addition to the control of carbapenem and colistin prescription are warranted in these settings. Future studies should target the extent of the fecal carriage of these organisms and their subsequent introduction into the common population and the community settings in Algeria.

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Chapter V

Annex

Description of Lachnoclostridium Nov. species.

Introduction

Study of the human microbiota is one of the major challenges encountered in the 21st century (1). In the past 30 years, bacterial species were mainly identified using microbial cultures that allow the study of their antibiotic susceptibility testing, genome sequencing and proteomic studies (2). Thereafter, metagenomic analysis and 16S rRNA sequencing were introduced. These latter have dramatically increased the knowledge on the diversity of the human gut microbiome (3). However, despite all this improvement, 80% of the bacterial species forming the human microbiota are still uncultured. Recently, microbial culturomics was introduced by the team of Pr.Raoult. Microbial culturomic allows the use of different temperatures, pH, mineral and nutrients to cultivate previously unculturable bacterial species (3).

In **Article 9** entitled "**Genome sequence and description of Lachnoclostridium phoceense isolated from a patient in Marseille"**, we report the isolation of a new bacterial species of the genus Lachnoclostridium. The strain was isolated using microbial culturomics from the urine sample of a patient admitted to the hospital in Marseille.

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Article 9

Genome sequence and description of Lachnoclostridium phoceense isolated from a patient after kidney transplantation in Marseille.

Iman Dandachi, Sami Brahimi, Jean-Christophe Lagier, Ziad Daoud, Jean-Marc Rolain.

To be submitted

Genome sequence and description of Lachnoclostridium phoceense isolated from a patient after kidney transplantation in Marseille.

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Abstract

Lachnoclostridium phoceense is a new specie in the genus of Lachnoclostridium. Lachnoclostridium phoceense is a Gram-positive anaerobic rod. The strain (CSUR = P3177) with the below described genome was isolated from the urine sample of an old women after kidney transplantation. The strain genome is of 3 500 754 bp long with 50.62 % GC content and consisting of a single scaffold.

Abbreviations

CSUR: collection de souches de l'unité des Rickettsies

MALDI-TOF MS: matrix-assisted laser-desorption/ionization time of flight mass spectrometry.

MEPHI: microbes evolution phylogeny and infections.

NRPS: non ribosomal peptide synthetase

PKS: polyketide synthase

Introduction

Lachnoclostridium phoceense (CSUR = P3177) is a Gram-positive, motile, strictly anaerobic rods that was isolated from the urine sample of a patient in Marseille using culturomics. New bacterial species are usually described using 16S rDNA sequencing, genome percent of GC content, phylogenetic analysis and DNA-DNA hypridization (1). However, nowadays, a new "polyphasic approach" has been developed. The polyphasic approach combines the phenotypic criteria with the genomic ones in order to describe and characterize newly isolated species (2).

The Lachnoclostridium genus includes a variety of bacterial species including organisms from the Lachnospiraceae genus Incertae Sedis in SILVA, the genus Clostridium XIVa in the RDP and clostridial cluster XIVa of Collins et al. It involves thirty validly described species with most of them being of the Lachnospiraceae family (3).

Here we present the phenotypic and genomic characteristics of a Lachnoclostridium novel specie isolated from a patient admitted to the hospital in Marseille.

Materials and Methods

Phenotypic and biochemical characterization

Lachnoclostridium phoceense Marseille-P3177 was isolated from the urine sample of a 51 years old woman after kidney transplantation. At 37°C, the urine sample was initially incubated for 96 hours in an anaerobic blood culture bottle (BACTEC Lytic/10 Anaerobic/F Culture Vials; Becton-Dickinson, Pont de Claix, France) supplemented with 5% of sterilized rumen. Thereafter, incubated sample was streaked on a 5% sheep blood Columbia agar medium and incubated for 5 days under anaerobic conditions at 37°C. Indeed, for the growth of Lachnoclostridium, three temperatures were first tested: 25, 30 and 37°C. However, the optimal growth was only observed at 37°C after five days of incubation. Colonies grown on the Colombia agar were translucent and whitish circular with a 250-350 nm ranging diameter. Gram-staining revealed that the strains were Gram-positive bacilli. Furthermore, motility test was positive.

The Isolated strain was subjected to MALDI-TOF MS (Bruker Daltonics, Bremen, Germany) identification as previously described (4). No significant MALDI-TOF score was obtained showing thus that this strain is an unknown bacterial specie. The spectrum was therefore added to our data-base.

Biochemical characteristics of isolated colonies were determined using API ZYM (BioMerieux, France) and (BioMerieux, France). Catalase assays (Biomerieux) and Oxydase

ones (Becton, Dickinson and company, Le pont de Claix France) showed that the strains are oxidase/catalase negative. Results of this part in addition to antibiotic susceptibility testing are pending.

16S rRNA gene sequencing and phylogenetic analysis

The Isolated strain was subjected to 16S rRNA sequencing. Using the maximum-likelihood method Mega 6 software and CLUSTALW, a phylogenetic tree (figure 1) was constructed showing that the isolated Lachnoclostridium phoceense has 94.6% similarity with Lachnoclostridium contortum strain ATCC 25540. This value is lower than the gene sequence threshold "98.7% 16S rRNA" recommended by Ebers and Stackebrandt to characterize an isolated strain as a new bacterial specie without DNA-DNA hybridization.

Genome properties

The strain bacterial genome is of 3 500 754 bp long with 50.62 % GC content (table 1). It is composed of 1 scaffold (composed of 1 contig). Of the 3 382 predicted genes, 3 315 were protein-coding genes and 67 were RNAs (4 genes are 5S rRNA, 4 genes are 16S rRNA, 4 genes are 23S rRNA, 55 genes are TRNA genes). A total of 2 328 genes (70.23%) were assigned as putative function (by cogs or by NR blast). 170 genes were identified as ORFans (5.13%). The remaining genes were annotated as hypothetical proteins (719 genes => 21.69%). Detailed properties and statistics are presented in Table 2. Genes distribution into COG functional categories are presented in figure 2. Genome assembly and annotation was performed by XEGEN (http://www.xegen.fr/).

Genome annotation

Using the Bio-Edit interface, a BLAST search was conducted against ARG-ANNOT, a database for acquired antibiotic resistance genes (ARGs). The BLAST search was done under an e-value of 10−5, moderately stringent conditions for in silico ARG prediction (5). ARG-ANNOT BLAST search revealed the presence of one resistance gene against tetracycline. The bacteriocin database available in our research unit (Bacteriocins of the URMITE database BUR) (http://drissifatima.wix.com/bacteriocins) was done via the collection of all available sequences from NCBI and databases. Protein sequences from the aforementioned database allow the identification of bacteriocins from the human gut microbiota via BLASTp methodology (6). Resistome analysis via this database showed the presence of 25 bacteriocins genes.

The presence of polyketide synthases and Nonribosomal peptide synthetases (PKS/NRPS) was analyzed by gene discrimination with large size using a database constructed in our laboratory, predicted proteins were compared against non-redundant (nr) GenBank database using blastp and were then examined using antiSMASH (7).

Description of Lachnoclostridium phoceense Nov sp.

Lachnoclostridium phoceense strain P3177 is a new specie in the genus of Lachnoclostridium that was isolated from a 51 years old women urine sample after kidney transplantation in Marseille. The specie's optimal growth conditions are at 37°C for 5 days under anaerobic conditions. The colonies are of 0.25-0.35 mm diameter on blood supplemented agar. Lachnoclostridium phoceense is a strictly anaerobic Gram-positive rod. It is also is catalase and oxidase negative.

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Transparency Declarations

No conflicts of interest or financial disclosure for all authors.

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Table 1. Genes and Nucleotides content of the Lachnoclostridium phoceense genome

Variant	Value	% of the total
Genome Size (bp)	3500754	100
Number of GC (bp)	1772172	50.62259293
Total number of genes	3382	100
Total number of protein genes	3315	98.01892853
Total number of RNA genes	67	1.981076241
Total number of TRNA Genes	55	1.626256704
Total number of RNA (5S, 16S, 23S) Genes	12	0.354819626
Coding sequence size	3152738	90.05883026
Coding sequence gene protein size	3130485	89.42316437
Coding sequence tRNA gene size	4148	0.118488759
Coding sequence (5S, 16S, 23S) gene size	18105	0.517174304
Number of protein coding gene	3315	100

% = percent, bp = base pair

Table 2. Number of genes associated with the 26 general COG functional categories

Code	Value	% of total	Description
[J]	195	5.882353	Translation
[A]	0	0	Rna processing and modification
[K]	201	6.0633483	Transcription
[L]	107	3.227753	Replication, recombination and repair
[B]	0	0	Chromatin structure and dynamics
[D]	40	1.2066365	Cell cycle control, mitosis and meiosis
[Y]	0	0	Nuclear structure
[V]	89	2.6847663	Defense mechanisms
[T]	101	3.0467572	Signal transduction mechanisms
[M]	101	3.0467572	Cell wall/membrane biogenesis
[N]	12	0.36199096	Cell motility
[Z]	0	0	Cytoskeleton
[W]	2	0.06033183	Extracellular structures
[U]	28	0.8446456	Intracellular trafficking and secretion
[O]	78	2.3529413	Posttanslational modification, protein turnover,chaperones
[X]	48	1.4479638	Mobilome: prophages, transposons
[C]	111	3.3484166	Energy production and conversion
[G]	191	5.761689	Carbohydrate transport and metabolism
[E]	165	4.9773755	Amino acid transport and metabolism
[F]	72	2.1719458	Nucleotide transport and metabolism
[H]	115	3.4690802	Coenzyme transport and metabolism
[I]	63	1.9004526	Lipid transport and metabolism
[P]	78	2.3529413	Inorganic ion transport and metabolism
[Q]	24	0.7239819	Secondary metabolites biosynthesis, transport and catabolism
[R]	179	5.3996983	General function prediction only
[S]	98	2.9562595	Function unknown
-	1410	42.533936	Not in COGs

% = percent

Figures Legends

Figure 1. Phylogenetic tree showing Lachnoclostridium phocaeense strain Marseille- P3177T relative to other phylogenetically close neighbours. GenBank accession numbers are indicated in parentheses. Sequences were aligned using CLUSTALW, and phylogenetic inferences were obtained using maximum-likelihood method within MEGA software. Numbers at nodes are percentages of bootstrap values obtained by repeating analysis 500 times to generate majority consensus tree. Only bootstrap scores of at least 90% were retained. Coprococcus comes was used as outgroup. Scale bar indicates 0.5% nucleotide sequence divergence (8).

Figure 2. Graphical circular map of the chromosome. From outside to the center: Genes on the forward strand colored by COG categories (only genes assigned to COG), genes on the reverse strand colored by COG categories (only gene assigned to COG), RNA genes (tRNAs green, rRNAs red), GC content and GC skew.

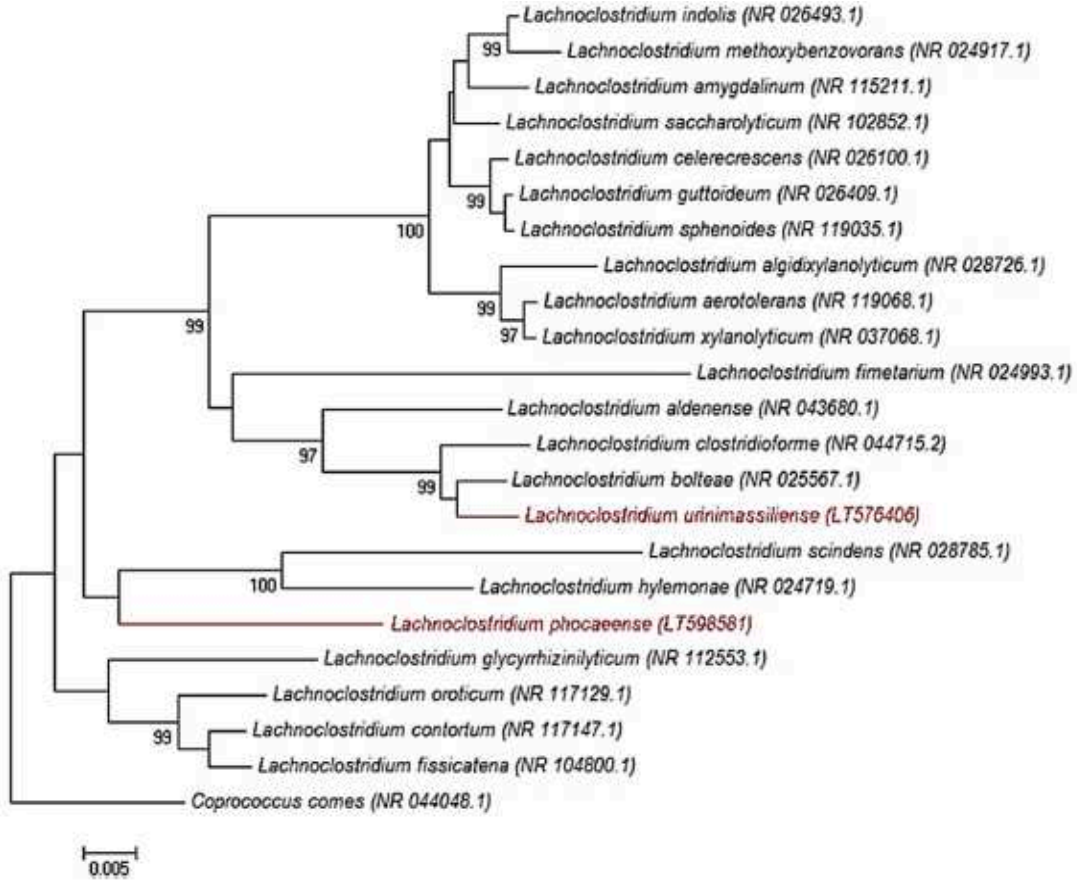

Figure 1. Phylogenetic tree showing Lachnoclostridium phocaeense strain Marseille- P3177T relative to other phylogenetically close neighbours. GenBank accession numbers are indicated in parentheses. Sequences were aligned using CLUSTALW, and phylogenetic inferences were obtained using maximum-likelihood method within MEGA software. Numbers at nodes are percentages of bootstrap values obtained by repeating analysis 500 times to generate majority consensus tree. Only bootstrap scores of at least 90% were retained. Coprococcus comes was used as outgroup. Scale bar indicates 0.5% nucleotide sequence divergence.

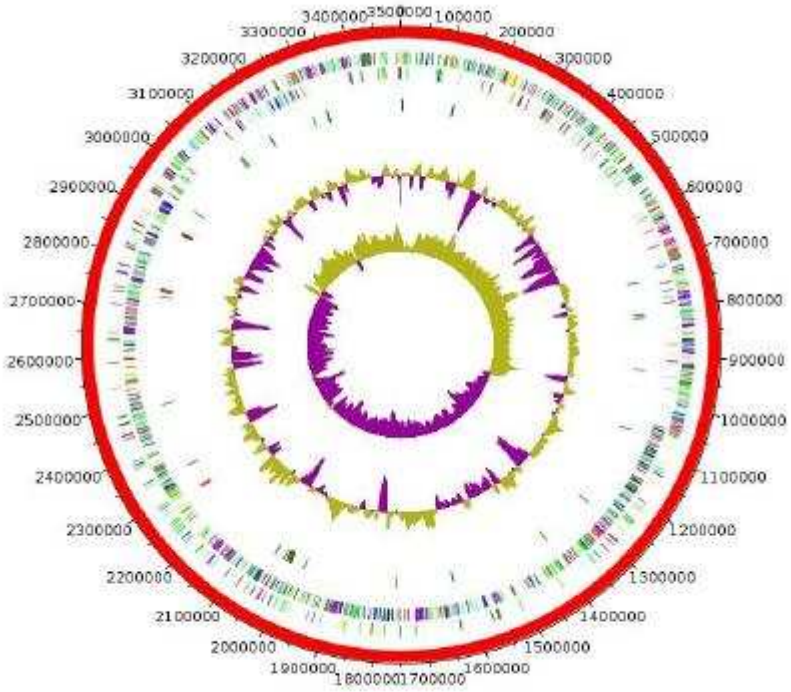

Figure 2. Graphical circular map of the chromosome. From outside to the center: Genes on the forward strand colored by COG categories (only genes assigned to COG), genes on the reverse strand colored by COG categories (only gene assigned to COG), RNA genes (tRNAs green, rRNAs red), GC content and GC skew.

Conclusion of Chapter V

The human body is a complex system composed of human cells and harboring trillions of bacteria and other microorganisms. It has been said that bacterial cells are 10 times out numbering the human cells (1). The term microbiota refers to the microorganisms that inhabit the mucosal and epithelial body surfaces exposed to the outside environment such as bacteria, archaea and yeasts.(2) The most complex bacterial community inside the human body is the gastrointestinal one (3). This latter has several functions inside the human body including the protection of the gut against the establishment of exogenous pathogenic bacteria, mediating differentiation and development of the intestinal epithelium, and producing enzymes that help in the digestion of nutrients and minerals absorption(4). In view of its complexity, it has been stated that only 20% of its composition has been determined (5). From the experience in our research unit, culturomics proved to be an efficient and promising tool for the identification of new bacterial species previously un-identified and un-cultured with other approaches.

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Chapter VI

Studies conducted in Lebanon during M2 and 1st year PhD Studies

Multi-drug Resistant organisms in Lebanese Nursing Homes.

Introduction

The spread of multi-drug resistance is among the most common public health addressed nowadays (1). The dissemination of multi-drug resistant organisms is sparked by the concern of causing infections with limited therapeutic options (2). In Lebanon, studies have shown the spread of ESBL as well as carbapenemase producers in the clinical settings (3)(4). However little is known about the prevalence of these organisms in the community settings such as in nursing homes.

In **Article 10** entitled **"Carriage of beta-lactamase-producing enterobacteriaceae among nursing home residents in north Lebanon"**, the fecal carriage of ESBL, ampC and carbapenemase producers was followed in 68 elderlies over a four month period. 76.5% of recruited nursing home residents were carriers of ESBL and/or carbapenemase producing Gram-negative bacilli. The carriage was dynamic and significantly related to a recent antibiotic intake.

Article 11 entitled **"Fecal carriage of MDROs in a population of lebanese elderly: Dynamics and impact on bacterial fitness"**, assesses the competitive growth of multi-drug resistant E. coli strains compared to sensitive E. coli, both isolated from nursing home residents. Sensitive E. coli strains out competed the resistant ones when grown in vitro.

Article 12 entitled **"Competition assays between ESBL-producing E. coli and K. pneumoniae isolates collected from Lebanese elderly: An additional cost on fitness"**, presents inter-species in vitro competitions assays between ESBL and sensitive E. coli and Klebsiella pneumoniae isolates. The results suggest that ESBL production in E. coli as well as in K. pneumoniae confer a fitness cost leading to a frequency decrease of these organisms in inter-species competitions.

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Article 10

Carriage of beta-lactamase-producing enterobacteriaceae among nursing home residents in north Lebanon.

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Carriage of beta-lactamase-producing *Enterobacteriaceae* among nursing home residents in north Lebanon

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SUMMARY

Background: Multidrug-resistant (MDR) *Enterobacteriaceae* can cause severe infections with high morbidity, mortality, and health care costs. Individuals can be fecal carriers of these resistant organisms. Data on the extent of MDR *Enterobacteriaceae* fecal carriage in the community setting in Lebanon are very scarce. The aim of this study was to investigate the fecal carriage of MDR *Enterobacteriaceae* among the elderly residents of two nursing homes located in north Lebanon.

Methods: Over a period of 4 months, five fecal swab samples were collected from each of 68 elderly persons at regular intervals of 3–4 weeks. Fecal swabs were subcultured on selective media for the screening of resistant organisms. The phenotypic detection of extended-spectrum beta-lactamase (ESBL), AmpC, metallo-beta-lactamase (MBL), and *Klebsiella pneumoniae* carbapenemase (KPC) production was performed using the beta-lactamase inhibitors ethylenediaminetetraacetic acid, phenylboronic acid, and cloxacillin. A temocillin disk was used for OXA-48. Multiplex PCRs were used for the genotypic detection of ESBL and carbapenemase genes, and sequencing was performed to identify CTX-M-15. The medical records of each subject were reviewed on a regular basis in order to assess the risk factors associated with MDR *Enterobacteriaceae* fecal carriage.

Results: Over the study period, 76.5% of the recruited elderly persons were at least one-time carriers. A total of 178 isolates were obtained. Phenotypic testing revealed that 91.5% of them were ESBL producers, 4% were AmpC producers, 2.8% were co-producers of ESBL and AmpC, and 1.7% were co-producers of OXA-48 and ESBL. Recent antibiotic intake was found to be the only independent risk factor associated with the fecal carriage of MDR *Enterobacteriaceae*.

Conclusions: The high prevalence of MDR *Enterobacteriaceae* detected in this study and the emergence of carbapenem resistance is alarming. Efficient infection control measures and antibiotic stewardship programs are urgently needed in these settings in order to limit the spread of resistant strains.

1. Introduction

Multidrug-resistant (MDR) *Enterobacteriaceae* are currently considered a major public health concern worldwide.[1,2] They can be transmitted easily among patients and healthy persons.[3] Studies have shown that after being selected by antibiotics, the cross-transmission of these organisms occurs frequently in the health care setting.[4] This dissemination will eventually lead to increased rates of MDR *Enterobacteriaceae* carriage. This carriage is often unrecognized and has been known to increase the risk of contracting infections caused by resistant agents.[5] The treatment of these cases is often challenging due to the limited therapeutic options; the antibiotic pipeline is drying up and no new antimicrobial agents targeted against MDR *Enterobacteriaceae* are foreseen in the near future.[6]

There is increasing evidence that nursing homes in the community are important reservoirs for MDR *Enterobacteriaceae*.[4,7] This is in major part due to the inappropriate use of antimicrobial agents in these facilities,[8] in addition to the difficulties particularly faced when establishing antibiotic stewardship and infection control programs.[9,10] The prevalence of MDR *Enterobacteriaceae* colonization in nursing homes varies according to the geographical location, patient population, and the level of care provided.[10]

In the Middle East, although several studies have been conducted to assess the prevalence of MDR *Enterobacteriaceae* in

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the hospital ward,[11] data on the prevalence of MDR *Enterobacteriaceae* among nursing home residents in these countries are very scarce. In Lebanon, clinical investigations have shown that the prevalence of MDR *Enterobacteriaceae* is on a continuous rise.[12] Local data reported in the form of flyers summarizing the susceptibility of bacteria at the Centre Hospitalier Du Nord in the north of Lebanon, show that between 2011 and 2013 the rate of extended-spectrum beta-lactamase (ESBL) production among clinical isolates increased from 24.6% to 30.4% and from 26.5% to 31.7% in *Escherichia coli* and *Klebsiella pneumoniae*, respectively (Ziad Daoud). Another recent study reported an increase of 1.2% in resistance and decreased susceptibility to ertapenem in clinical isolates of *Enterobacteriaceae*. This resistance was mainly attributed to the production of OXA-48 beta-lactamase.[12]

In an attempt to understand the situation of carriage in the nursing homes of the country and to shed light on this important issue, the present research group conducted a study in Lebanon in which it was found that 71.6% of the recruited elderly subjects were at least one-time carriers during the study period.[13] The plan was to study the situation in the north of Lebanon, where the extent of the spread of bacterial resistance in the community is not well documented. The socio-cultural as well as economic and educational levels in the north of Lebanon are also very particular to this area of the country. These include the level of poverty and the absence of basic governmental services such as public sanitation and infrastructure, as most of the services are concentrated in the capital Beirut. Unfortunately, all of these data are anecdotal and based on impressions, since official statistics are not available in the country.

The aim of this study was thus to investigate the fecal carriage of MDR *Enterobacteriaceae* among the residents of two major nursing homes located in the north of Lebanon through the determination of the prevalence, dynamics, and risk factors for MDR *Enterobacteriaceae* fecal carriage among elderly subjects. In addition, it was sought to determine whether CTX-M-15, the predominant ESBL gene in the Lebanese population,[14] was also the major ESBL genotype carried among these elderly people.

2. Materials and methods

2.1. Ethics, consent, and permissions

The Research Committee of the University of Balamand and the Project Management Unit at the Lebanese Ministry of Agriculture approved this study. The patient or his/her legal guardian or family member signed a consent form for their participation in the study. The privacy of participants and transparency of the ethical process were guaranteed.

2.2. Study design and population

This was a cross-sectional study conducted in two major nursing homes located in Tripoli in the north of Lebanon. Candidates for this study were elderly residents aged >60 years. A total of 68 individuals were recruited. Fifty-seven were chosen randomly from nursing home 1. This facility has around 60 rooms and a capacity of 200 beds. Eleven elderly persons were recruited randomly from nursing home 2. This facility offers around 20 rooms with a capacity of 50 beds.

2.3. Data collection

The medical records of each elderly person were reviewed with the help of the nurse responsible. Age, sex, number of roommates, mobility status (ambulant/in a wheelchair or bedridden), and the date of admission were all recorded. In addition, urinary/fecal incontinence, the presence of wounds or ulcers, and the previous or current use of a urinary catheter were also reported. Furthermore, the recruited elderly persons were checked for comorbidities (MDR bacterial infections, diabetes, cancer, pulmonary, cardiovascular, renal, or neurological diseases, and urogenital pathologies), hospital admission during the last year, and whether they had undergone any surgeries, as well as their antibiotic intake during the last 3 months.

2.4. Collection of fecal swabs and isolation of resistant Enterobacteriaceae

Between December 2013 and April 2014, five samples (fecal swabs) were obtained from each of 68 elderly persons at regular intervals of 3–4 weeks. A total of 262 samples were collected: 59 at collection 1, 51 at collection 2, 57 at collection 3, 51 at collection 4, and 44 at collection 5. The fecal swabs were subcultured on MacConkey agar supplemented with cefotaxime (2 μg/ml) for the screening of MDR *Enterobacteriaceae*. From each selective plate, different colonies presenting with different morphologies were picked up separately and suspended in Luria broth. After overnight incubation, each bacterial suspension was subcultured again on a selective plate. The following day, if the plate contained colonies with single morphologies, the isolate was preserved in 20% glycerol aliquots at −20 °C for further testing; if more than one type was observed, a re-isolation was performed for further purification.

An elderly subject was defined as a carrier if an MDR *Enterobacteriaceae* was isolated from his/her fecal sample. If the patient was found to be a carrier at all five collections, he/she was considered a 'permanent carrier'. If the resistant bacterium was isolated at fewer than five collections, the subject was defined as an 'intermittent carrier'. Finally, if no MDR *Enterobacteriaceae* was isolated during the five collections, the subject was considered a 'never carrier'. All isolates were identified using biochemical gallery tests (API 20E; bioMérieux).

2.5. Phenotypic tests

Antimicrobial susceptibility testing was performed for 178 isolates using the Kirby–Bauer disk diffusion method. Interpretation of the results was performed in accordance with the Clinical and Laboratory Standards Institute (CLSI) guidelines 2014.[15] Fifteen antimicrobial agents were tested (Table 1). The amoxicillin–clavulanic acid disk was placed in the center between cefepime, ceftazidime, and aztreonam, in order to detect a possible 'keyhole effect'. AmpC beta-lactamase and carbapenemase production was suspected when resistance to cefoxitin and ertapenem, respectively, was observed. Unfortunately, resistance to cefoxitin is not sufficient to distinguish between constitutive and plasmid-mediated AmpC, therefore it was considered that both types of AmpC were detected by this test. In order to confirm these phenotypically, ethylenediaminetetraacetic acid (EDTA), phenylboronic acid (PBA), and cloxacillin were used as beta-lactamase inhibitors.[16–18] In addition, temocillin susceptibility testing was performed as a presumptive test for the detection of the OXA-48 enzyme.[19]

2.6. Detection of ESBL type using multiplex PCR

In order to identify the type(s) of ESBL present in the clinical isolates, multiplex PCR was performed on a representative sample of 18 isolates chosen based on their profile of resistance. Bacterial DNA was prepared by suspending one or two colonies of each test isolate in 200 μl of distilled water and heating the solution at 95 °C for 10 min. The presence of $bla_{\text{CTX-M}}$, bla_{SHV}, bla_{TEM}, and bla_{OXA} genes was tested using previously published primer sets and

Table 1
Rates of susceptibility of different *Enterobacteriaceae* isolates

Antimicrobial agent	Number of susceptible isolates (%)			
	Escherichia coli (*n* = 159)	*Klebsiella pneumoniae* (*n* = 5)	*Klebsiella oxytoca* (*n* = 9)	*Citrobacter diversus* (*n* = 5)
Ampicillin	0 (0)	0 (0)	0 (0)	0 (0)
Aztreonam	7 (4.4)	0 (0)	2 (22.2)	1 (20)
Cefoxitin	138 (86.8)	5 (100)	8 (88.8)	4 (80)
Cefotaxime	0 (0)	0 (0)	0 (0)	0 (0)
Ceftazidime	16 (10)	0 (0)	1 (11.1)	0 (0)
Cefepime	9 (5.6)	0 (0)	0 (0)	0 (0)
Amoxicillin–clavulanic acid	61 (38.3)	2 (40)	1 (11.1)	3 (60)
Piperacillin–tazobactam	68 (42.7)	1 (20)	4 (44.4)	0 (0)
Meropenem	156 (98.1)	5 (100)	9 (100)	5 (100)
Imipenem	156 (98.1)	5 (100)	9 (100)	5 (100)
Ertapenem	156 (98.1)	5 (100)	9 (100)	5 (100)
Tigecycline	118 (100)[a]	3 (100)[a]	6 (100)[a]	5 (100)
Trimethoprim–sulfamethoxazole	59 (37.1)	0 (0)	1 (11.1)	0 (0)
Ciprofloxacin	65 (40.8)	1 (20)	2 (22.2)	0 (0)
Gentamicin	83 (52.2)	1 (20)	4 (44.4)	2 (40)

[a] Only 118 *E. coli*, three *K. pneumoniae*, and six *K. oxytoca* isolates were tested for tigecycline susceptibility.

conditions.[20] Each reaction tube contained 10 μl of master Mix (Qiagen), 4 μl of primers, and 1 μl of DNA, and was made up to a total volume of 20 μl with sterile distilled water. The PCR reaction conditions consisted of a 15 min denaturation step at 95 °C, followed by 30 amplification cycles of 30 s at 94 °C, 90 s at 62 °C, and 60 s at 72 °C, with a final extension step of 10 min at 72 °C.[20]

The primer sequences and expected amplicon sizes of the target ESBL genes were as follows: for bla_{SHV}: F-CTTTATCGGCCCTCACTCAA, R-AGGTGCTCATCATGGGAAAG (327 bp); bla_{TEM}: F-CGCCGCATACAC-TATTCTCAGAATGA, R-ACGCTCACCGGCTCCAGATTTAT (445 bp); bla_{CTX-M}: F-ATGTGCAGYACCAGTAARGTKATGGC, R-TGGGTRAAR-TARGTSACCAGAAYCAGCGG (593 bp); bla_{OXA}: F-ACACAATACATAT-CAACTTCGC, R-AGTGTGTTTAGAATGGTGATC (813 bp).

In order to visualize the PCR amplicons, samples were mixed with 4 μl of Thermo Scientific loading dye and loaded into the wells of a 1.5% agarose gel in 1× Tris–acetate–EDTA (TAE) buffer. The gel was run at 130 V for 60 min. Amplicons were visualized using an ultraviolet transilluminator system (DIGI DOC-IT System) for analysis. The gel had one well containing a DNA ladder (100 bp; Thermo Scientific) in order to be able to estimate the size of the DNA amplicons.

2.7. Plasmid sequencing and analysis

Plasmid DNA was extracted as described above, quantified using Qubit, and sequenced using the Illumina NGS platform. The sequence data were downloaded from the GenBank database and each sequence file was compared to a number of reference plasmid replicon sequences present in the Plasmid Finder database using BLASTn. Circular representations of the plasmid sequence were created using Unipro UGENE software, and the sequenced plasmids were aligned and compared to reference replicon sequences using BioEdit software.

2.8. Detection of carbapenemase genes using PCR

Another multiplex PCR was conducted for the detection of the carbapenem resistance genes *Klebsiella pneumoniae* carbapenemase (KPC), New Delhi metallo-beta-lactamase (NDM), OXA-48, IMP, SPM, and VIM. DNA extraction was performed as described in the previous section. The presence of the carbapenem resistance genes was tested using universal primers.[21] PCR amplification reactions were performed in a volume of 20 μl containing 10 μl of Taq PCR Master Mix, 5 μl of sterile water, 4 μl of the primer mix, and 1 μl of the extracted DNA. The conditions of the PCR reaction were as follow: 94 °C for 10 min, then 36 cycles of 30 s at 94 °C, 40 s at 52 °C, and 50 s at 72 °C for amplification, then 5 min at 72 °C for the final extension.[21] Amplified DNA products were subjected to electrophoresis on a 1.5% agarose gel in 1× TAE buffer. The gel was run at 130 V for 1 h. The visualization of amplicons was performed using an ultraviolet transilluminator system (DIGI DOC-IT System) for analysis.

The primer sequences and expected amplicon sizes of target carbapenemase genes were as follows: bla_{KPC}: F-CGTCTAGTTCTGCTGTCTTG, R-CTTGTCATCCTTGTTAGGCG (798 bp); bla_{NDM}: F-GGTTTGGCGATCTGGTTTTC, R-CGGAATGGCTCATCACGATC (621 bp); bla_{OXA-48}: F-GCGTGGTTAAGGATGAACAC, R-CATCAAGTT-CAACCCAACCG (438 bp); bla_{IMP}: F-GGAATAGAGTGGCTTAAYTCTC, R-GGTTTAAYAAAACAACCACC (232 bp); bla_{SPM}: F-AAAATCTGGG-TACGCAAACG, R-ACATTATCCGCTGGAACAGG (271 bp); bla_{VIM}: F-GATGGTGTTTGGTCGCATA, R-CGAATGCGCAGCACCAG (390 bp).

2.9. Statistics and data analysis

For univariate analysis, classical descriptive methods were used according to each site separately. Furthermore, the distributions of variables according to carriage status were compared by conducting a bivariate analysis. A p-value of ≤ 0.05 was considered statistically significant. Furthermore, risk factors with a p-value of ≤ 0.15 were subjected to multivariate analysis. IBM SPSS Statistics version 20.0 (IBM Corp., Armonk, NY, USA) was used for all statistical calculations.

3. Results

3.1. Demographics and prevalence of MDR Enterobacteriaceae fecal carriage

The demographic characteristics of the elderly subjects are presented in Table 2. For both nursing homes, the prevalence of fecal carriage was as follow: 32 elderly subjects (54.2%) were fecal carriers at the first collection, 33 (64.7%) at the second collection, 24 (42.1%) at the third collection, 24 (47%) at the fourth collection, and 25 (56.8%) at the fifth collection. Overall, 76.5% of the recruited residents were at least one-time carriers, while 23.5% of them were never carriers.

3.2. Dynamics of MDR Enterobacteriaceae fecal carriage

In this study, 262 samples were collected, of which 138 were positive for MDR *Enterobacteriaceae* (52.6%). From these

Table 2
Characteristics of nursing home residents recruited in this study[a]

	NH1	NH2
Total number	57	11
Sex		
Male	19 (33.3)	5 (45.5)
Female	38 (66.7)	6 (54.5)
Age, years, mean (SD)	78 (7.8)	75.82 (9.3)
LOS, days, mean (median)	1016 (818)	402.36 (598)
Room accommodation		
Single	3 (5.3)	10 (90.9)
Double	13 (22.8)	1 (9.1)
Triple	1 (1.8)	0 (0)
Quadruple	4 (7)	0 (0)
More than 4 beds/room	36 (63.2)	0 (0)
Mobility status		
Ambulant	11 (19.3)	4 (36.4)
Wheelchair	46 (80.7)	4 (36.4)
Bedridden	0 (0)	3 (27.3)
Urinary catheter	4 (7)	4 (36)
Urinary/fecal incontinence	43 (75.4)	7 (63.6)
Wounds/ulcers	6 (10.5)	2 (18.2)
Recent surgery during last 3 months	2 (3.5)	3 (27.3)
Recent hospitalization during last year	8 (14)	4 (36.4)
Recent antibiotic intake during last 3 months	27 (47.4)	7 (63.6)
Multidrug-resistant bacterial infections	2 (3.5)	0 (0)
Diabetes	6 (10.5)	4 (36.4)
Cancer	2 (3.5)	0 (0)
Pulmonary diseases	6 (10.5)	3 (27.3)
Cardiovascular diseases	24 (42.1)	4 (36.4)
Neurological diseases	24 (42.1)	2 (18.2)
Urogenital pathologies	11 (19.3)	4 (36.4)
Renal diseases	1 (1.8)	0 (0)

NH, nursing home; SD, standard deviation; LOS, length of stay.
[a] All data are presented as the number (%) unless stated otherwise.

138 positive samples, 178 isolates were obtained. The number of elderly subjects versus the number of isolates was not 1 to 1, since more than one isolate was obtained for some residents. Overall, 159 isolates (89%) were identified as *E. coli*, 14 (8%) as *Klebsiella spp*, and five (3%) as *Citrobacter spp*.

The fecal carriage among elderly subjects varied from one collection to another (Figure 1). From collection 1 to 2, the carriage of MDR *Enterobacteriaceae* disappeared for four subjects (12.5%), while it appeared in 10 (37%). Between collections 2 and 3, the carriage disappeared in 13 subjects (38.2%), while it appeared in two (11.8%). Between collections 3 and 4, six carriers (25%) became non-carriers, while eight non-carriers (24.2%) became carriers. Between collections 4 and 5, the carriage disappeared in six subjects (25%), while it appeared in 10 (37%). Overall, out of the 52 elderly subjects who were at least one-time carriers, eight (15.4%) were permanent carriers, while 44 (84.6%) were intermittent carriers. *E. coli* was the most stable resistant colonizer isolated at the five collections, while *Klebsiella spp* and *Citrobacter spp* were only isolated at four and three of the collections, respectively.

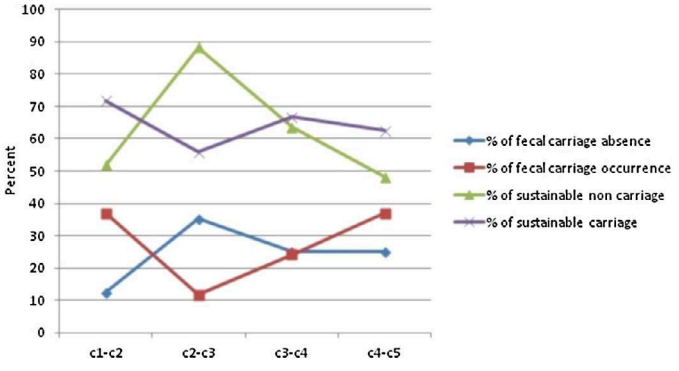

Figure 1. Dynamics and stability of multidrug-resistant *Enterobacteriaceae* fecal carriage.

3.3. ESBL, AmpC, and OXA-48 detection

The antimicrobial susceptibility testing results are summarized in Table 3. Phenotypic testing revealed that out of 178 isolates, 163 (91.5%) were ESBL producers. Five isolates (2.8%) were found to be co-producers of ESBL and AmpC. Seven isolates (4%) were considered AmpC producers. Furthermore, 46% of the isolated ESBL and/or AmpC producers were co-resistant to at least two other non-beta-lactam antimicrobial agents, 38% were co-resistant to only one non-beta-lactam, and 16% showed no co-resistance. The detailed susceptibility rates for each category are presented in Table 3. As an average of the five collections, 89.5% of ESBL production was detected in *E. coli*, while only 8.5% and 1.8% were detected in *Klebsiella spp* and *Citrobacter spp*, respectively. A 71.4% AmpC production was observed in *E. coli*; however, the methodology used does not distinguish between the constitutive and plasmid-mediated resistance due to AmpC. The simultaneous production of ESBL and AmpC, as well as ESBL and OXA-48, was observed at only the first and second collections; in both cases these were produced by isolates of *E. coli* (Table 4). Three isolates of *E. coli* were carbapenem-non-susceptible. Two of these were isolated from the same patient during the first and second collections, while the third was isolated from another patient during the first collection. In the subsequent collections, no carbapenem-resistant isolates were detected. Phenotypic tests suggested an OXA-48 probably co-produced with ESBL. In this regard, temocillin disks were used for the three isolates (Figure 2).

3.4. Genotypic detection of resistance and occurrence of CTX-M-15

Multiplex PCR analysis performed on 18 isolates revealed the presence of the TEM gene in 17 of them, CTX-M in 16, OXA in four, and SHV in two. Eleven isolates showed coexistence of CTX-M and TEM genes, four showed coexistence of three or four genes, and three isolates harbored only one gene (Figure 3). The 16 isolates harboring the CTX-M gene were all positive for CTX-M-15 after

Table 3
Rates of susceptibility of MDR *Enterobacteriaceae* isolates

Antimicrobial agent	Number of susceptible isolates (%)		
	ESBL producers (n = 163)	ESBL and AmpC co-producers (n = 5)	AmpC producers (n = 7)
Ampicillin	0 (0)	0 (0)	0 (0)
Aztreonam	7 (4.2)	0 (0)	3 (42.8)
Cefoxitin	152 (93.2)	0 (0)	0 (0)
Cefotaxime	0 (0)	0 (0)	0 (0)
Ceftazidime	14 (8.5)	0 (0)	0 (0)
Cefepime	2 (1.2)	0 (0)	7 (100)
Amoxicillin–clavulanic acid	67 (41.1)	0 (0)	0 (0)
Piperacillin–tazobactam	68 (41.7)	2 (40)	3 (42.8)
Meropenem	163 (100)	5 (100)	7 (100)
Imipenem	163 (100)	5 (100)	7 (100)
Ertapenem	163 (100)	5 (100)	7 (100)
Tigecycline	122 (100)[a]	3 (100)[a]	7 (100)
Trimethoprim–sulfamethoxazole	55 (33.74)	3 (60)	2 (28.5)
Ciprofloxacin	61 (37.4)	2 (40)	2 (28.5)
Gentamicin	83 (50.9)	1 (20)	6 (85.7)

MDR, multidrug-resistant; ESBL, extended-spectrum beta-lactamase.
[a] Only 122 ESBL producers and three ESBL and AmpC co-producers were tested for tigecycline susceptibility.

Table 4
Prevalence of MDR *Enterobacteriaceae* in different species over the five collections

	Species	Number of isolates	Phenotypic mechanism of resistance
Collection 1	*Escherichia coli*	35	ESBL
		3	ESBL/AmpC
		1	AmpC
		2	OXA-48/ESBL
	Klebsiella oxytoca	5	ESBL
Collection 2	*Escherichia coli*	36	ESBL
		2	ESBL/AmpC
		1	AmpC
		1	OXA-48/ESBL
	Klebsiella oxytoca	3	ESBL
	Klebsiella pneumoniae	3	ESBL
Collection 3	*Escherichia coli*	25	ESBL
		1	AmpC
	Citrobacter diversus	1	ESBL
Collection 4	*Escherichia coli*	26	ESBL
		1	AmpC
	Klebsiella pneumoniae	1	ESBL
	Citrobacter diversus	1	ESBL
Collection 5	*Escherichia coli*	24	ESBL
		1	AmpC
	Klebsiella oxytoca	1	AmpC
	Klebsiella pneumoniae	1	ESBL
	Citrobacter diversus	2	ESBL
		1	AmpC

MDR, multidrug-resistant; ESBL, extended-spectrum beta-lactamase.

DNA extraction and sequencing, therefore showing a high occurrence of this enzyme in the ESBL population. In the phenotypic testing, 17 out of the 18 isolates showed a keyhole effect and were therefore identified as ESBL producers (Table 5).

Regarding the three carbapenem-resistant isolates, multiplex PCR analysis showed that all of them harbored an OXA-48 gene (Figure 4), thereby confirming the phenotypic results.

In view of the low number of isolates selected for genotypic testing, these results cannot be generalized, and tests addressing a larger number of isolates should be performed in the future to confirm that this is true on a larger scale.

3.5. Risk factors associated with fecal carriage of MDR Enterobacteriaceae

The associations between MDR *Enterobacteriaceae* fecal carriage and different factors are presented in Table 6. Univariate analysis revealed that recent antibiotic intake during the last 3 months and urogenital pathologies were the only risk factors associated with the fecal carriage of MDR *Enterobacteriaceae* ($p = 0.03$ and $p = 0.015$, respectively). The percentage of residents who had a recent antibiotic intake was 59.6% (31/52) among the at least one-time carriers and 18.8% (3/16) among the never carriers. For urogenital pathologies, the prevalence was 28.8% (15/52) in carriers versus 0% (0/16) in never carriers. In the multivariate analysis, three factors were included: recent antibiotic intake ($p = 0.03$), urogenital pathologies ($p = 0.015$), and diabetes ($p = 0.102$). This final analysis revealed that recent antibiotic

Figure 2. Temocillin test for the phenotypic detection of OXA-48 production. (A) Negative results (sensitivity) with non-OXA-48 producing isolates. (B) Positive results (resistance) with the three carbapenem-resistant *Enterobacteriaceae* isolates producing OXA-48 isolated in this study.

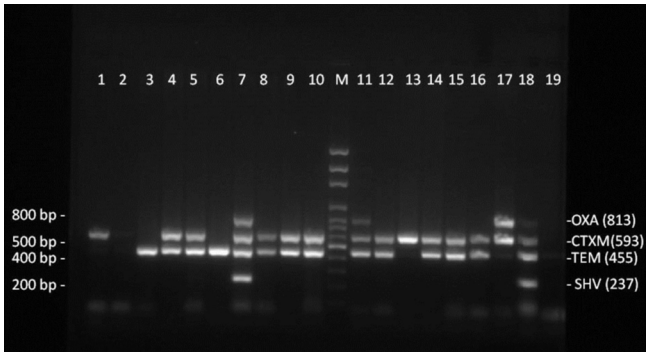

Figure 3. Detection of the beta-lactamase genes SHV, TEM, CTX-M, and OXA in multidrug-resistant *Enterobacteriaceae* isolates obtained from nursing home residents, using multiplex PCR. Lanes 1–18 represent the multidrug-resistant *Enterobacteriaceae* isolates tested. Lane 19 corresponds to the positive control (TEM 455 bp). Lane M is a 1.2-kb DNA ladder. The molecular size of the band in question is indicated in parentheses on the right of the image.

Table 5
Genotypic detection of beta-lactamase genes versus phenotypic identification

Species	Number of isolates	Phenotypic mechanism of resistance	Genes harbored
Escherichia coli	10	ESBL	TEM, CTX-M
Escherichia coli	2	ESBL	TEM, CTX-M,OXA
Escherichia coli	2	ESBL	TEM
Escherichia coli	1	ESBL	CTX-M
Citrobacter diversus	2	ESBL	TEM, SHV, CTX-M, OXA
Citrobacter diversus	1	AmpC	TEM, CTX-M

ESBL, extended-spectrum beta-lactamase.

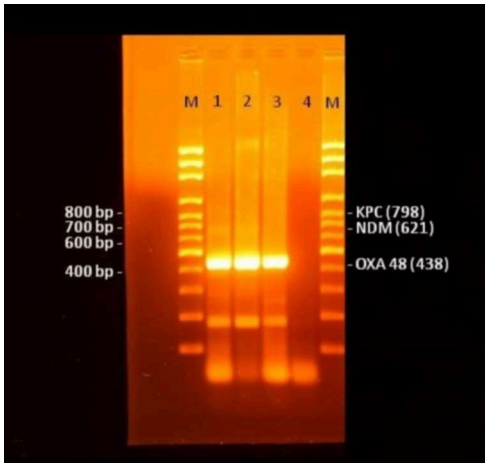

Figure 4. Detection of carbapenemase genes OXA-48, NDM, and KPC in carbapenem-resistant *Enterobacteriaceae* isolates obtained from nursing home residents, using multiplex PCR. Lanes 1–3 represent the carbapenem-resistant *Enterobacteriaceae* isolates. Lane 4 corresponds to the negative control. Lane M is a 1.2-kb DNA ladder. The molecular size of the band in question is indicated in parentheses on the right of the image.

intake was the only independent risk factor associated with MDR *Enterobacteriaceae* fecal carriage.

4. Discussion

Although several studies have addressed the issue of MDR *Enterobacteriaceae* in Lebanon, data on the spread of bacterial resistance in the community are very scarce. Only one recent study has been carried out in nursing homes in Beirut, and that study was performed by the present research group. In that study, it was found that 71.6% of the recruited elderly subjects were at least one-time carriers.[13] Similar results were found in the present study implemented in the north of Lebanon (76.5%). These results, however, are relatively high when compared to those from similar studies conducted in long-term care facilities worldwide: 70.3% in Italy,[22] 41.3% in Japan,[23] and 14.7% in Australia.[24] Differences in sample size, medical care, and hand hygiene practices at each site, in addition to differences in the microbiological screening methods used in each study might have influenced the results and therefore have yielded some variations.[25] Another important factor to consider when comparing these results is that the majority of the studies were conducted at one time-point only.

As shown in the present study, the carriage status of an elderly person should not be assumed on the basis of only one fecal sampling; rather, multiple screening samples are needed. According to Filius et al., differences in colonization rates could arise as a result of antibiotic consumption that has decreased the number of MDR *Enterobacteriaceae* to an undetectable level in the stool sample.[26]

Table 6
Association between different factors and MDR *Enterobacteriaceae* fecal carriage[a]

	At least one-time carrier	Never carrier
Total number	52 (76.5)	16 (23.5)
Sex		
Male	19 (36.5)	5 (31.2)
Female	33 (63.5)	11 (68.8)
Age, years, mean (SD)	77.81 (7.7)	77.63 (9.3)
LOS, days, mean (median)	900.7 (610)	1629 (829.5)
Room accommodation		
Single/double	22 (42.3)	5 (31.2)
Triple and more	30 (57.7)	11 (68.8)
Mobility status		
Ambulant	12 (23)	3 (18.8)
Wheelchair/bedridden	40 (77)	13 (81.2)
Urinary catheter	7 (13.5)	1 (6.2)
Urinary/fecal incontinence	37 (71.2)	13 (81.2)
Wounds/ulcers	7 (13.5)	1 (6.2)
Recent surgery during last 3 months	5 (9.6)	0 (0)
Recent hospitalization during last year	11 (21)	1 (6.2)
Recent antibiotic intake during last 3 months	31 (59.6)[b]	3 (18.8)[b]
Multidrug-resistant bacterial infections	2 (3.8)	0 (0)
Diabetes	10 (19.2)[c]	0 (0)[c]
Cancer	2 (3.8)	0 (0)
Pulmonary diseases	7 (13.5)	2 (12.5)
Cardiovascular diseases	23 (44.2)	5 (312)
Neurological diseases	19 (36.5)	7 (43.8)
Urogenital pathologies	15 (28.8)[b]	0 (0)[b]
Renal diseases	1 (1.9)	0 (0)

MDR, multidrug-resistant; SD, standard deviation; LOS, length of stay.
[a] All data are presented as the number (%) unless stated otherwise.
[b] *p*-Value ≤0.05.
[c] *p*-Value ≤0.15.

The fecal carriage of AmpC producers among the recruited residents is an important finding in this study. AmpC-producing *Enterobacteriaceae* strains have previously been reported in clinical samples from Lebanon.[27,28] However, the present study appears to be the first to report the prevalence of these MDR bacteria in a community setting. AmpC beta-lactamases are cephalosporinases that can be chromosomally mediated with inducible expression or plasmid-mediated with constitutive expression.[29,30] Along with ESBLs, the non-recognition of these mechanisms by clinical laboratory personnel leads to inappropriate reporting of the antibiogram to the physician responsible. This in many cases may lead to therapeutic failures.[31] Nevertheless, the present study might have suffered some limitations due to the use of phenotypic tests to incriminate the corresponding mechanisms of resistance. As is well known, these tests are very helpful for clinical microbiology laboratories; however, their specificities and sensitivities are questionable.

The detection of OXA-48 producers is a major and alarming issue. These beta-lactamases are plasmid-mediated class D oxacillinases that convey resistance to penicillins and have moderate hydrolyzing activity to carbapenems.[32] In this study, the phenotypic confirmation of OXA-48 production was performed using temocillin disks. High-level resistance to temocillin is not restricted to OXA-48 producers; metallo-beta-lactamases (MBLs) and KPCs can also be highly resistant to temocillin.[33] Therefore, temocillin resistance is considered a phenotypic confirmation of OXA-48 only in cases where other carbapenem resistance mechanisms are excluded.[34] It is important to note that the three ertapenem-resistant isolates in this study were intermediate to meropenem and imipenem and were isolated from two different elderly subjects who had no history of recent hospitalization; however, recent antibiotic treatment with amoxicillin–clavulanic acid was reported for one of them.

Of interest, it was found that in spite of the considerable socio-economic and cultural differences between Beirut and Tripoli, the results of this study were, to a certain extent, similar to those obtained in the study previously undertaken by this research group in Beirut.[13] In this context, there is agreement between these two studies on the frequency of carriage of ESBL-producing organisms (*E. coli* 82.7% in Beirut and 89% in Tripoli, *K. pneumoniae* 9.7% in Beirut and 8% in Tripoli). In addition, 80.7% of elderly subjects in Beirut were at least one-time carriers and 19.3% never carriers, while these percentages were found to be 76.5% and 23.5%, respectively, in elderly persons in Tripoli. However, although both studies agree that recent antibiotic intake is a significant risk factor, it was found that recent urinary tract pathologies and diabetes were risk factors only among Tripoli nursing homes residents. In addition, carbapenem-resistant *Enterobacteriaceae* were not isolated from the Beirut population.

Obviously other factors played a role in this relatively high prevalence. One possibility is the cross-transmission with resistant bacteria, since 38.5% of elderly subjects who were at least a one-time carrier had no history of recent antibiotic intake. In nursing homes, modes of transmission of MDR *Enterobacteriaceae* usually result from non-adherence to infection control measures; environmental surfaces are not frequently decontaminated, waste is often disposed of incorrectly, and hand hygiene practices are far from optimal in these settings.[10] In 2011, a randomized controlled trial was undertaken in Hong Kong long-term care facilities in order to determine the effectiveness of a hand hygiene infection control program. During the study period, adherence to hand hygiene increased significantly and the occurrence of serious infections decreased from 1.42 cases to 0.65 cases per 1000 resident-days.[35]

In conclusion, this study demonstrated that the prevalence of fecal carriage of MDR *Enterobacteriaceae* in north Lebanon is high and shows different patterns (one-time carriage, constant carriage, never carriage, etc.). The screening of newly admitted residents for the fecal carriage of MDR *Enterobacteriaceae* becomes a crucial task. The emergence of carbapenem resistance in the community is alarming; training of clinical laboratory technologists on the appropriate detection of the different mechanisms of resistance is essential. The prevalence of MDR *Enterobacteriaceae* fecal carriage among elderly nursing home residents (76.5%) is noteworthy and underlines the importance of nursing homes as reservoirs of resistance in the Lebanese community. The fecal carriage of MDR *Enterobacteriaceae* is dynamic and changes with time. In the majority of the isolates obtained, multidrug resistance was mediated by ESBL production. CTX-M-15 was present in 16 out of the 18 tested ESBL-producing isolates. This does not differ from the average CTX-M-15 in the Lebanese population, although the number of genotypically tested isolates in this study was relatively low. It is well known that phenotypic tests are not as accurate as genotypic methods; however, these are the best available way to detect resistance and incriminate the corresponding mechanism of resistance with an acceptable level of certainty in the clinical laboratories of the country.

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Article 11

Fecal carriage of MDROs in a population of Lebanese elderly: Dynamics and impact on bacterial fitness.

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Fecal carriage of MDROs in a population of Lebanese elderly: Dynamics and impact on bacterial fitness

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ABSTRACT

Muti-Drug Resistant Organisms (MDROs) are problematic all over the world, especially in Lebanon. High fecal carriage rates of MDR *Enterobacteriaceae* were reported from Lebanese nursing homes. Some studies show that MDROs have a fitness cost as compared to sensitive isolates. In this study, the competitive growth of MDR *Escherichia coli* obtained from fecal samples from elderly is assessed.

Fecal swabs from ten elderly patients from a Lebanese nursing home were obtained between June and December, 2015. Isolates were identified by API 20E and antimicrobial susceptibilities were determined. Production of ESBL (extended spectrum β lactamase), MBL (metallo β lactamse), AmpC and KPC (*Klebsiella pneumonia* carbapenemase) was detected phenotypically by the use of EDTA, PBA, cloxacillin, and DDSTs. *In-vitro* competition assays were performed using *E. coli* isolates with different combinations of bacterial resistance.

A total of 117 isolates was obtained with 71.8% *E. coli*, 7.7% of which were ESBL and 5.1% AmpC producers. Sensitive *E. coli* isolates out-competed all other isolates when in competition, followed sequentially by ESBL, AmpC, and OXA-48 (oxacillin) producers.

This study shows an advantage of sensitive *E. coli* strains obtained from fecal samples to out-compete resistant strains in specific *in-vitro* conditions. This ability could be exploited in the elimination of MDR organisms from the gut flora, after further investigation.

Introduction

The rapid emergence and spread of bacterial resistance is considered a major public health concern [1]. Bacterial resistance may develop in the gastro-intestinal tract in several ways. For instance, the excessive use of antimicrobial agents eliminates the sensitive bacteria of the human gut normal flora, facilitating colonization by resistant organisms [2]. Multi-Drug Resistant Organisms (MDROs) could also be acquired from dietary sources and colonize the gut [3]. These MDROs could increase the risk of endogenous infections by resistant bacteria and reduce the efficiency of available treatment options [4,5].

Beta-lactamase production is one of several mechanisms by which bacteria develop resistance [6]. The most common β-lactamases in *Enterobacteriaceae* are Extended Spectrum Beta-Lactamases (ESBLs), AmpCs and carbapenemases [7]. ESBLs are usually plasmid mediated and confer resistance to penicillins, monobactams and extended spectrum cepholasporins, yet show *in-vitro* susceptibility to cephamycins and amoxicillin-clavulanic acid [8]. AmpCs are also present as chromosomal or plasmidic and they are additionally able to hydrolyze cephamycins [9]. Carbapenemases have the ability to hydrolyze carbapenems and relay high-level resistance to beta-lactams [10].

Varying rates of fecal carriage of resistant *Enterobacteriaceae* were observed in different communities. In the Far East, a study conducted in the Chinese Shandong province showed a 42% fecal carriage rate of β-lactamase producing *Enterobacteriaceae* [11]. A similar study covering seven nursing homes in Shanghai reported a rate of 46.92% [12]. Also, a one year study in Nara, Japan identified an 8.5% carriage rate in the community, while the rate went as high as 19.6% among elderly in Japanese nursing homes [13,14]. In European countries such as Germany, a three-year study focusing on resistant *Escherichia coli* fecal carriage identified a 6.4% carriage rate in the community [15]. A different study involv-

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ing thirty one nursing homes in Bavaria recorded a rate of 14.7% [16]. Moreover, simultaneous investigations in two Swedish cities revealed an 8.7% carriage rate of β-lactamase producing *Enterobacteriaceae* in the community and 11% among nursing home residents [17]. In Lebanon, a study conducted on children over a period of three months in 2013 reported a 24.8% carriage rate of MDR organisms [18]. However, higher rates reaching 71.6% and 76.5% of β-lactamase producing *Enterobacteriaceae* were detected in nursing homes in Beirut and Tripoli, respectively [19,20]. These studies also noted that resistant isolates were not consistently recovered from the patients at all the time points chosen.

Many studies showed an association between bacterial resistance and a fitness cost incurred on the bacterium [21]. This association could possibly lead to the loss of resistant strains as they become outgrown by sensitive strains in the gut. One study performed on tigecycline resistant *E. coli* showed a lower total yield of this strain as compared to the parenteral isolate. The reduction was most probably attributed to the acquired resistance [22]. Although several studies have investigated the fitness cost of certain resistant strains, the co-existence of MDR *Enterobacteriaceae* having different mechanisms of resistance has not been investigated.

In this study, fecal samples from elderly residing in a nursing home in north Lebanon were screened for resistant *Enterobacteriaceae* over a period of six months. The *in-vitro* competition among *E. coli* isolates with different phenotypic susceptibility profiles from these samples was then evaluated.

Materials and methods

Study design and population

A cross sectional study was conducted on 10 elderly patients residing in a nursing home located in Tripoli, north Lebanon.

Criteria for selection: patients were randomly selected from a pool of elderly patients in a nursing home previously identified by our group as carriers of MDR organisms. In addition, patients who received antibiotics within the 10 days prior to the initiation of the study were not recruited, and patient who received antibiotics during the period of the study were supposed to be discarded (however, none of the patients fulfilled this criterion).

Collection of fecal swabs and bacterial identification

One fecal swab was collected from each elderly at a regular monthly interval between July and December, 2015. Sensitive bacterial isolates were collected from MacConkey agar plates whereas resistant isolates were collected from MacConkey agar plates supplemented with 2 mg/ml cefotaxime. API20E strips (BioMérieux) were used for identification. Individual isolates were preserved in Luria Bertani broth supplemented with 20% glycerol at −20 °C.

Phenotypic detection of resistance

The Kirby-Bauer diffusion method was used to determine the antimicrobial susceptibility of all the collected isolates. The results were interpreted according to the Clinical Laboratory Standards Institute (CLSI) guidelines [23]. In this study, Multi-Drug-Resistant-Organism (MDRO) was defined as any ESBL, AmpC, and/or Carbapenemase producing *Enterobacteriaceae*. The Double Disk Synergy Test (DDST) was used for the phenotypic determination of ESBL production. In this test, amoxicillin-clavulanic acid disks surrounded by cefepime, ceftazidime and aztreonam disks were placed on a lawn of the test isolate on Mueller Hinton Agar (MHA). After overnight incubation at 37 °C, the detection of a "keyhole" was indicative of ESBL production. Additional tests were performed for all isolates showing reduced susceptibility towards cefoxitin or carbapenems. The additional tests consisted of determination of the changes in the inhibition zones of antibiotic disks by the use of: MHA plates impregnated with 5 mM ethylenediaminetetraacetic acid (EDTA) for the detection of Metallo beta-lactamases (MBLs); MHA plates impregnated with10 g/L phenylboronic acid (PBA) for the detection of *Klebsiella pneumonia* Carbapenemase (KPC); and MHA plates embedded with 270 mg/L cloxacillin for the detection of AmpC [24–26]. Temocillin disks were also used for the detection of OXA-48 production [27].

In-vitro *competition assays*

In-vitro competition assays were performed as described by Lopez-Rojas et al., with minor modifications [28]. Nine combinations were used in the competition assays that included: ESBL, AmpC, or OXA-48 producers on one hand and sensitive *E. coli* isolates on the other; two MDR *E. coli* isolates with different mechanisms of resistance in competition with each other; two MDR *E. coli* isolates with different mechanisms of resistance and a sensitive isolate in competition with each; three MDR *E. coli* isolates with different mechanisms of resistance in competition with each other; and three MDR *E. coli* isolates with different mechanisms of resistance with one sensitive isolate in competition with each other. The OXA-48-producing *E. coli* isolate was provided by Miss Iman Dandachi from a previous study on fecal swabs from elderly patients for inclusion in our study.

Inocula were adjusted to 1.5×10^6 CFU/mL and were used in order to prepare single cultures and mixed cultures for the selected combinations. Single cultures consisted of the strains that were in competition without the presence of any other organism. Single and mixed cultures for the same strains were performed at the same time. For single cultures, 1:10 serial dilutions in sterile distilled water were performed. At each time point, including the moment of inoculation, 20 μL from each dilution was then spread on MHA agar plates in duplicates and the plates were incubated overnight at 37 °C. The same was performed for mixed cultures that were subsequently spread on both MHA and selective MHA (containing 16 mg/L Gentamycin, 2 μg/mL Ciprofloxacin or 2 μg/mL from a 10^5 μg/mL cefotaxime solution; depending on the susceptibility profile of the isolate). In parallel, the OD_{580} was measured at each time point for all the cultures. The following days, viable colonies were counted and the concentrations of the Colony Forming Units per mL (CFU/mL) of each strain in the initial suspension were determined. Competition Indexes (CI) were calculated from mixed cultures. The following formula was used for calculating the CI at each time point: [(*number of isolates A recovered*)/(*number of isolates B recovered*)]/[(*number of isolates A inoculated*)/(*number of isolates B inoculated*)], where isolates "A" and "B" were determined for each combination that was used individually [28]. Growth rates and doubling times were also calculated from the counts and ODs of single cultures, respectively [29,30].

Statistics and data analysis

Semi quantitative and qualitative analysis were conducted using SPSS 20.0 software.

Ethics, consent, and permissions

A consent form regarding the participation in the study was signed by each recruited patient, his/her legal guardian, or an entitled member from his/her family. The privacy of participants and transparency of the ethical process were guaranteed. IRB approval was obtained.

Table 1
Dynamics of β-lactamase producing *Enterobacteriaceae* carriage among recruited elderly. Six fecal swabs for collected from elderly patients and phenotypic determination of the mechanism of the resistant isolates was performed. "AmpC" and "ESBL" stand for the detection of these enzymes among the isolates, "-" indicates that all the isolates were sensitive, "ND" stands for not determined, and "D" indicates that the patient died.

Patient	Collection 1	Collection 2	Collection 3	Collection 4	Collection 5	Collection 6
P1	AmpC	AmpC	–	–	–	–
P2	ESBL	–	–	–	ND	–
P3	–	–	–	–	–	–
P4	–	–	–	–	ESBL	–
P5	ESBL	–	ESBL	ND	ESBL	–
P6	AmpC & ESBL	ESBL	AmpC	–	AmpC	–
P7	ESBL	ESBL	–	–	–	ND
P8	–	–	–	–	AmpC	–
P9	–	–	–	–	AmpC	–
P10	ESBL	–	D	D	D	D

Results

Prevalence and dynamics of resistant Enterobacteriaceae

Nine out of the ten recruited elderly showed at least one time fecal carriage of resistant *Enterobacteriaceae*. Six out of these ten (60%) were fecal carriers in the first collection, three out of ten (30%) in the second, two out of nine (22.2%) in the third, none out of eight (0%) in the fourth, five (62.5%) out of eight the fifth and none (0%) out of seven in the sixth. Table 1 shows the dynamics of collecting the β-lactamase producing enzymes over the six collections. Since more than one isolate was obtained from each recruited elderly, the ratio of fecal samples and isolates collected was not 1:1 and the collected isolates totaled 117. *E. coli* was predominant among the collected isolates (71.8%). Four *K. pneumoniae*, three *Acinetobacter baumannii*, one *Enterobacter cloacae*, one *Proteus mirabilis*, and one *Pseudomonas aeruginosa* isolates were also among the collected isolates.

Of the 117 isolates, 100 (85.5%) were sensitive to all the tested antimicrobial agents and 14.5% were resistant to more than two classes of antimicrobial agents and therefore considered MDR. Non—*E. coli* isolates showed very high susceptibility to all the tested antimicrobial agents. Of the 84 *E. coli* isolates, 94% were susceptible to amoxicillin-clavulanic acid, 91.7% to piperacillin-tazobactam and cefoxitin, 86.9% to ceftazidime, 88.1% to cefepime, 89% to aztreonam, 90.9% to gentamycin, 84.5% to ciprofloxacin, and 76.2% to trimethoprim-sulfamethoxazole. None of the isolates was resistant to carbapenems.

Phenotypic detection of β-lactamase production showed that nine (7.7%) *E. coli* isolates Produced ESBL. AmpC production was detected in six (5.1%) isolates where five were identified as *E. coli* and one as *E. cloacae*. However, the phenotypic test used in this study does not differentiate between chromosomal or plasmidic AmpC production. MBL, KPC, and OXA-48 were not detected phenotypically in any of the isolates.

In-vitro *competition assays*

Competition between a non-β-lactamase producer and β-lactamase producing E. coli

The Competition Indexes (CIs) obtained from *in-vitro* competition assays showed that, in the majority of the cases; the sensitive *E. coli* out-competed the β-lactamase producing *E. coli* strains. When in competition with sensitive *E. coli* strains, two ESBL producers showed a decreased growth at 8 and 72 h with CIs equal to 0.268 and 0.245, respectively. Three AmpC producers also showed a reduced growth after 8 h with CIs ranging between 0.853 and 0.375. Moreover, a continuous decrease in growth of the OXA-48 producer was observed after a CI equal to 0.036 was detected after 8 h of incubation.

Competition between β-lactamase producing E. coli

The growth of AmpC producing *E. coli* exhibited a decrease in growth after 8 h with a CI equal to 0.430 when grown with ESBL producing *E. coli*. Moreover, a CI equal to 0.125 after 8 h was noticed for the OXA-48 producer when competing with ESBL producing *E. coli*. On the other hand, when competing with an AmpC producer, the CI of the OXA-48 producing strain was 0.226 after 24 h of incubation. Furthermore, in one assay where the three types of β-lactamase producing *E. coli* were co-cultured, the OXA-48 producer was outcompeted by both ESBL and AmpC producers (at 8 h, the CI was equal to 0.732 and 0.417, respectively). In the same experiment, AmpC had a weaker growth than ESBL since after 24 h a CI of 0.750 was obtained.

When the ESBL, AmpC, and sensitive *E. coli* strains were put in competition, ESBL producers (CIs: 0.347 and 0.491) and non-beta-lactamase producers (CIs: 0.922 and 0.128) out-competed AmpC producers after 8 h of incubation in two separate assays. ESBL producers exhibited, twice, a reduced growth after 8 h of co-culture (CIs: 0.966 and 0.260) when compared to the sensitive *E. coli*. However, at 48 h, a superior growth for the ESBL producers was noticed (CIs: 1.749 and 3.600). When a combination of all the phenotypes was placed in competition, the OXA-48 producer exhibited the slowest growth after 8 h of incubation as compared to the AmpC (CI: 0.119) and ESBL (CI: 0.033) producers, as well as the sensitive(CI: 0.052) strain. A decrease in growth after 8 h was also noticed for the AmpC producer as compared to the ESBL (CI: 0.275) producer and the sensitive (CI: 0.440) strain. It was not until 48 h after incubation that a decrease in growth was noted for the ESBL (CI: 0.989) producing isolate when compared to the sensitive isolate. Graphs for representatives of the aforementioned competition assays are presented in Figs. 1a and b.

Growth rates

The growth rates obtained from single cultures and the doubling time of these isolates are presented in Table 2. In general, the doubling times of the sensitive and the β-lactamase producing strains were different, leading therefore to greater growth rate constants in the sensitive isolates *versus* the β-lactamase producers. Moreover, similarly to what was observed in the competition assays, ESBL producers exhibited higher growth rate constants while in single cultures than both AmpC and OXA-48 producers. AmpC producers also had higher growth rate constants than OXA-48 producers (Table 2).

Discussion

High fecal carriage rates of resistant *Enterobacteriaceae* were identified in the Lebanese community, more specifically in nursing homes [19,20]. In this study, the fecal carriage rate of β-lactamase producing *Enterobacteriaceae*, as detected at least once during the study period, among 10 elderly patients residing in a nursing home

reached 90%. In accordance with other studies, *E. coli* was the predominant species among both resistant and sensitive isolates [14,19,20]. Also in conformity with earlier studies, the present results showed dynamic carriage of β-lactamase producing *Enterobacteriaceae* among residents of Lebanese nursing homes [19,20].

A possible explanation for this dynamic carriage would be the fitness cost exerted on the bacteria by the resistance mechanisms. In fact, available literature reported that in an antibiotics-free environment, resistant genes confer a fitness cost to the bacteria, leading to its decrease in fitness and frequency [21]. This was shown in our study where isolates with certain resistance mechanisms had slower growth rates when cultured alone without antibiotic stress as compared to strains with other susceptibility profiles (Table 2). Shin and Ko also identified a fitness cost in CTX-M producing *E. coli* when co-cultured with a non ESBL producing isolate from the same species [31]. In addition, AmpC and carbapenemase producing *E. coli* isolates were found to exhibit a reduced fitness cost in the presence of their parenteral isolates [32,33]. In our study,

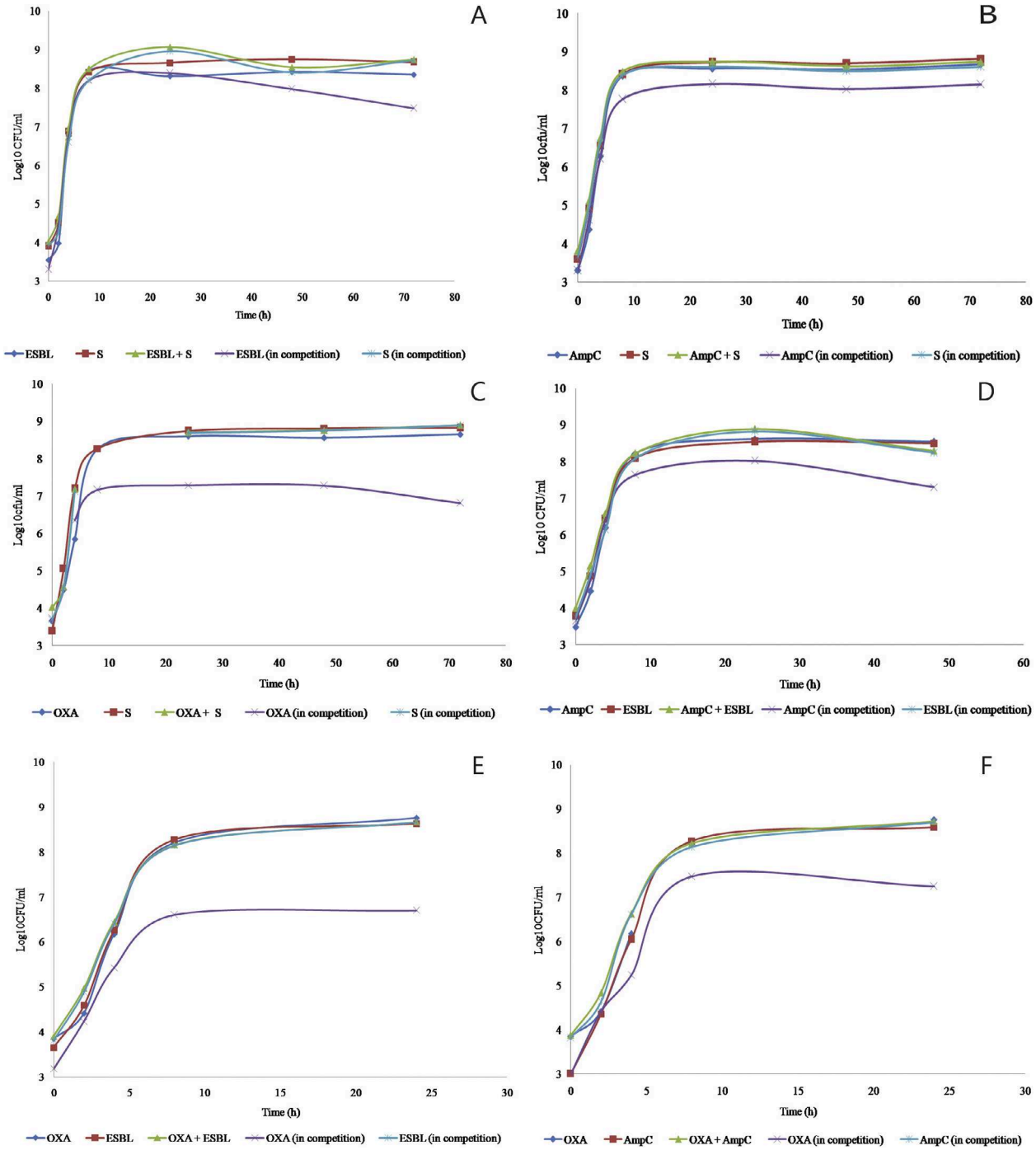

Fig. 1. (a) *In-vitro* competition assays between *E. coli* of different susceptibility profiles. Parts A, B, and C show the growth of *E. coli* isolates having one type of β-lactamase placed in competition with sensitive *E. coli* strains. Parts D, E, and F represent the competition assays between two different *E. coli* isolates producing different types of β-lactamases. (b) *In-vitro* competition assays between *E. coli* of different susceptibility profiles. Part G shows the competition assay between an AmpC producer, an ESBL producer and a sensitive *E. coli* strain. Part H shows the competition between three types of β-lactamase producing *E. coli* (OXA-48, AmpC and ESBL). Part I shows the *in-vitro* competition assay between an OXA-48 producer, AmpC producer, ESBL producer and a sensitive *E. coli* isolate.

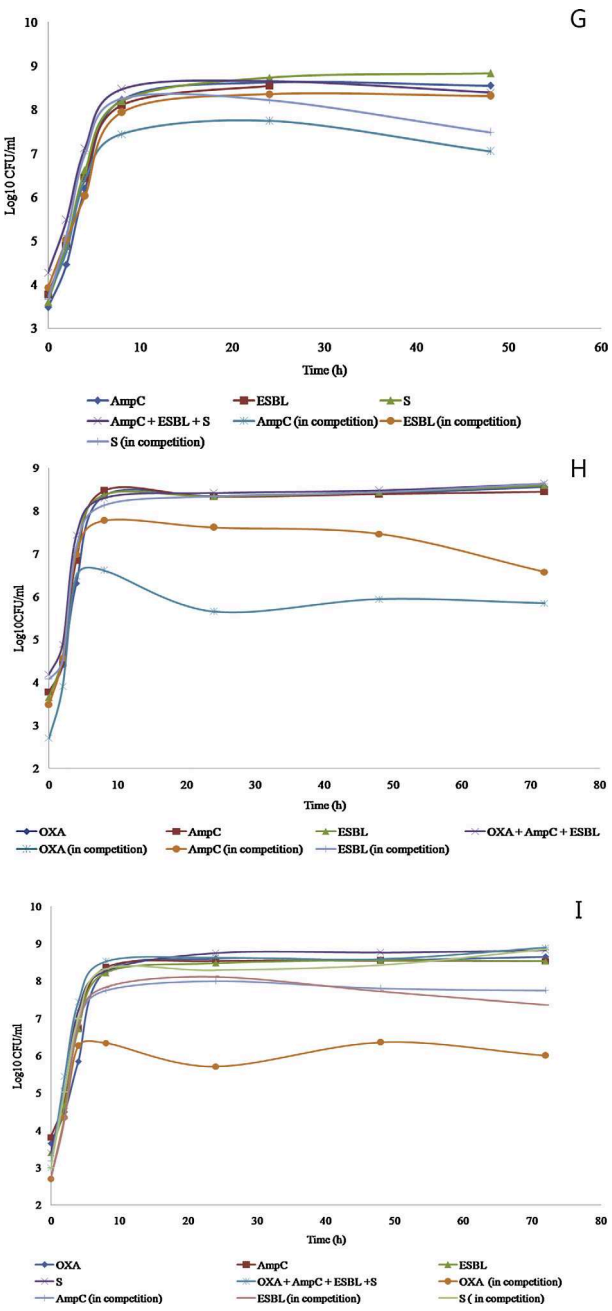

Fig. 1. (a) (*Continued*)

in accordance to what was previously published, ESBL, AmpC and OXA-48 producing *E. coli* isolates exhibited a slower growth rate in the presence of a sensitive strain of *E. coli*. When competition assays were done in presence of *E. coli* producing different types of beta-lactamases, a higher growth rate was exhibited by ESBL producers over AmpC and Oxa-48 producers, and a higher growth rate was shown by AmpC producers over Oxa-48 producers. This suggests that the type of the β-lactamase produced might affect direct or indirectly the ability of the isolate to grow in presence of another bacterium, affecting therefore bacterial fitness.

To the best of our knowledge, this is the first study to conduct competition assays on isolates of *E. coli* producing different types of β-lactamases. Our results indicate that, among the tested organisms, sensitive isolates of *E. coli* were found to be the least affected by the presence of other bacteria, and therefore, to compete the most in this context. Interestingly, a greater fitness cost could be associated with the OXA-48 producing *E. coli*, followed respectively by the AmpC and ESBL producers. These findings could possibly be one of the reasons as to why ESBL harboring *E. coli* isolates are more frequently encountered in fecal samples than those producing AmpC and OXAs [19,20]. It also explains why MDR organisms were not consistently isolated from the same patient over time. Moreover, this could be a trigger for future studies that would explore the possibility of causing the resistant organisms to be lost from the gut flora by putting them in competition with susceptible ones.

In conclusion, high rate of MDROs were detected in fecal samples of elderly residents in a Lebanese nursing home. Moreover, resistance through the production of β-lactamases in *E. coli* seems to confer a fitness cost on the bacterium, as detected by our *in-vitro* competition assays. The specific type of β-lactamase results in a different fitness cost where OXA-48 seems to exert the greatest toll on the bacterial cell, and ESBLs the least. In view of the increased car-

Table 2
Growth rates and doubling times of all the used isolates. The growth rate and the doubling time of each isolate used in the *in-vitro* competition assay were calculated during the exponential phase in single cultures.

Isolate	Growth rate constant	Doubling time (min)
ESBL producers *versus* sensitive strain		
P4S5D122/2 (ESBL)	0.714	27.98
P4S5D122/1 (sensitive)	0.785	29.04
P5S5D122/1 (ESBL)	0.701	30.13
P5S5D122/2 (sensitive)	0.735	28.31
AmpC producers *versus* sensitive strain		
P6S5D122/1 (AmpC)	0.510	29.41
P6S5D122/2 (sensitive)	0.510	30.64
N48t (AmpC)	0.578	12.76
P9S4D90/1 (sensitive)	0.655	16.66
P6S5D122/1 (AmpC)	0.535	12.06
P4S5D122/1 (sensitive)	0.775	11.81
OXA-48 producer *versus* sensitive strain		
Z6t2 (OXA-48)	0.502	14.75
P9S4D90/1 (sensitive)	1.133	15.69
AmpC *versus* ESBL producers		
N48t (AmpC)	0.598	11.83
N36b (ESBL)	0.724	13.88
OXA-48 *versus* ESBL producers		
Z6t2 (OXA-48)	0.597	14.22
N31 (ESBL)	0.601	13.81
OXA-48 *versus* AmpC producers		
Z6t2 (OXA-48)	0.597	14.22
N48t (AmpC)	0.543	13.50
AmpC and ESBL producers with sensitive strain		
P6S5D122/1 (AmpC)	0.465	12.26
P4S5D122/2 (ESBL)	0.478	14.49
P4S5D122/1 (sensitive)	0.598	13.27
N48t (AmpC)	0.598	11.83
N36b (ESBL)	0.724	13.88
P9S4D90/1 (sensitive)	0.769	15.08
OXA-48, AmpC, and ESBL producers		
Z6t2 (OXA-48)	0.594	13.44
N48t (AmpC)	0.747	11.86
N31 (ESBL)	0.928	13.38
OXA-48, AmpC, and ESBL producers with sensitive strain		
Z6t2 (OXA-48)	0.502	14.75
N48t (AmpC)	0.742	13.93
N31 (ESBL)	0.810	14.39
P9S4D90/1 (sensitive)	1.140	15.69

riage of MDROs among members of the intestinal flora, our findings are important for the understanding of how these microorganisms interact with each other promoting or decreasing the spread of resistance in this environment. The systematic use of antimicrobial agents to eradicate or limit the spread of resistant organisms has many drawbacks and contributes to the collateral damage. In this context, the understanding of the composition of the normal flora and the different mechanisms of resistance can be a valuable tool in the decision whether to use an antibiotic or not in specific categories of patients. A bigger pool of patients and the inclusion of different types of bacteria could shed further light on this matter and future studies should include a bigger sample of patients in order to validate any conclusion.

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Competing interests

None declared.

Ethical approval

Not required.

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Article 12

Competition assays between ESBL-producing E. coli and K. pneumoniae isolates collected from Lebanese elderly: An additional cost on fitness.

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Competition assays between ESBL-producing *E. coli* and *K. pneumoniae* isolates collected from Lebanese elderly: An additional cost on fitness

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ABSTRACT

The dissemination of Multi Drug Resistant Organisms (MDROs) is one of the major public health problems addressed nowadays. High fecal carriage rates of MDR *Enterobacteriaceae* were reported from Lebanese nursing homes. Studies have shown that the acquisition of resistance genes by bacteria might confer a fitness cost detected as a decrease in the frequency of these bacteria as compared to sensitive isolates. In this study, the competitive growth of MDR *Enterobacteriaceae* isolated from elderly is assessed.

Sensitive and ESBL-producing *Escherichia coli* and *Klebsiella pneumoniae* isolates were identified. Interspecies *in-vitro* competition assays were conducted in different combinations.

ESBL-producing *K. pneumoniae* presented a fitness cost when competing against sensitive *E. coli*. On the other hand, resistant *E. coli* only showed a fitness cost when growing in presence of two sensitive *K. pneumoniae* isolates. These results suggest that ESBL-production genes in *E. coli* and *K. pneumoniae* may confer a fitness cost that leads to the decrease in frequency of these bacteria in interspecies competitions. Culturing bacteria in a medium with more diverse isolates can provide better insights into bacterial competition and resistance dynamics, which can be exploited in the search for alternative therapeutic approaches towards the colonization of resistant bacteria.

Introduction

The dissemination of Multi Drug Resistant Organisms (MDROs) is one of the major public health issues being addressed nowadays [1]. Infections with MDROs can lead to increased morbidity, mortality and health care costs [2]. In this context, members of the *Enterobacteriaceae* family have developed complex mechanisms of resistance, chiefly the production of extended spectrum beta lactamase (ESBLs), AmpC beta lactamases and carbapenemases; these enzymes provide the bacterium with resistance toward the majority of the therapeutic options available in the clinical market [3,4]. The antibiotic pipeline is drying up and no new antibiotics are seen in the near future for the treatment of infections caused by these MDROs [2].

The human intestinal microbiota is currently recognized as an epicenter for gene resistance and horizontal gene transfer among bacterial species [5]. This is mainly due to the intestinal high exposure to antimicrobial agents driven by the over usage of antibiotics [6]; in addition to its rich abundance in nutrients, attachment sites and high cell density. The over usage of antibiotics drives a selective pressure that favors resistant bacteria over the sensitive ones; in addition, it creates a favorable environment for the transfer and development of resistance genes [6]. Accordingly, high fecal carriage levels of resistant *Enterobacteriaceae* were detected in the Lebanese community. For instance, a study examining carriage among children between 1 and 5 years old presented a rate of 24.8% [10]. Moreover, studies on Lebanese nursing homes revealed remarkably higher rates of fecal carriage of 71.6% and 76.5% in samples collected from Beirut and Tripoli, respectively [11,12]. Notably, these studies also reported that resistant isolates were not consistently retrieved from the patients at all the tested time points.

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Table 1
Primers used in this study.

Target(s)	Primer	Sequence (5′ to 3′)	Size (bp)
TEM	TEM-F	CGC CGC ATA CAC TAT TCT CAG AAT GA	445
	TEM-R	ACG CTC ACC GGC TCC AGA TTT AT	
SHV	SHV-F	CTT TAT CGG CCC TCA CTC AA	237
	SHV-R	AGG TGC TCA TCA TGG GAA AG	
OXA	OXA-F	ACA CAA TAC ATA TCA ACT TCG C	813
	OXA-R	AGT GTG TTT AGA ATG GTG ATC	
CTXM	CTXM-F	ATG TGC AGY ACC AGT AAR GTK ATG GC	593
	CTXM-R	TGG GTR AAR TAR GTS ACC AGA AYC AGC GG	

When present in an antibiotic free environment, studies have shown that the acquisition of resistance genes by bacteria interferes with their biological functions and might confer a fitness cost detected as a decrease in the frequency of these bacteria [7].

The hypothesis stating that the decreased and controlled antibiotic usage can lead to a reduction in intestinal carriage of bacterial resistance remains to be proved. Studies conducted in order to investigate the fitness alterations caused by the acquisition of antibiotics resistance genes were mainly targeting sensitive bacterial species and their resistant counterpart [8–10]. However, inter-species competitions are not given attention.

In this study, resistant *Enterobacteriaceae* isolated previously by Challita et al. [11] from fecal samples collected from elderly residing in nursing homes in North Lebanon were put in competition. Hence, the aim was to evaluate the fitness alterations conferred by the production of β-lactamases, more specifically ESBLs, in *Escherichia coli* and *Klebsiella pneumoniae* isolates through in-vitro competition assays between sensitive and resistant isolates. Fitness alterations conferred by the production of ESBL, in the presence of more than one competing sensitive strains was also investigated.

Methodology

Bacterial isolates

A total of 4 strains of *E. coli* and 4 strains of *K. pneumoniae* were used for the competitions assays. For *E. coli*, as well as for *K. pneumonia*, the strains consisted of 2 sensitive and 2 ESBL producers. These strains were isolated from fecal swabs of elderlies, residents in a nursing home situated in Tripoli, North of Lebanon.

Identification of chosen isolates was done using API 20E strips (BioMérieux). Antibiotic Susceptibility Testing was performed using the Kirby-Bauer diffusion technique. Interpretation of the results was performed according to the Clinical Laboratory Standards Institute (CLSI) guidelines (2014) [12]. For the determination of ESBL production, the Double Disk Synergy Test (DDST) was used. Briefly, in this test, a disk of amoxicillin-clavulanic acid was placed in the center between ceftazidime, cefepime and aztreonam disks on the surface of a Mueller Hinton Agar plate inoculated with the tested organism. The detection of a key-hole effect after an overnight incubation at 37◦C was the phenotypic confirmation of ESBL production.

Multiplex PCR analysis for CTX-M, TEM, SHV and OXA genes detection

For the genotypic confirmation of beta lactamases production, a multiplex PCR was conducted. Universal primers, previously described were used for bla_{CTX-M}, bla_{TEM}, bla_{SHV} and bla_{OXA} genes (Table 1) [13]. DNA extraction was done by suspending 2 colonies of the test isolate in 200 μL of sterile distilled water and heating the solution at 95 °C for 10 min. Thereafter, a multiplex PCR was carried on under the following reaction conditions: 15 min of initial denaturation step at 95 °C, followed by 30 amplification cycles of 30 s at 94 °C, 90 s at 62 °C, and 60 s at 72 °C, with a final extension step of 10 min at 72 °C. Amplified PCR products were run on a 1.5% agarose gel at 130 V for 1 h. DNA amplicons visualization was done using a digital Gel documentation system (Biorad).

In-vitro competition assays

In-vitro competition assays were performed as described previously [14]. Six different combinations were used: one sensitive *E. coli* and one sensitive *K. pneumonia*; one ESBL-producing *E. coli* and one ESBL-producing *K. pneumoniae*; one sensitive *E. coli* and one ESBL-producing *K. pneumoniae*; one sensitive *K. pneumoniae* and one ESBL-producing *E. coli*; one ESBL-producing *K. pneumoniae* and two different sensitive *E. coli*; one ESBL-producing *E. coli* and two different sensitive *K. pneumoniae*. To note that assays were conducted in duplicates and taking into account all possible combinations within each group.

For each bacterial isolate used in single culture or in combination with another competing isolate, an initial inoculum of 0.5 McFarland (equivalent to 1.5×108 CFU/mL) was prepared; thereafter, 1:100 dilution in SDW was preformed to reach a final concentration of 1.5×106 CFU/mL. In each combination, single cultures contained the *E. coli* or *K. pneumoniae* isolate alone in the medium, while the mixed cultures contained competing isolates all together. For single cultures, 1:10 serial dilutions in SDW were performed. Thereafter, 20 μL from each dilution was spread on MHA agar plates in duplicates and incubated overnight at 37 °C at each time point, including the moment of inoculation. For mixed cultures, same procedure was performed; however, 20 μL from each dilution was spread on both MHA agar plates and selective MHA plate containing cefotaxime (2 μg/mL). To note that the selective plates were only for the combinations including sensitive and resistant isolates; whereas those containing only sensitive or resistant strains in competition, the spread was done on Uriselect medium plates in order to differentiate between *E. coli* and *K. pneumoniae* competing isolates. At each time point, the OD_{580} was measured. The concentrations of Colony Forming Units per mL (CFU/mL) of the original suspensions at each time point, was calculated by counting the viable colonies on the agar plates after overnight incubation at 37 °C.

Furthermore, competition indexes (CI) were calculated from mixed cultures using the following formula at each time point:

$$\frac{\text{number of isolates (A) recovered/number of isolates (B) recovered}}{\text{number of isolates (A) inoculated/number of isolates (B) inoculated}}$$

"A" and "B" isolates are determined for each combination that was used individually [14]. Counts and ODs of single cultures were used for the calculations of growth rates and doubling times respectively [15,16].

Statistical analysis

IBM SPSS Statistics version 20.0 (IBM Corp., Armonk, NY, USA) was used for the qualitative and semi-quantitative analysis calculations.

Results

Genotypic detection of beta lactamase genes

Multiplex PCR analysis revealed that the 2 ESBL producing *E. coli* chosen in this study harbored the CTX-M and CTX-M/TEM genes respectively. Regarding the ESBL producing *K. pneumoniae* strains: one had the CTX-M, SHV and TEM genes while the second one contained all 4 genes: TEM, SHV, CTX-M and OXA. *E. coli*

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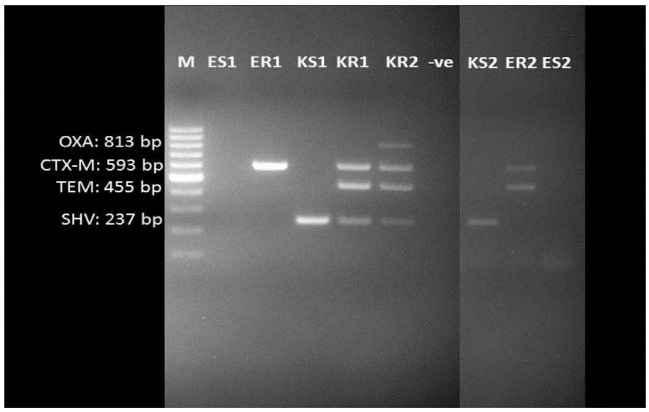

Fig. 1. Detection of beta lactamase genes: OXA, CTXM, SHV and TEM in *E. coli* and *K. pneumoniae* isolates used in this study. ES1/ES2 and ER1/ER2 correspond the sensitive and resistant *E. coli* isolates respectively. KS1/KS2 and KR1/KR2 correspond the sensitive and resistant *K. pneumoniae* isolates respectively. M is a 1.2-kb DNA ladder. Molecular size of the bands in question is indicated in parentheses on the right of the picture.

susceptible isolates were negative for beta lactamase genes while the *K. pneumoniae* ones harbored only the SHV gene (Fig. 1). SHV gene is universally present in *K. pneumoniae*, evolved first as a chromosomal gene encoding for naturally produced penicillinases in *Klebsiella* spp.; this gene have spread to other enterobacterial species through its incorporation into plasmids [17].

In-vitro competition assays

Competition between a sensitive E. coli *and a sensitive* K. pneumoniae

The competition indexes obtained from in vitro competition assays showed that the sensitive *K. pneumoniae* out-competed the sensitive *E. coli* isolate when grown in the same medium; this was suggested by a CI of 0.72 and 0.73 after 8 and 48 h respectively for the two sensitive *K. pneumoniae* strains tested.

Competition between an ESBL producing E. coli *and an ESBL producing* K. pneumoniae

After 8 h of incubation, no difference in growth of the ESBL producers was detected. However, after 24 h, ESBL producing *E. coli* out-competed the ESBL producing *K. pneumoniae*, represented by a CI bigger than 2.

Competition between an ESBL producing E. coli *and one or two sensitive* K. pneumoniae

When an ESBL producing *E. coli* was put in the same medium with one sensitive *K. pneumoniae* the following result was obtained: out of 4 combination possibilities performed, 2 showed no difference in growth between the competing isolates; whereas 2 showed competition indices (1.25 and 2.59 after 8 and 48 h respectively) favoring the ESBL producing *E. coli* compared to the sensitive *K. pneumoniae*. On the other hand, when the ESBL producing *E. coli* was in competition with two sensitive *K. pneumoniae* isolates, it showed a decrease in growth after 8 h of incubation, represented by a CI equal to 0.85.

Competition between an ESBL producing K. pneumoniae *and one or two sensitive* E. coli

When an ESBL producing *K. pneumoniae* was put in competition with one or two sensitive *E. coli* isolates, the CI was always in favor of the sensitive *E. coli* strains (CI <1 after 8 h). Representative graphs for all aforementioned results are presented in Fig. 2.

Table 2
Doubling times and growth rates of all used isolates. These were calculated during the exponential phase in single cultures.

Isolates	Doubling time (min)	Growth rate
Sensitive *E. coli* v/s sensitive *K. pneumoniae*		
E. coli (S1)	46.48	2.384
K. pneumoniae (S1)	45.08	2.158
E. coli (S2)	53.11	2.116
K. pneumoniae (S2)	47.21	2.423
ESBL *E. coli* v/s ESBL *K. pneumoniae*		
E. coli (R1)	46.93	2.662
K. pneumoniae (R1)	45.78	2.289
E. coli (R2)	35.93	2.191
K. pneumoniae (R2)	36.77	2.233
ESBL *K. pneumoniae* v/s sensitive *E. coli*		
E. coli (S1)	47.76	1.992
K. pneumoniae (R1)	39.38	2.252
E. coli (S2)	35.19	2.923
K. pneumoniae (R2)	40.58	2.375
E. coli (S2)	53.18	2.880
K. pneumoniae (R1)	55.11	2.545
E. coli (S1)	18.66	1.446
K. pneumoniae (R2)	27.33	1.236
ESBL *E. coli* v/s sensitive *K. pneumoniae*		
K. pneumoniae (S1)	45.32	2.110
E. coli (R1)	46.23	2.582
K. pneumoniae (S2)	38.84	2.329
E. coli (R2)	38.54	2.014
K. pneumoniae (S1)	40.45	2.888
E. coli (R2)	42.98	3.677
K. pneumoniae (S2)	41.22	2.122
E. coli (R1)	43.97	2.865
ESBL *K. pneumoniae* v/s two sensitive *E. coli*		
E. coli (S1)	30.45	2.761
E. coli (S2)	31.37	2.822
K. pneumoniae (R1)	45.98	1.471
ESBL *E. coli* v/s two sensitive *K. pneumoniae*		
K. pneumoniae (S1)	28.39	3.233
K. pneumoniae (S2)	27.12	2.818
E. coli (R1)	48.71	2.142

Growth rates

The doubling times and Growth rates calculated from single cultures of each isolate used are presented in Table 2. Overall, the results of growth rates and doubling times were compatible with the competition indices. Sensitive *K. pneumoniae* isolates had lower doubling time and higher growth rates compared to the sensitive *E. coli*. In addition, considering the in-vitro competition assays of ESBL producers and sensitive isolates; in these latter, except for one case (ESBL *E. coli* versus sensitive *K. pneumoniae*), sensitive *E. coli* and *K. pneumoniae* isolates had always a lower doubling time and a higher growth rate compared to the ESBL producer.

Discussion

The fecal carriage of MDROs has been thought as a risk factor for infections with limited therapeutic options and causing increased morbidity and health care costs [18]. Studies addressing this issue all agreed that the fecal carriage of resistant organisms is always dynamic i.e. variable over time [19]. One possible cause of this dynamicity is that resistance genes when acquired by a bacterium, temper with the normal growth and confer a fitness cost for the hosting organism [20]. Among other factors, the fitness cost is manifested by an increased doubling time and a lowered growth rate. These properties can be inferred when culturing resistant isolates alone and in the presence of sensitive strains [7]. While many studies addressed this issue by performing in-vitro competition assays between sensitive and resistant isolates of the same species, inter species competitions has been given little attention. Our study

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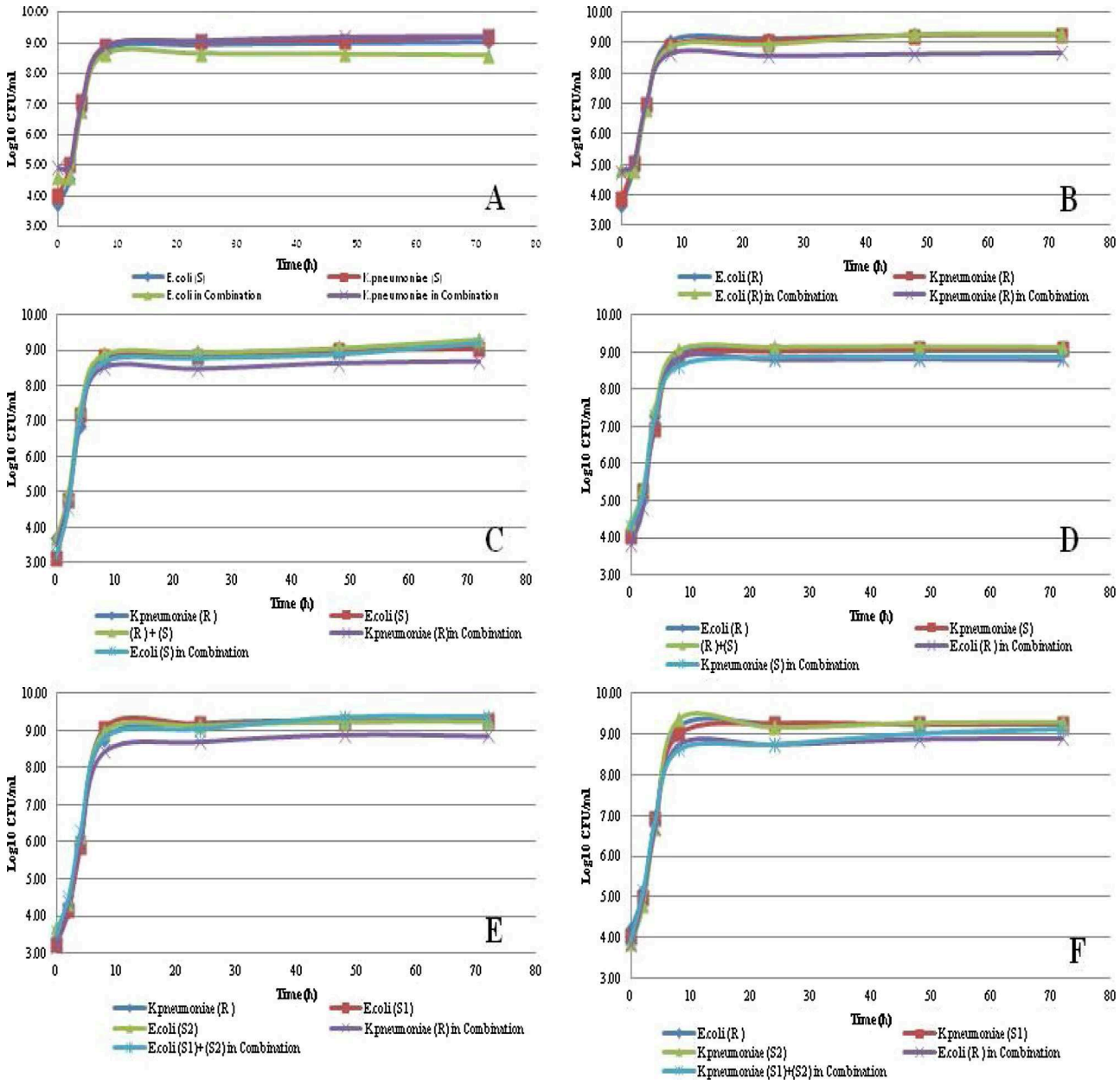

Fig. 2. In vitro competition assays between (A) sensitive *E. coli* and *K. pneumoniae* (B) ESBL-producing *E. coli* and *K. pneumoniae* (C) (E) ESBL-producing *K. pneumoniae* with one and two sensitive *E. coli* respectively (D) (F) ESBL-producing *E. coli* with one and two sensitive *K. pneumoniae* respectively.

has shown that when present in the same medium, sensitive *K. pneumoniae* is more fit then its *E. coli* counterpart. However, when both are ESBL producers, *E. coli* is the out-competitor. This phenomenon can have two explanations: first is that the acquisition of ESBL genes induces a fitness advantage in *E. coli* while it causes a fitness cost in *K. pneumoniae*. Second, as shown in Fig. 1, ESBL producing *K. pneumoniae* isolates harbored more beta lactamase genes than the ESBL producing *E. coli* strains. Whether a higher number of resistance genes can cause a higher fitness cost in the hosting bacterium remains to be tested on a larger number of samples containing a wider variety of resistance genes. On the other hand, the ESBL producing *E. coli* showed also a fitness advantage when competed with a sensitive *K. pneumoniae*. This in part shows that an ESBL producing *E. coli* has a fitness advantage in the presence of one *K. pneumoniae* isolate whether this latter is an ESBL producer or not. However, when present with more than one sensitive *K. pneumoniae* isolate (Fig. 1C), it pays a fitness cost depicted by significantly higher doubling time and growth rate. Therefore, it can be deduced that not only the resistance characteristics of the competitor strains are important but also their numbers in the medium.

Regarding the competition assay involving ESBL producing *K. pneumoniae* and sensitive *E. coli* isolates, our results showed that the sensitive strains are always out-competing resistant isolates, even if these latter belong to different species. Given the fact that antibiotics target essential bacterial functions, it seems plausible that newly acquired bacterial resistance, which results from alterations of cellular functions and enzymes production, imposes changes on bacterial fitness; hence, inducing alterations in competition outcomes [7]. One study conducted by Linkevicius et al has shown that competition assays between wild type *E. coli* and tigecycline resistant isolates harboring mutations in the efflux regulatory network (ERN) *lon* and *marR* genes, and LPS genes had 13%, 0.3% and 24% fitness decrease for *lon*, *marR* and LPS mutants respec-

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tively [21]. As for carbapenem resistance genes, plasmid-mediated NDM-1 exhibits a fitness reduction in *K. pneumoniae* and *E. coli* [8] and VIM-2 in *Salmonella enteric* [22] when cultured with their sensitive counterparts. Recently, a study conducted by Challita et al. showed that among different beta lactamase producing *E. coli*; OXA-48 producers exhibited the greater fitness cost, followed by AmpC then ESBL producers as compared to the sensitive isolates when co-cultured altogether [11].

In conclusion, to the best of our knowledge, our study is the first to expose two different gastro-intestinal tract colonizers in inter-species competitions. Increasing the number of studied isolates, in addition to the usage of additional types of bacterial species in competition would mimic the diverse composition of intestinal normal flora and gives better insights about bacterial competition and resistance dynamics. Furthermore, in accordance with other previous studies, it became plausible that a possible way of managing GIT colonization with ESBL producers is the reduction and controlled usage of antibiotics. Alternatively, another suggested employment of these findings may be the application of "Fecal Microbiota Transplantation" (FMT). Since antibiotics consumption disrupts the GIT microbiota and enables opportunistic pathogens to cause infections, FMT from a healthy donor can re-establish the normal flora by competition [23].

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Competing interests

None declared.

Ethical approval

Not required.

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Chapter VI conclusion

The prevalence of multi-drug resistant organisms in the nursing homes of north Lebanon is elevated (1). The fecal carriage appears to be dynamic and significantly associated with a recent antibiotic intake (2). Infection control measurement including the screening of newly admitted residents for multi-drug resistance is needed in these settings. Furthermore, antibiotic stewardship programs are crucial to control the over-use of antibacterial agents in these areas.

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CONCLUSION AND FUTURE PERSPECTIVES

For many years, multi-drug resistant organisms were thought to be confined to the hospital settings (1). However, recent studies have demonstrated the presence of an external reservoir of resistance in "animal sector" from which multi-drug resistant organisms can be transferred to humans (1). ESBL, carbapenem and colistin resistant Gram-negative bacilli are nowadays heavily reported in livestock worldwide (2) (3). The zoonotic transmission of multi-drug resistant organisms is sparked by the concern of causing infections with limited therapeutic options (4). The first step toward controlling the diffusion and emergence of resistance in animals is by determining the extent of the dissemination of multi-drug resistant organisms in a country via surveillance studies; then deciphering the driver factors that have contributed to the observed situation and for which infection control measures will be implemented accordingly.

In Lebanon, the extent of ESBL and colistin resistant organisms' dissemination in food producing animals was unknown. This research work provides an original description on the current epidemiology of ESBL and ampC producing Gram-negative bacilli in chicken and pigs over the Lebanese territory. Furthermore, this work reports for the first time in this country the detection of mcr-1 in poultry, swine, feed, litter but also in farmers. Previous studies In Lebanon targeted mainly the prevalence of beta lactamase producers in the clinical settings (5). On the other hand, the mechanism of colistin resistance was described by a single study that reported the detection of three colistin resistant K. pneumoniae in a hospital in Beirut; colistin resistance in these latter where due to mutations in the phoP/Q, pmrA/B and mgrB genes (6). ESBL/ampC producing Gram-negative bacilli are heavily disseminated in poultry and swine. Over a two years period, the prevalence of ESBL producers has increased significantly by 32% in the south of Lebanon. A significant increase was also observed at the level of CTX-M and TEM genes. The detection of different sequence types in addition to the random distribution of isolated strains in the MSP dendrogram reveals a multi-clonal dissemination of multi-drug resistant organisms and suggests rather the diffusion of plasmids carrying resistance genes. Gentamicin and colistin are among the most common antibiotics administered for poultry in Lebanon. Personal communication with a worker in one of the visited swine farms revealed that enrofloxacin is given for pigs. Indeed, it has been suggested that unregulated use of antibiotics is the main driver for the emergence of resistance in animals (7). However, unfortunately, other factors are involved such as poor sanitation and crowding (8). In Lebanon, this mostly applies to the swine farms where we have found during our surveillance poor feed quality, questionable hygienic measures and waste management.

Studies have shown that contaminated waste water, soils, air dust and feed are all possible routes of resistance transmission from animals to their surroundings and vice versa (9).

The detection of beta lactamase and mcr-1 positive Gram-negative bacilli in the chicken feed in Lebanon is questionable. Are these due to contamination from the housing environment or that antibiotics are hiddenly used as feed additives? In the literature, it has been suggested that the detection of bacteria resistant to antibiotics used as feed additives in animals is a possible evidence that antibiotics use as growth promoters is a contributor of the emergence and dissemination of multi-drug resistance in food producing animals (10). An example for this is the use of avoparcin (a vancomycin analogue not used in humans) as a feed additive in livestock. The use of this antibiotic as a growth promoter in animals was associated with the emergence and dissemination of vancomycin resistant Enterococci (VRE) in humans In Europe in the early and mid-1990s (11). As a consequence, European Union banned avoparcin administration for animals in 1995 (11). Thereafter, surveillance studies have shown that avoparcin ban was accompanied with a significant decrease in the prevalence of vancomycin resistant Enterococci in animals and subsequently in humans (11). This is unlike the US, where avoparcin use in the veterinary sector continued and as a result no change in the dissemination of VRE has occurred. A recent review paper conducted by O'Driscoll et al in 2015 showed that the prevalence of VRE is significantly lower in Europe compared to the one in the US and Latin America (12). Therefore, in Lebanon, in view of the heavy dissemination of mcr-1 strains observed during our investigations, it becomes crucial to ban colistin use in animals. This will definitely lead to a decrease in the prevalence of mcr-1 in the territory as the aforementioned experience of avoparcin ban in Europe has shown. Colistin ban and control of antibiotic usage in the Lebanese veterinary sector can be compensated in the future by the use of vaccines against the most common bacteria causing infections in the Lebanese Livestock. This could be achieved by first conducting surveillance studies on resistant bacteria in diseased animals. By knowing the most common bacterial agents causing infections in the Lebanese farm animals with their profiles of resistance; vaccines against these latter can then be implemented. One example could be the use of the ASN-4 monoclonal antibody against mcr-1 E. coli (13). Guachalla et al showed that ASN-4 retained its bactericidal activity against positive mcr-1 ST131 E. coli strains as compared to the mcr-1 negative ones (13).

Moreover, as previously reported for humans (14), unfortunately antibiotics are not the sole contributors to the emergence of resistance in animals; indeed colistin ban in Lebanon should be accompanied by the implementation of strict infection control measures in farm animals.

Farm's owners and workers should be trained to ensure continuous proper disinfected areas. Disinfection of farmers' boot upon entry and exit from the chicken house in addition to gloves wear, devoted clothing and footwear are warranted. Furthermore, risk factors associated with the acquisition of colistin resistance in animals in Lebanon, beside colistin use and hygienic measures should be explored in future studies.

In conclusion, our work has contributed to a better knowledge of the epidemiology and risk factors of the acquisition of multi-drug resistant bacteria in animals in Lebanon. In the "one health" concept this work re-emphasizes the need to have global intervention measures to avoid dissemination of antibiotic resistance in humans, animals and environment.

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