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Présentée par

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RECHERCHE DE BACTERIES MULTI RESISTANTES DANS LES FECES DES ANIMAUX DU BASSIN MEDITERRANEEN.

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Mr. Le Professeur Philippe Colson	**Président de jury**
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AVANT PROPOS

Le format de présentation de cette thèse correspond à une recommandation de la spécialité Maladies Infectieuses et Microbiologie, 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.

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RÉSUMÉ

La résistance aux antibiotiques est devenue une priorité mondiale en matière de santé publique et animale. L'utilisation des antibiotiques chez les animaux est un facteur de risque de sélection et de transmission des bactéries résistantes à l'homme. Notre travail de thèse a porté sur la recherche des bactéries multirésistantes zoonotiques dans les fèces des animaux du bassin méditerranéen, région pour laquelle peu de données sont disponibles. Cette thèse s'articule autour de quatre objectifs principaux; (1) épidémiologie et prévalence des bactéries productrices de β-lactamases et de carbapénèmases chez les poulets d'Algérie, (2) étude et détection de la résistance plasmidique à la colistine chez les animaux d'Algérie, (3) recherche de vecteurs et/ou de réservoirs de bactéries zoonotiques résistantes à la colistine, (4) épidémiologie des bactéries résistantes à la colistine chez les humains en France, au Laos et au Liban. Au départ de cette thèse, nous avons rédigé deux revues de synthèse bibliographique portant sur l'épidémiologie de la résistance aux antibiotiques (notamment les β-lactamines et la colistine) chez les bactéries à Gram-négatif isolées à partir des animaux et sur l'utilisation en milieu vétérinaire de certains antibiotiques de derniers recours utilisés chez l'homme. Dans une première revue, nous avons voulu mettre en parallèle l'utilisation de la colistine dans l'élevage et l'émergence de la résistance à la colistine d'origine plasmidique chez les animaux dans le monde afin de soulever la question de l'utilisation de cet antibiotique utilisé en thérapeutique humaine et les risques de transmission de la résistance à la colistine chez l'homme. La deuxième revue a porté sur la prévalence et l'émergence des bactéries résistantes aux β-lactamines, dont les carbapénèmes, et à la colistine chez les animaux dans le bassin méditerranéen. Puis nous avons mené un certain nombre de travaux scientifiques pour étudier et décrire la résistance aux antibiotiques chez les animaux.

La deuxième partie de cette thèse a porté sur la recherche et la caractérisation des bactéries résistantes aux β-lactamines chez les poulets de 8 régions d'Algérie et du sud de la France. A travers cette investigation, nous avons trouvé une forte prévalence et une grande diversité des gènes codants pour des β-lactamases à spectre étendu (BLSE) et des carbapénèmases dans les fèces de volailles d'Algérie.

Du fait de la découverte récente de la résistance à la colistine médiée par un transposon, le gène mcr-1, une partie de notre travail a porté sur la détection des réservoirs animaliers de bactéries

porteuses du gène mcr-1 afin d'identifier de nouveaux vecteurs potentiels de ces bactéries résistantes à la colistine. Nous avons détecté en premier lieu la présence du gène mcr-1 dans des souches d'Escherichia coli isolées à partir de selles humaines provenant du Laos et de Thaïlande ainsi que dans des souches d'E. coli isolées de fèces de porcs du Laos et de poulets d'Algérie. Il s'agit de la première description de mcr-1 en Afrique. Dans le même axe, nous avons ensuite désigné des systèmes de détection rapide par PCR en temps réel et PCR standard afin de détecter et caractériser le gène mcr-1. Ces systèmes ont été appliqués sur des collections de fientes de poulets et de fèces de bovins, ovins et caprins d'Algérie, ainsi que sur des fientes de goéland de Marseille. L'étude phénotypique (culture, isolement, MALDI TOF, antibiogramme…) et génotypique (PCR en temps réel, PCR standard, MLST et séquençage) sur l'ensemble du matériel biologique déjà cité nous a permis de détecter mcr-1 sur des fientes de poulets et des fèces de caprins.

A travers ces investigations nous avons montré l'émergence et la dissémination du gène mcr-1 chez les animaux (poulets et caprins) dans plusieurs sites en Algérie. Nous avons également rapporté la détection de souches résistantes à la colistine dans des isolats humains en France et au Liban. Ces travaux ont participé à la description de l'épidémiologie du gène mcr-1 dans le monde chez les animaux et les Hommes et soulèvent la question du devenir de l'usage de la colistine chez l'homme et l'animal. Pour faire face à ces risques, nous pensons que des mesures de restriction d'usage des antibiotiques (y compris de la colistine) chez les animaux doivent s'appliquer, notamment en Algérie, pour limiter la propagation de ces gènes de résistance à l'homme. Il apparait donc nécessaire et urgent de mettre en place des enquêtes de surveillance de l'usage des antibiotiques chez les animaux notamment en Algérie pour éviter la propagation de souches résistantes chez l'homme.

Mot clés : gène mcr-1, colistine, BLSE, carbapénèmases, Algérie, Marseille.

ABSTRACT

Antibiotic resistance has become a global priority in public and animal health. On this light, our PhD work focused on the research for multidrug-resistant zoonotic bacteria in animal feces in the Mediterranean basin. This thesis is divided into 6 chapters with four objectives; (1) the epidemiology and prevalence of β-lactamases in chickens from Algeria, (2) contribution to the study and detection of plasmid resistance to colistin in animals from Algeria, (3) vectors/ reservoirs of zoonotic bacteria resistant to colistin, and (4) Epidemiology of bacteria resistant to colistin in humans in France, Laos and Lebanon. At the start of this thesis, we have prepared two bibliographic synthesis reviews on the epidemiology and dissemination of Gram-negative bacteria resistant to colistin and other antibiotics worldwide. The first review was devoted to the study of the use of colistin and the emergence and dissemination of plasmid resistance in animals around the world, while the second review focused on the prevalence and emergence of bacteria resistant to carbapenems, β-lactams and colistin in animals from the Mediterranean basin. A first part focused on the research and characterization of bacteria resistant to β-lactams and carbapenems in chickens from 8 regions in Algeria and in the south of France. Through this investigation, we found a high prevalence and diversity of ESBL and carbapenemases genes in feces from poultry in Algeria. Due to the recent emergence of plasmid resistance to colistin mcr-1, we focused on the characterization of the emergence and dissemination of this plasmid resistance gene in Algerian animals as well as in search of new vectors and reservoirs of bacteria carrying mcr-1. First, we detected the presence of mcr-1 in E. coli isolated from human stools from Laos and Thailand as well as from a swine from Laos and chickens from Algeria. In the same axis, we were able to design a rapid detection system, including real-time PCR and standard PCR to detect and characterize mcr-1 gene in samples, mainly from animal origin. These systems have been applied on a collection of chicken droppings and feces from cattle, sheep and goats from Algeria, as well as droppings from gulls from Marseille. The phenotypic (culture, isolation, MALDI TOF, antibiogram, MIC ...) and genotyping (real-time PCR, standard PCR, MLST and sequencing) studies on all the biological material already mentioned allowed us to detect mcr-1mainly on chicken droppings and goat feces. Throughout these investigations, we can conclude that there is an emergence and spread of mcr-1 in animals (chickens and goats) in several sites in Algeria. We also noted the detection of colistin-resistant bacteria in human isolates in France and in Lebanon.

This indicates a spread of mcr-1 and colistin resistance genes worldwide in animals and humans and raises the question on the colistin usage in animals. To prevent this end, we believe that restrictions on the use of antibiotics (including colistin) should be applied, particularly in Algeria, to limit the spread of these resistance genes. Control of the use of colistin should be routinely performed in humans and animals through colistin resistance surveys in animals and humans.

Key words: mcr-1 gene, colistin, ESBL, carbapenemases, Algeria, Marseille.

INTRODUCTION

Depuis la découverte établie par **Fleming** en 1928 de l'antibiotique « une pénicilline ; métabolite fongique » et son développement ultérieur par **Chain** et **Florey** pendant la deuxième guerre mondiale, nous avons assisté à une vraie révolution dans le domaine de l'antibiothérapie (**Rolain et al., 2016**). Rapidement après la découverte de la pénicilline, plusieurs autres antibiotiques ont été décrits, suivis par la fabrication de molécules antibactériennes synthétiques et semi-synthétiques. La découverte des antibiotiques, notamment la pénicilline a sans doute été l'une des avancées thérapeutiques les plus importantes du vingtième siècle. Rapidement, un arsenal thérapeutique important a été disponible pour faire face aux agents infectieux. La définition d'un antibiotique a été adoptée comme étant « une substance à faible poids moléculaire produite par un microorganisme qui peut à faible concentration inhiber où tuer d'autres microorganismes ».

Les antibiotiques sont utilisés chez les humains et les animaux d'élevage, ainsi que dans l'aquaculture comme agents thérapeutiques, prophylactiques et comme facteurs de croissance (probiotiques) (**Rolain et al., 2013 ; Roca et al., 2015**). Pendant plus de 50 ans, les antibiotiques utilisés comme facteurs de croissance semblaient n'avoir qu'un effet positif qui était de favoriser la croissance des animaux destinés à la consommation humaine. Cette utilisation à grande échelle des antibiotiques a malheureusement conduit à l'émergence de la résistance aux antibiotiques. Les premières bactéries résistantes ont été identifiées dès les années 1940, avec notamment l'émergence de souches de Staphylococcus aureus résistantes à la pénicilline dès 1947 (**Hallet Collis., 1998**).

Depuis la découverte de la pénicilline, la résistance aux antibiotiques a été liée principalement à l'utilisation exagérée des antibiotiques par l'exercice d'une pression de sélection sur les bactéries (**Cassir et al., 2014**). Cependant, des études récentes ont montré que la résistance aux antibiotiques était un mécanisme biologique ancien et que l'existence de gènes de résistance était antérieure à

notre utilisation actuelle des antibiotiques. Ainsi, des échantillons de pergélisol bactérien ont montré l'existence de gènes de résistance depuis plus de 30.000 ans (**Olaitan et al., 2016** ; **D'Costa et al., 2011**).

La résistance aux antibiotiques a été décrite peu après l'introduction des antibiotiques en thérapeutique clinique et animale, non seulement dans la flore commensale des animaux et de l'homme, mais surtout dans des agents pathogènes pour l'Homme d'origine animale (**Olaitan et al., 2014**). Le passage de ces bactéries zoonotiques chez l'homme permettrait le transfert de gènes de résistance à des espèces anthropophiles, et la propagation de la résistance aux antibiotiques chez l'Homme. Les liens existants entre le développement de la résistance chez l'animal et chez l'Homme posent la question du devenir de l'utilisation et de l'efficacité des antibiotiques en milieu vétérinaire et en santé publique.

La viande blanche et rouge ainsi que les œufs sont les principales sources alimentaires pour les humains. La consommation mondiale de protéine d'origine animale et produits dérivés des animaux dans les marchés mondiaux ne cessent d'augmenter au cours des dernières années (**Landoni et Albarellos., 2015**). Le problème de la résistance aux antibiotiques représente un réel défi pour les vétérinaires, les pharmaciens, les médecins et les chercheurs car il a un impact sévère sur la santé humaine et animale. Le développement de la résistance peut être maitrisé à condition qu'un certain nombre de mesures soient appliquées pour prolonger la durée de vie de ces antibiotiques à la fois en médecine humaine et vétérinaire.

Cette thèse a été menée pour donner suite à la description croissante de bactéries à Gram-négatif multirésistantes d'origine animale. Les phénomènes de transmission des bactéries résistantes aux humains, à partir des animaux et de l'environnement sont de plus en plus décrits dans la littérature (**Rolain et al., 2013**). Ce travail de thèse a donc porté sur l'étude et la recherche des bactéries

multirésistantes zoonotiques dans les fèces des animaux du bassin méditerranéen à travers deux objectifs principaux :

-Recherche et caractérisation de l'émergence et de la dissémination de la résistance plasmidique à la colistine chez les animaux,

-Recherche et caractérisation des gènes codants pour la résistance aux carbapénèmes et aux β-lactamines chez les animaux.

Le chapitre I est une synthèse bibliographique de deux revues de la littérature sur l'épidémiologie et la dissémination mondiale des bactéries à Gram-négatif résistantes à la colistine et aux autres antibiotiques. Une première revue a été consacrée à l'utilisation de la colistine en médecine vétérinaire et aux liens qui pouvaient exister avec l'émergence et la dissémination de la résistance à la colistine médiée par le gène transférable mcr-1 chez les animaux à travers le monde (**Article N° 1)**. Nous détaillons dans cette revue la propagation mondiale de la résistance à la colistine à médiation plasmidique chez les animaux et le risque de transmission de cette résistance de l'animal à l'Homme. Une deuxième revue de la littérature a été réalisée sur la prévalence et l'émergence de bactéries résistantes aux β-lactamines, et notamment aux carbapénèmes, et à la colistine chez les animaux dans le bassin méditerranéen (**Article N° 2)**. Dans cette deuxième revue, nous décrivons l'épidémiologie et la prévalence des bactéries productrices de β-lactamases, et de carbapénèmases, ainsi que les bactéries d'origine animale résistantes à la colistine dans le bassin méditerranéen.

Dans le chapitre II, nous avons étudié l'épidémiologie et la prévalence des bactéries productrices de β-lactamases et de carbapénèmases chez les poulets d'Algérie. Dans ce volet, nous nous sommes intéressés à la recherche, l'isolement et la caractérisation des bactéries résistantes aux carbapénèmes et aux autres β-lactamines dans 8 régions d'Algérie et du sud de la France. A travers cette investigation, nous avons trouvé une forte prévalence et une grande diversité des gènes

codants pour des β-lactamases à spectre étendu (BLSE) et des gènes codants pour des carbapénèmases dans les fèces des volailles d'Algérie, tandis qu'aucun des échantillons de Marseille n'étaient positifs pour ces gènes (**Article N° 3**). Une autre étude a été menée en Algérie sur la prévalence de souches de Salmonella productrices de BLSE chez les poulets de chair dans la région nord-est de l'Algérie. L'objectif était d'évaluer le profil de résistance aux antibiotiques des isolats aviaires et humains afin de rechercher une potentielle relation clonale entre ces souches de sources différentes (**Article N° 4**).

Dans le chapitre III, du fait de la découverte récente de la résistance à la colistine médiée par un transposon, le gène mcr-1 (**Liu et al., 2016**), ce chapitre a porté sur la détection des réservoirs animaliers de bactéries porteuses du gène mcr-1 afin d'identifier de nouveaux vecteurs potentiels de ces bactéries résistantes à la colistine. Nous nous sommes particulièrement intéressés à la détection de la résistance plasmidique à la colistine chez les animaux d'Algérie, notamment dans des bactéries isolées à partir de poulets, de bovins, d'ovins et de caprins. En premier lieu, nous avons réalisé un travail incluant la première description du gène mcr-1en Afrique (**Article N° 5)**. Du fait de l'émergence rapide et de la dissémination mondiale du gène mcr-1 (**Liu et al., 2016**) qui a pu être constatée par l'intérêt et le nombre de publications concernant la détection de ce gène dès les premiers mois après la parution du papier princeps, il nous a paru nécessaire de désigner plusieurs systèmes de détection rapide. Deux systèmes de PCR en temps réel ont été désignés, testés et appliqués dans notre travail. L'ensemble de ces résultats afait l'objet de l'**Article N° 6**. Après avoir décrit pour la première fois la présence du gène mcr-1 dans une collection de fientes de poulets en Algérie (**Article N° 5)**, nous avons réalisé une deuxième étude portant sur la détection du gène mcr-1 dans des fientes de poulets provenant d'autres régions d'Algérie. L'objectif était de connaître la prévalence du portage digestif de ce gène chez les volailles en Algérie (**Article N° 7)**.

Enfin, nous avons participé à l'établissement du répertoire des réservoirs animaliers qui peuvent abriter des bactéries porteuses du gène mcr-1, à travers la détection de ce gène dans les fèces de différents animaux, notamment de bovins, d'ovins et de caprins, ainsi que dans des écouvillons de surface d'élevage en Algérie (**Article N° 8)**.

Le chapitre IV a porté sur la description de nouveaux réservoirs de bactéries porteuses du gène mcr-1, cette fois-ci chez des animaux sauvages. Nous avons isolé dans un premier temps des bactéries résistantes à la colistine et caractérisé le support moléculaire de cette résistance dans des fientes de goélands prélevés à Marseille, France (**Article N° 9)**. Une autre étude (Annexe : collaboration) s'est intéressée à la caractérisation des bactéries multirésistantes isolées à partir des oiseaux urbains de la ville de Marseille notamment à partir des pigeons et goélands (**Article N° 13)**. Un dernier travail a permis de mettre en évidence la présence du gène mcr-1 dans des tiques Ixodes ricinus collectées en Algérie dans une zone où le gène mcr-1 avait été détecté dans les fèces de poulets. La détection du gène mcr-1 dans des vecteurs différents des animaux destinés à la consommation humaine permet d'envisager d'autres modes de transmission de ce gène à l'Homme, et montre l'étendu du réservoir du gène mcr-1 dans le monde vivant (**Article N° 10)**.

Le chapitre V inclut des travaux de collaboration effectués sur l'épidémiologie des bactéries résistantes à la colistine chez les humains en France, au Laos et au Liban (**Article N° 11**) et (**Article N° 12**).

Le chapitre VI est une annexe est composé de deux articles, l'**Article N° 14** et l'**Article N° 15**. Dans **l'article N° 14** nous avons décrit l'émergence de souches d'origine clinique d'Enterococcus faecium résistantes à la vancomycine au Liban. L***'article* N° 15** fait partie du projet de culturomique réalisé à l'IHU (Institut Hospitalo-Universitaire) qui a permis de découvrir un grand nombre de nouvelles espèces bactériennes et qui sont soumis à l'étude taxonogénomique. Dans

cette étude nous avons décrit le génome de Clostridium niameyense sp. Nov., bactérie qui a été isolée à partir d'un échantillon fécal recueilli chez un patient souffrant d'anorexie et de marasme vivant au Nigeria.

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I -ÉPIDEMIOLOGIE DES BACTÉRIES A GRAM-NEGATIF RÉSISTANTES A LA COLISTINE ET AUX AUTRES ANTIBIOTIQUES A TRAVERS LE MONDE : USAGE DES ANTIBIOTIQUES CHEZ LES ANIMAUX ET ÉMERGENCE DE LA RÉSISTANCE.

Les β-lactamines, notamment les céphalosporines de 3ème génération ont été largement utilisées au cours des dernières décennies dans le traitement des infections à germes variés du fait de leur large spectre d'action. Les carbapénèmes sont eux utilisés en dernière ligne dans le traitement des infections à germes multirésistants causés par Pseudomonas aeruginosa, Acinetobacter baumannii, Klebsiella pneumoniae ou E. coli (**Lee et Baker., 2018 ; Baron et al., 2016**). Cependant, cette large utilisation non contrôlée a créé une pression de sélection sur ces bactéries, menant à l'émergence de la résistance à différentes classes d'antibiotiques (**Patel et Bonomo., 2013**). Au cours des dernières années, les bactéries à Gram-négatif productrices de β-lactamases et de carbapénèmases se sont répandues dans les hôpitaux, les milieux communautaires et dans l'environnement (**Rubin et Pitout., 2014**). Face à l'émergence de bactéries extrêmement résistantes (XDR) ou panrésistantes (PDR), la recherche de nouvelles molécules et l'évaluation de l'efficacité d'antibiotiques anciens et délaissés sont devenus une priorité mondiale.

Actuellement, les polymyxines, dont la colistine, sont réutilisées en pratique clinique (**Holmes et al., 2016**) comme antibiotiques de « dernier recours » pour le traitement des infections dues aux bactéries à Gram-négatif multirésistantes (**Mendelson et al., 2018**). La colistine est un polypeptide antimicrobien cationique qui se lie au lipide A du lipopolysaccharide des bactéries, perturbant l'équilibre hydroélectrolytique de la membrane et conduisant à sa rupture et à la lyse cellulaire (**Falagas et al., 2005**). Actuellement à travers le monde, la colistine est largement utilisée chez les animaux pour le traitement des maladies infectieuses, dans la prophylaxie des maladies et aussi comme facteur de croissance (**Biswas et al., 2012**).

La colistine est suradministrée en particulier dans l'élevage des volailles et du porc, pour faire face aux infections à E. coli et à Salmonella, connues pour causer des effets négatifs sérieux tels que

diarrhées et septicémies conduisant à des pertes économiques importantes (**Kempf et al., 2016 ; Fernandes et al., 2016 ; Catry et al., 2015**). L'utilisation excessive de la colistine principalement en médecine vétérinaire a conduit à l'émergence de bactéries résistantes à la colistine (**Shen et al., 2016**).

En novembre 2015, **Liu et al**. a rapporté pour la première fois en Chine un nouveau gène de résistance à la colistine à médiation plasmidique, le gène mcr-1 codant pour une phospho-éthanolamine transférase (**Liu et al., 2016**). Par la suite, le gène mcr-2 a également été décrit, (**Mendelson et al., 2018**) d'abord été détecté chez des animaux, puis dans des isolats humains et dans des aliments provenant d'animaux (**Al-Tawfiq et al., 2017**). Depuis, une diversité de variants de ces gènes de résistance à la colistine a été rapportée dans le monde (mcr-1.2, mcr-1.3, mcr1.4,…, mcr-1.12) (**Sun et al., 2018**).

L'objectif de cette première partie était de réaliser l'épidémiologie de la résistance à la colistine et autres antibiotiques chez les animaux dans le monde et d'essayer d'établir un lien entre cette résistance et la consommation d'antibiotiques et notamment de colistine par pays.

La première revue de ce chapitre s'intitule « **Colistin use in animals : a two-side weapon against multi drug resistant organisms »** (**Article 1**). Cette revue a été divisée en quatre parties : (**1**) description de l'émergence de la résistance à la colistine à médiation plasmidique chez les animaux dans le monde entier, (**2**) dissémination des variants chez les animaux, (**3**) causes de cette dissémination mondiale de la résistance en traitant les utilisations de la colistine chez les animaux et (**4**) risque de transmission de cette résistance des animaux aux humains. A travers cette revue, nous avons rapporté l'émergence et la dissémination mondiale de la résistance plasmidique mcr-1 chez les animaux dans 4 continents du monde (Afrique, Europe, Amérique et Asie). Cette

dissémination mondiale de la résistance à la colistine mcr-1 était suivie par une diversité de découverte de variants mcr-2/3/4/5 chez les animaux et dans le monde. Nous avons résumé par la suite l'impact de l'utilisation de la colistine en médecine vétérinaire dans le monde entier pour lier cette consommation à la prévalence de la résistance à la colistine chez l'animal et chez l'homme. En outre, nous avons discuté le risque de propagation de bactéries résistantes à la colistine provenant d'animaux à l'homme. A travers cette revue nous avons recommandé à la fin de restreindre la consommation de colistine chez les animaux. Cela peut se réaliser avec la mise en place d'enquêtes de surveillance sur la consommation de colistine dans le monde entier, particulièrement en Afrique et en Asie car la prévalence des gènes de résistance à la colistine chez les bactéries à Gram-négatif chez les animaux ne cesse d'augmenter et soulève la question de l'efficacité réelle de l'administration de la colistine chez les animaux et le rôle des animaux dans le transfert de la résistance chez les humains (**Article N° 1**).

La deuxième revue de cette thèse s'intitule **« Prevalence and emergence of ESBLs, carbapenemases and colistin resistant Gram-negativebacteria in animals of the Mediterranean basin »** (**Article N° 2**). Cette revue, dont l'objectif était de réaliser une synthèse de la résistance aux β-lactamines et aux polymyxines, est scindée en trois parties : (**1**) prévalence des bactéries porteuses de BLSE chez les animaux dans le bassin méditerranéen, (**2**) prévalence des bactéries productrices de carbapénèmases chez les animaux dans le bassin méditerranéen, (**3**) prévalence de la résistance à la colistine chez les animaux dans le bassin méditerranéen. A travers cette synthèse bibliographique, nous avons rapporté la prévalence des BLSE et des producteurs d'AmpC chez les animaux dans le bassin méditerranéen notamment chez les poulets et les produits de consommation d'origine avicole, les bovins, les ovins, les porcs, les animaux de compagnie, les

oiseaux sauvages et les animaux domestiques. Parmi les bactéries porteuses de BLSE le groupe CTX-M 1 semble prédominer chez les animaux de cette zone, suivi par SHV-12 et CTX-M groupe 9. Par la suite nous nous sommes intéressés à la description de la prévalence et de la dissémination des bactéries productrices de carbapénèmases. Quant à la résistance à la colistine chez les animaux, la prévalence mondiale de la résistance aux polymyxines représente environ 10% des bactéries à Gram-négatif, avec les taux les plus élevés observés dans les pays méditerranéens et en Asie du Sud-est. A travers cette revue il en ressort que les bactéries multirésistantes isolées dans cette zone étaient souvent co-résistantes aux antibiotiques, fréquemment utilisés en médecine vétérinaire comme traitement, promoteurs de croissance, en prophylaxie, et en médecine humaine à des fins thérapeutiques. Les antibiotiques utilisés en médecine vétérinaire comprennent principalement la tétracycline, les aminoglycosides, les fluoroquinolones et les polymyxines. En effet, il semble que l'émergence de bactéries productrices de BLSE et de carbapénèmases chez les animaux soit liée à la pression de sélection appliquée par l'utilisation excessive d'antibiotiques. Le niveau de consommation d'antibiotiques chez les animaux devrait donc être reconsidéré dans la région méditerranéenne, en particulier en Afrique du Nord, et également en Asie occidentale, où aucune donnée précise n'est disponible sur le niveau de consommation d'antibiotiques chez les animaux.

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REVUE DE LA LITTÉRATURE 1

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

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Outline:

Introduction

The worldwide spread of plasmid mediated colistin resistance in animals

MCR- variants

Use of colistin in animals

Risk of transmission of colistin resistance from animals to humans

Conclusion

Table 1: Colistin use and emergence of plasmid mediated resistance in animals worldwide

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

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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.

1. 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.

2. 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.

3. 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.

4. 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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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)

"-" = unknown

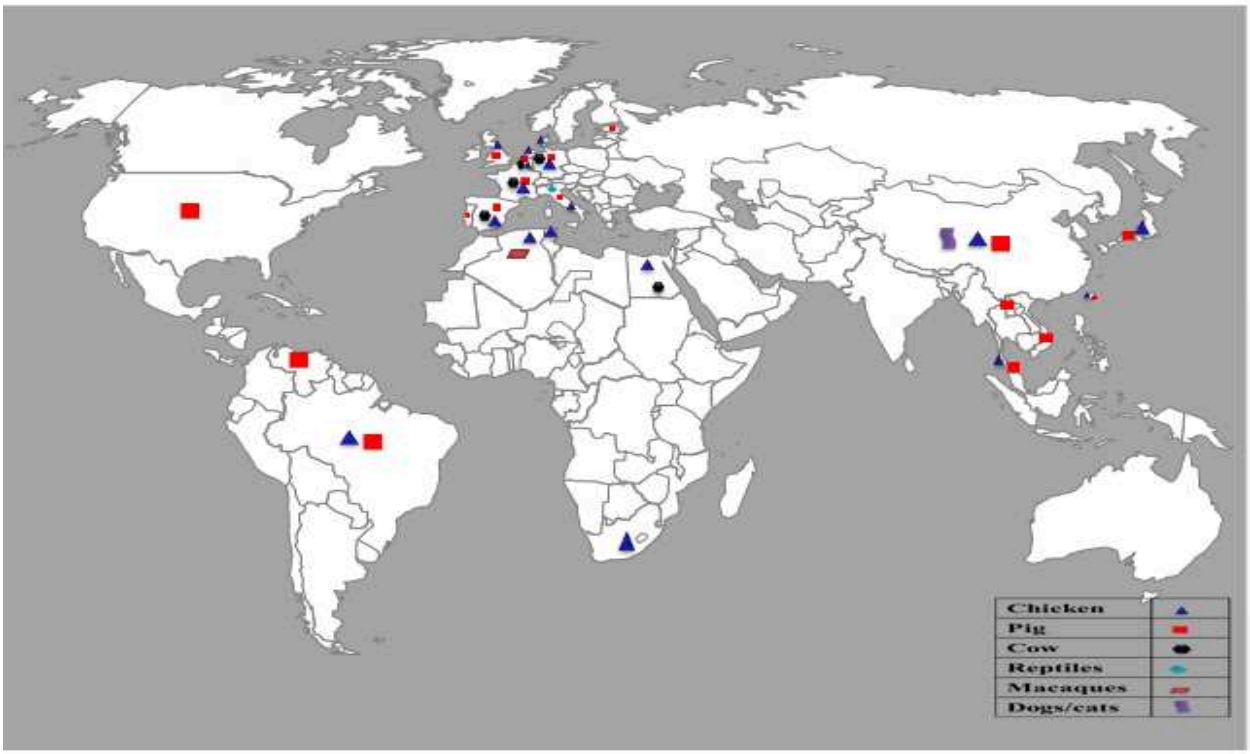

Figure 1: The Worldwide spread of plasmid mediated colistin resistance in animals.

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Prevalence and emergence of ESBLs, carbapenemases and colistin resistant Gram-negative bacteria in animals of the Mediterranean basin.

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Frontiers in Microbiology journal

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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Keywords

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Abstract

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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.

In review

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.

In review

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).

In review

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-

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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).

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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

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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

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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).

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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

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411 distribution was observed: in E. coli (CTX-M-1, CTX-M-15, CTX-M-32, CTX-M-3, CTX-M-
412 14) (Poirel et al. 2013)(Haenni et al. 2014) (Melo et al. 2017), in K. pneumoniae (CTX-M-
413 15/DHA) (Poirel et al. 2013), in E. cloacae (CTX-M-15, SHV-12) (Haenni et al. 2016), in P.
414 mirabilis (CMY-2) and in Proteus rettgeri (CTX-M-1) (Schultz et al. 2017). The dissemination
415 of extended-spectrum-cephalosporin resistance in companion animals in France necessitates
416 studies addressing the risk factors responsible for the acquisition of these strains in pets as well
417 as novel approaches to control the spread of resistance in these animals. Furthermore, the
418 contribution of the pet animals to the spread of resistance in the common population in France
419 should be also investigated. Moreover, France is the only Mediterranean country in which
420 studies reporting ESBL and/or AmpC-producing bacteria in horses are available. Between 2010
421 and 2013, E. cloacae harboring CTX-M-15, CTX-M-1 and SHV-12 were isolated from clinical
422 samples of horses. These genes were located on IncHI2 and IncP plasmids and were harbored by
423 strains of various sequence types such as ST127, ST372, ST145, ST114, ST135, ST118, ST268,
424 ST107 (Haenni et al. 2016). Later on, VEB-6 carrying P. mirabilis were isolated from healthy
425 horses (Schultz et al. 2017). In Greece, CMY-2 carried on IncI1 plasmid and harbored by ST212
426 E. coli strains were isolated from diseased canines in 2011 (Vingopoulou et al. 2014). More
427 recently, a study conducted in Greek households revealed the detection of extended-spectrum-
428 cephalosporin-resistant E. coli isolates. The strains presented with different sequence types
429 including the human pandemic ST131 clone which suggests a possible from humans to animals
430 and vice-versa (Liakopoulos et al. 2018).
431
432 In Egypt, CTX-M beta-lactamases have been detected in E. coli recovered from cats' rectal
433 swabs. In this same study, CTX-M-producing E.coli, K. pneumoniae and P. mirabilis were
434 isolated from dogs (Abdel-Moein and Samir 2014). In Algeria, only one study reported the
435 detection of E. coli strains carrying blaCTX-M-1, blaCTX-M-15 in cats and blaCTX-M-1,
436 blaCTX-M-15, blaSHV-12 in dogs (Yousfi et al. 2016b). In Tunisia, CTX-M-1 carrying E. coli
437 were isolated from cats; while from dogs CTX-M-1, CTX-M-15 and CMY-2-producing E. coli
438 were detected (Sallem et al. 2013) (Grami et al. 2013). CTX-M-1 was mostly carried on IncI1
439 plasmid where as CTX-M-15 on IncFII (Grami et al. 2013). The blaCTX-M-1 and CMY-2 genes
440 were also found associated with the ISEcp1. Indeed it appears that the insertion sequence ISEcp1
441 might be also responsible for the dissemination of CMY-2 AmpC genes apart from the blaCTX-
442 M ones.
443
444 **Wild Birds and domestic animals**
445 Besides companion and food producing animals, scattered reports exist on the isolation of ESBL
446 from domestic animals such as wild birds and dromedaries in the Mediterranean. For instance,
447 CTX-M-producing E. coli was isolated from wild birds in Algeria (Meguenni et al. 2015),
448 Turkey (Yilmaz and Guvensen 2016), blaCTX-M-1 in addition to blaCTX-M-15 carrying E.
449 cloacae in France (Bonnedahl et al. 2009). Furthermore, in France, CTX-M-1 and CTX-M-15
450 were detected in ST93, ST124 and ST10 E. coli strains recovered from tawny owls/rock pigeons
451 and domestic geese, respectively. In addition, a CTX-M-15/DHA-producing ST274 K.
452 pneumoniae was isolated from a hedgehog living in the same city (Poirel et al. 2013). Rooks
453 carrying CTX-M-14 type ESBL in E. coli have been described in Italy and Spain (Jamborova et
454 al. 2015). Furthermore, in Spain, E. coli and K. pneumoniae harboring CTX-M-14, CTX-M-1,
455 CTX-M-32, CTX-M-9, CTX-M-15, CTX-M-14b, CTX-M-3, and CTX-M-8 were recovered
456 from the fecal samples of gulls (Stedt et al. 2015). In rabbits, CMY-2-producing E. coli and

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457 CTX-M-14, CTX-M-9-producing E. cloacae were isolated (Blanc et al. 2006)(Mesa et al. 2006).
458 More recently, blaCTX-M-1 was identified in E. coli isolated from the fecal sample of a deer
459 living in the Los Alcornocales natural park in southern Spain (Alonso et al. 2016). In Algeria,
460 blaCTX-M-15 and blaCTX-M-9 genes were detected in E. coli isolated from the gut and gills of
461 fish caught in the Mediterranean across Bejaia city (Brahmi et al. 2016). In this study, it has been
462 suggested that the presence of beta-lactamase producers is due to contamination of the fish from
463 river water and the rising amount of untreated waste that is released into the Mediterranean Sea
464 from the agricultural as well as the industrial operations (Brahmi et al. 2016). These findings
465 emphasizes on the importance of the natural environment in the dissemination of resistance from
466 humans to animals and vice versa. Furthermore, Bachiri et al. also reported the detection of
467 CTX-M-15-producing ST584 K. pneumoniae in Barbary macaques situated in national parks in
468 the north of Algeria (Bachiri et al. 2017). In both Tunisia and Egypt, CTX-M beta-lactamases
469 were detected in E. coli and Pseudomonas aeruginosa recovered from dromedaries and camels,
470 respectively (Ben Sallem et al. 2012) (Elhariri et al. 2017). In Croatia, the only study
471 investigating the prevalence of ESBL in animals was conducted in 2009-2010 in mussels caught
472 in the Adriatic Sea. In this study, 18 Aeromonas species carrying SHV-12, CTX-M-15, FOX-2
473 and PER-1 were identified (Maravic et al. 2013).
474
475 **Prevalence of carbapenemase producers in livestock and domestic animals**
476 Carbapenems are beta-lactam antibiotics often considered as the last resort antimicrobial agent
477 against multi-drug resistant organisms (Temkin et al. 2014). Carbapenems are active against
478 ESBL and AmpC-producing Gram negative bacilli. Due to the wide dissemination of multi-drug
479 resistant organisms, these antimicrobials recently became heavily used in human medicine. As a
480 result, the emergence of carbapenem resistance has accelerated and it is now a normal
481 phenomenon encountered in hospital settings and, to a lesser extent, community settings. The
482 production of hydrolyzing enzymes called "carbapenemases" is one of the mechanisms by which
483 carbapenem resistance is mediated in Gram negative bacilli. These include a) class A
484 carbapenemases (KPC, GES, SME, IMI, NMC-A) b) class B metallo beta-lactamases "MBL"
485 (NDM, VIM, IMP and TMB) and c) class D oxacillinases (Martinez-Martinez and Gonzalez-
486 Lopez 2014).
487
488 In the Mediterranean basin, in Egypt, OXA-48 and OXA-181 carbapenemases were detected in
489 E. coli strains recovered from dairy cattle farms (Braun et al. 2016). In the poultry production
490 system, one study reported the isolation of K. pneumonia and K. oxytoca harboring NDM
491 metallo beta-lactamases (Abdallah et al. 2015). Another study described the identification of K.
492 pneumoniae carrying OXA-48, NDM and KPC type carbapenemases. Isolated strains were
493 recovered from the liver, lungs and trachea of broiler chicken (Hamza, Dorgham, Hamza 2016).
494 In Algeria, NDM-1 and NDM-5 were observed, respectively, in ST85 Acinetobacter baumannii
495 and ST1284 E. coli originating from raw milk in the west and north of the country (Chaalal et al.
496 2016) (Yaici et al. 2016). In E. coli, NDM-5 was located on an IncX3 plasmid (Yaici et al.
497 2016). In broilers, OXA-58 was identified (Chabou et al. 2017) while in pigeons, in addition to
498 OXA-58 and OXA-23 were detected (Morakchi et al. 2017). In terms of companion animals,
499 NDM-5 and OXA-48-producing E. coli were reported from healthy dogs Algeria (Yousfi et al.
500 2015) (Yousfi et al. 2016a). The NDM-5 was harbored by an E. coli strain having the same
501 sequence type ST1284 previously described in cattle (Yaici et al. 2016) (Yousfi et al. 2015).
502 OXA-48 was further detected in healthy and diseased cats in the same city (Yousfi et al. 2016a).

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503 Furthermore, in this same country, two A. baumannii producing OXA-23 were isolated from fish
504 (Brahmi et al. 2016). In Lebanon, A. baumannii with different sequence types (ST294, ST491,
505 ST492, ST493) were detected in a horse's mouth carrying OXA-143 (Rafei et al. 2015), and in
506 pigs and cattle carrying OXA-23(Al Bayssari et al. 2015a). Furthermore, in cattle, a VIM-2-
507 producing P. aeruginosa was isolated (Al Bayssari et al. 2015a). In fowl, Bayssari et al. reported
508 the detection of OXA-23 and OXA-58 harboring A. baumannii and OXA-48-producing E. coli as
509 well as VIM-2 producing P. aeruginosa (Al Bayssari et al. 2015b). VIM-2 producers in fowl
510 and cattle were of different sequence types suggesting the presence of plasmid that is mediating
511 the spread of this resistance gene. In France, OXA-23-producing Acinetobacter species were
512 described in cows and dogs (Poirel et al. 2012) (Herivaux et al. 2016). Melo et al reported the
513 detection of OXA-48 located on an IncL plasmid and carried by an ST372 E. coli strain from
514 dogs in France (Melo et al. 2017). In contrast, in Spain, only one study reported the isolation of a
515 VIM-1-producing ST2090 K. pneumoniae from a dog's rectal swab (Gonzalez-Torralba et al.
516 2016) (Figure 2).
517

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

520 The different phylogroups and sequence types of beta-lactamase and mcr-1 positive strains as
521 well as the type of plasmids carrying ESBL, AmpC, carbapenemase and mcr-1 genes detected in
522 all animal sources in the Mediterranean region are summarized in table 2. In this area of the
523 world, it appears that multi-drug resistance in the veterinary sector is mediated by the spread of
524 different phylogroups and sequence types with the main ones being A, B and D phylogroups
525 (table 2). The detection of ST10 in CTX-M producers in poultry, cattle, pets and domestic
526 animals in Algeria, Tunisia, Lebanon and France is of special interest. ST10 was often described
527 in the literature as being common to ESBL E. coli strains of human and avian origin worldwide
528 such as in Germany (Belmar Campos et al. 2014), Denmark (Huijbers et al. 2014), Vietnam
529 (Nguyen et al. 2015) and Chile (Hernandez et al. 2013). ST10 was suggested as being associated
530 with the spread of CTX-M ESBL types and mcr-1 genes in humans, animals and environments
531 (Monte et al. 2017). Another distinct finding is the detection of ST101 in dogs and cats in Italy.
532 ST101 is an international sequence types frequently detected in pigs (El Garch et al. 2017),
533 broilers (Sola-Gines et al. 2015) as well as in the clinical settings. In several countries, ST101
534 was associated to NDM-1 E. coli strains isolated from the clinical settings of Germany, Canada,
535 Australia, UK and Pakistan (Yoo et al. 2013) implying thus that ST101 is a candidate for the
536 zoonotic transmission to the human population.
537

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

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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

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595 beta-lactam antibiotics. Molecular analysis targeting the co-localization of ESBL and mcr genes
596 along with the ones mediating resistance toward non beta-lactams is however warranted in order
597 to validate this hypothesis. Also in Tunisia, two colistin resistant E. coli strains positive for mcr-
598 1 and harboring the CMY-2 gene were recently detected in chicken. Both strains shared the same
599 sequence type "ST2197" in addition to their PFGE patterns. The mcr-1 gene in these latter was
600 associated with the ISApl1 and was carried by IncP plasmid while the CMY-2 gene was located
601 on an IncI1 plasmid type (Maamar et al. 2018). Furthermore, in this same country, a recent study
602 revealed the absence of mcr-1 and mcr-2 positive Gram-negative bacilli in camel calves in
603 southern Tunisia (Rhouma et al. 2018). Likewise, in Egypt, mcr-1 was detected in E. coli
604 isolated from diseased chickens as well as from cows displaying subclinical mastitis (Khalifa et
605 al. 2016) (Lima Barbieri et al. 2017). The emergence of mcr-1 in Egypt can be related to the use
606 of colistin in animal agriculture, and its ready application as a therapeutic agent for colibacillosis
607 as well as other infections, in rabbits and calves (Lima Barbieri et al. 2017). In Southeast Asia,
608 Dandachi et al. reported the detection of the mcr-1 plasmid mediated colistin resistance gene in
609 E. coli in poultry in the south of Lebanon (Dandachi et al. 2018). This strain had a sequence type
610 of ST515 that was not reported before in mcr-1 E. coli strains of poultry origin (Dandachi et al.
611 2018).
612
613 Of the European countries bordering the Mediterranean, Spain was the first to report the
614 detection of mcr-1 in E. coli and Salmonella enterica isolated from farm animals (Quesada et al.
615 2016). This could be related to the fact that Spain is one of the countries were colistin is
616 extensively used in veterinary medicine (de Jong et al. 2013). More recently, mcr-1 co-existing
617 with mcr-3 on the same non mobilizable IncHI2 plasmid was detected in an E. coli strain
618 recovered from cattle feces in a slaughterhouse (Hernandez et al. 2017). In France, as part of
619 routine surveillance by the French agricultural food sector, mcr-1 was identified in four
620 Salmonella spp isolated from sausage, food of poultry origin and boot swabs taken from broiler
621 farms (Perrin-Guyomard et al. 2016) (Webb et al. 2016). E. coli harboring mcr-1 was also
622 isolated in France from pig, broiler and turkey samples (Haenni et al. 2016). Haenni et al.
623 reported the identification of unique IncHI2/ST4 plasmid co-localizing mcr-1 and ESBL genes in
624 an E. coli strain isolated from French veal calves (Haenni et al. 2016). In Italy, Carnevali et al.
625 reported the detection of mcr-1 in Salmonella spp strains isolated from poultry and pigs
626 (Carnevali et al. 2016). Subsequently, mcr-1 was further detected in E. coli of swine origin. In
627 the aforementioned report, mcr-1 was co-existent with the carbapenemase OXA-181 in the same
628 bacterium and was carried on an IncX4 plasmid type (Pulss et al. 2017). In the Mediterranean
629 basin, likewise ESBL producers, mcr positive strains belong to different phylogroups and appear
630 to be not clonally related; however, they were not associated to a common plasmid or an
631 insertion sequence type. This questions the molecular mechanism by which the mcr genes are
632 being disseminating in this region of the world. More molecular work is warranted in this area
633 especially that mcr genes are often located on plasmids carrying ESBL and/or carbapenemase
634 genes.

635 **Antibiotic use in animals and potential impact on public health**

636 For many years, the use of antibiotics in the veterinary medicine has increased animal health via
637 lowering mortality and the incidence of infectious diseases (Hao et al. 2014). However, in view
638 of the heavy dissemination of resistant organisms namely ESBL, AmpC and carbapenemase
639 producers in addition to the emergence of colistin resistance in livestock and animals with
640 frequent contacts with human; the efficiency of antibiotic administration to animals has been

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641 reconsidered. Indeed, antibiotic use in animals is not controlled, in that these latter are not only
642 prescribed for treatment, but are also given for prophylaxis and as growth promoters (Economou
643 and Gousia 2015). In its recent publication, the world health organization recommended a
644 reduction but an overall restriction of the use of medically important antibiotics for prophylaxis
645 and growth promotion in farm animals (WHO 2017 2017). According to the world health
646 organization list of Critically Important Antimicrobials for Human Medicine (WHO CIA list),
647 these include mainly extended spectrum cephalosporins, macrolide, ketolides, glycopeptides and
648 polymixins (WHO CIA 2017 2017). The control of antibiotic use in the veterinary sector aims to
649 reduce the emergence of resistance in addition to preserving the efficacy of important classes for
650 treatment in the human medicine.
651
652 In the Mediterranean region, tetracyclines, aminoglycosides, sulfonamides, fluoroquinolones and
653 polymixins are the most common antimicrobial classes prescribed in the veterinary sector (table
654 1). The usage level of each antibiotic class in addition to its real purpose of administration apart
655 from treatment is limited and not well understood in this area of the world. In fact, it is nowadays
656 accepted that the over-use of antibiotics in animals is the main driven for the dissemination of
657 multi-drug resistance (Barton 2014). As shown in table 1, ESBL, AmpC and carbapenemase
658 producers are often co-resistant to non beta-lactam antibiotics with the most common being
659 gentamicin, streptomycin, tetracycline, trimethoprim-sulfamethoxazole, nalidixic acid and
660 ciprofloxacin. One study conducted in healthy chicken in Tunisia showed the presence of tetA,
661 tetB, sul1 and sul2 on the same plasmids carrying the blaCTX-M genes (Maamar et al. 2016).
662 Another study in Egypt, reported the detection of tetB, qnrB2, qnrA1, aadA1 on the same gene
663 cassette along with the blaCMY-2 AmpC beta-lactamase gene (Ahmed, Shimamoto, Shimamoto
664 2013). In Italy, strA/B, tetD, qnrB, aadA1, sulI genes were associated with the blaCTX-M and
665 blaSHV ESBL genes types in companion animals (Donati et al. 2014). Furthermore, in this same
666 country, aminoglycoside modifying enzymes (aadA1, aadA2), quinolone resistance genes
667 (qnrS1), florfenicol/chloramphenicol resistance gene (floR), in addition to tetracycline and
668 sulfonamide resistance genes (tetA, sul1, sul2, sul3) were found associated with OXA-48/181
669 and OXA-48/181/ CMY-2 /mcr-1 positive E. coli strains isolated from pigs (Pulss et al. 2017). In
670 Salmonella enterica, Franco et al reported the detection of a megaplasmid harboring the
671 blaCTX-M-1 ESBL gene along with tetA, sulI, dfrA1 and dfrA14 conferring thus additional
672 resistance towards tetracycline, sulfonamide and trimethoprim (Franco et al. 2015). Beta-
673 lactamase producing Gram-negative bacilli appear thus to be selected by the co-selective
674 pressure applied by the use of non beta-lactam antibiotics in livestock and companion animals.
675 Surveillance studies addressing the types, purpose and level of antibiotic classes' administration
676 in animals of the Mediterranean region are warranted in order to develop approaches that control
677 the use of antibiotics while preserving animal's health. This is especially in Syria, Cyprus,
678 Albania, Montenegro, Bosnia, Herzogovina, Monacco, Morocco and Libya where even no data
679 exists on the prevalence and epidemiology of multi-drug resistant organisms in animals.
680
681 The spread of multi-drug resistant organisms of animal origin is sparked by the concern of being
682 transmitted to humans; these latter can then be causative agents for infections with limited
683 therapeutic options (Bettiol and Harbarth 2015). The transfer of resistant organisms from animals
684 to humans can occur either via direct contact or indirectly via the consumption of
685 under/uncooked animals products (Dahms et al. 2014). Recent studies have also highlighted the
686 importance of the farms surrounding environment in the transmission chain. Air (von Salviati et

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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

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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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764 **Table 1.** Non Beta-lactam resistance in MDR of animal origin versus antibiotic consumption in the Mediterranean Basin
765

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)

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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)

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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)

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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, 3rd/4th 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, 1st/2nd/3rd/4th Cephalosporins, Sulfonamides, Trimethoprim, Macrolides, Lincosamides, Fluoroquinolones, Aminoglycosides, Polymixins, Pleuromutilins	(EMA/ESVAC, 2014)

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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	

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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, 3rd/4th 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)

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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.

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787 **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			

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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		

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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)

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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)

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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			

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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)

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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		

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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)

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

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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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Figure 1.JPEG

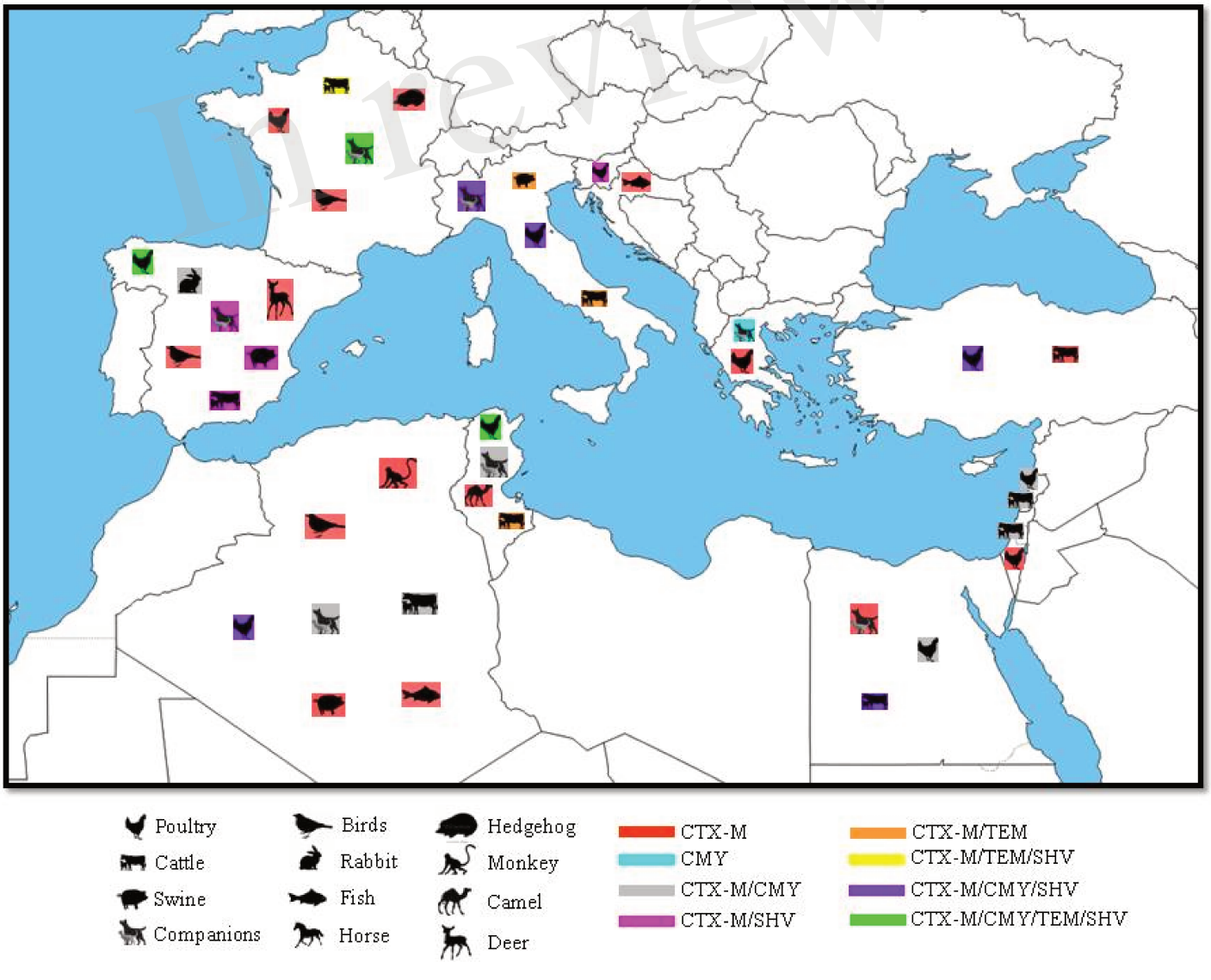

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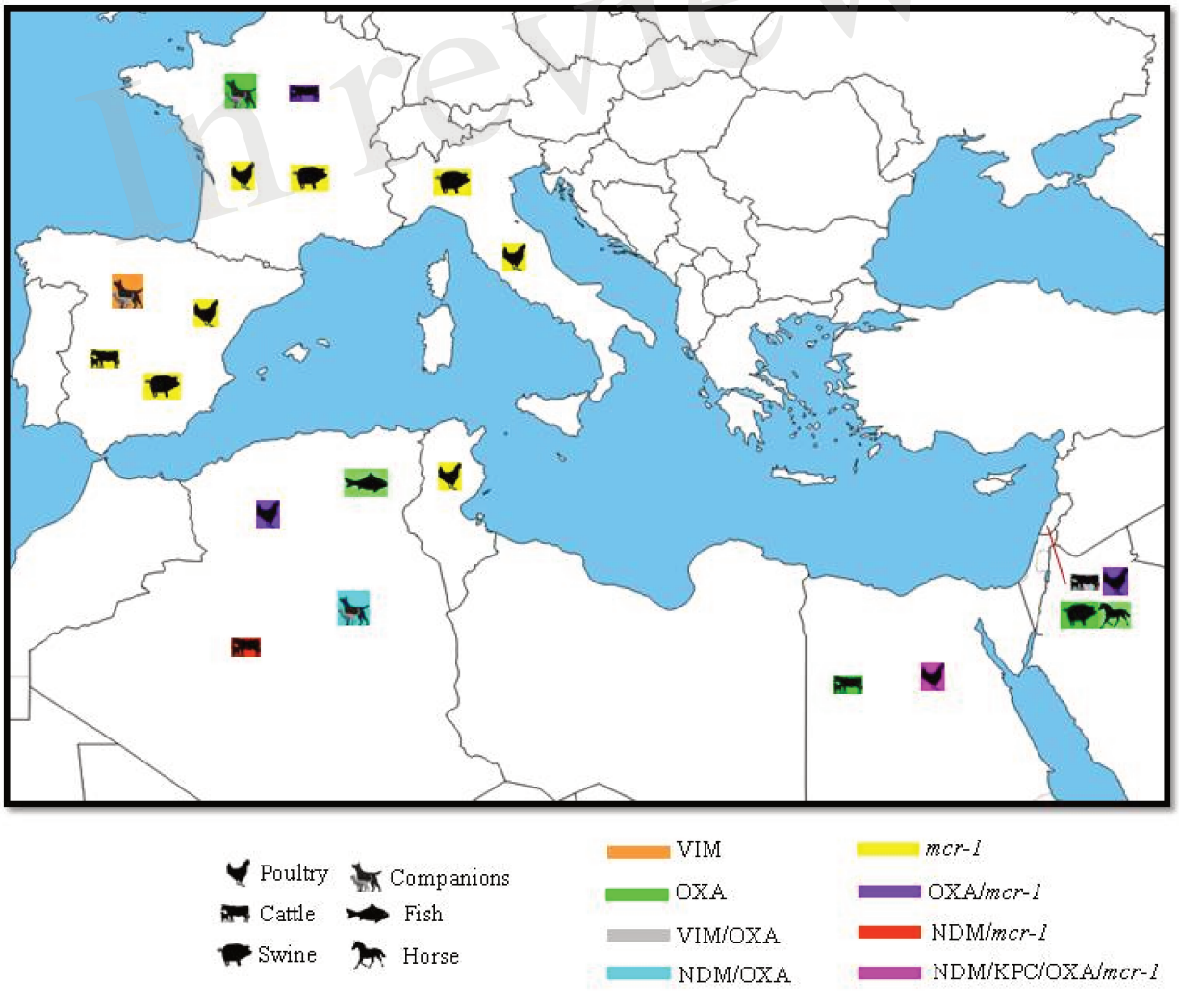
Figure 2.JPEG



In review

II-EPIDEMIOLOGIE ET PREVALENCE DES BACTERIES PRODUCTRICES DE BETA-LACTAMASES ET DE CARBAPENEMASES CHEZ LES POULETS D'ALGERIE.

De grandes quantités d'antimicrobiens sont administrées à des animaux destinés à la consommation humaine, en particulier dans les aliments pour animaux, afin de traiter et prévenir les maladies animales (**Scott et al., 2018**). Cette pratique excessive semble participer à l'augmentation de la résistance aux antimicrobiens chez les humains (**Page et al., 2012**)

La résistance aux agents antimicrobiens a en effet été largement rapportée dans le monde, et l'utilisation irrationnelle des antimicrobiens dans la production animale pour prévenir et traiter les maladies infectieuses et améliorer la croissance des animaux semble être un facteur favorisant la dissémination de cette résistance (**WHO., 2017**).

Malgré le manque de preuves, il semble biologiquement plausible que l'utilisation d'antimicrobiens chez les animaux puisse entraîner la sélection et la dissémination de gènes de résistance au sein des microbiotes animaliers, y compris dans leurs carcasses, leurs viandes et les produits d'origine animale destinés à la consommation humaine, entrainant un risque potentiel non négligeable de transmission de cette résistance à l'homme, avec des conséquences sanitaires et économiques potentiellement dévastatrices (**Tang et al., 2017 ; Scott et al., 2018**).

Les bactéries productrices de β-lactamases à spectre étendu (BLSEs) sont connues comme pathogènes nosocomiaux et sont résistantes à presque tous les antibiotiques de la classe des β-lactamines (**Al-Bayssari et al., 2015**). Les β-lactamases les plus courantes comprennent les TEM, SHV et CTX-M, qui sont les plus fréquentes des BLSE (**Giedraitiene et al. 2011 ; Al-Bayssari et al., 2015**). L'augmentation de la prévalence des bactéries productrices de BLSE en pathologie humaine a incité les cliniciens à utiliser les carbapénèmes, β-lactamines à large spectre réservées normalement en dernière intention, parfois en tant que traitement empirique de 1ère ligne, conduisant ainsi à l'émergence de la résistance à cette famille (**Mathlouthi, et al., 2017**).

Dans la première étude de ce chapitre (**Article N° 3)**, nous avons évalué la prévalence et la diversité des gènes qui codent pour la production de β-lactamases et de carbapénèmases chez les volailles d'Algérie et de Marseille (France). A partir de 503 fientes de poulets collectées dans 8 régions d'Algérie et de 330 fientes collectées à Marseille, nous avons recherché la présence de BLSE (TEM, SHV, and CTX-M) et de carbapénèmases (NDM, OXA48, OXA58) par des approches phénotypiques (culture, isolement et identification) et génotypiques (PCR standard, qPCR et séquençage). Toutes les fientes de poulets de Marseille étaient négatives pour la recherche de BLSE et de carbapénèmases. En revanche, parmi les 503 échantillons prélevés en Algérie, 128/503 étaient positifs pour le gène blaTEM, 83/503 pour blaSHV, 46/503 pour blaCTX-M et 132/503 étaient positifs pour le gène blaOXA58. Cette étude montre la grande diversité et la forte prévalence des gènes codants pour les BLSE dans les différents sites étudiés en Algérie. Cela est justifié par l'administration irrationnelle des antibiotiques chez les animaux en Algérie. Des mesures de restriction d'usage des antibiotiques doivent s'appliquer afin de limiter le transfert de gènes de résistance des bactéries d'origine animale à l'homme.

La deuxième étude (**Article N° 4)**, a porté sur l'étude de la prévalence et de la clonalité des souches de Salmonella productrices de BLSE chez les volailles d'Algérie ainsi que leurs relations avec les souches humaines. Quarante-cinq souches de Salmonella isolées à partir de fientes de poulets et 37 souches d'origine humaines de Salmonella ont été étudiées. Cinq sérotypes de S. enterica ont été trouvés dans les isolats aviaires et 9 dans les isolats humains. 51,11% et 26,6% des isolats d'origine aviaire étaient résistants à la ciprofloxacine et au céfotaxime, respectivement, alors que les isolats d'origine humaine présentaient une résistance plus faible à ces antibiotiques (13,5% pour la ciprofloxacine et 16,2% pour la céfotaxime). Dix-huit souches (12 aviaires et 6 humaines) étaient porteuses de BLSE [Bla CTX-M-1 (n = 12), Bla CTX-M-15 (n = 5) et Bla TEM (n = 8)]. De façon intéressante, sept des souches productrices de BLSE (5 aviaires et 2 humaines) appartenait au même ST (ST15). A travers cette étude nous

avons montré la présence de bactéries d'origine commune entre l'animal et l'homme et prouve le risque de transfert de gènes de résistance de l'animal à l'homme.

ARTICLE 3

Title: Prevalence of extended-spectrum β-lactamase- and carbapenemase-encoding genes in poultry feces from Algeria and Marseille, France.

Selma Chabou, Hamza Leulmi, Bernard Davoust, Atef Aouadi and Jean Marc Rolain.

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Prevalence of extended-spectrum β-lactamase- and carbapenemase-encoding genes in poultry faeces from Algeria and Marseille, France

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ABSTRACT

Objectives: The aim of this study was to evaluate the prevalence of extended-spectrum β-lactamase (ESBL)- and carbapenemase-encoding genes in poultry from Algeria and Marseille, France.
Methods: Samples consisted of faeces collected from broilers in France and Algeria between 2014 and 2015. DNA extraction and quantitative PCR were performed on 833 faecal samples to screen for the presence of genes encoding ESBLs (bla_{TEM}, bla_{SHV} and bla_{CTX-M}) and carbapenemases (bla_{NDM}, bla_{VIM}, bla_{KPC}, bla_{OXA-23}, bla_{OXA-24}, bla_{OXA-48} and bla_{OXA-58}). To characterise bacteria carrying antimicrobial resistance genes, positive results were simultaneously sequenced.
Results: All chicken faeces from Marseille were negative for ESBL- and carbapenemase-encoding genes. However, of the 503 faecal samples collected in Algeria, 128 (25.4%) were positive for bla_{TEM}, 83 (16.5%) for bla_{SHV}, 46 (9.1%) for bla_{CTX-M} and 132 (26.2%) for bla_{OXA-58}. Using a sequencing reaction, a high diversity of ESBL genes was observed throughout the sites studied.
Conclusions: This study shows a high prevalence and diversity of ESBL and carbapenemase genes in poultry faeces from Algeria, whereas none of the samples from Marseille were positive for these genes. The high prevalence of ESBLs in Algeria is in contrast to the observation that no chickens were found to be positive in France.
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1. Introduction

Resistance to antimicrobial agents has been widely reported as a result of widespread use of antimicrobials in animal husbandry to prevent and treat disease and to improve growth [1].

The widespread use of antimicrobials in livestock and food-producing animals has been linked to the emergence and spread of antimicrobial-resistant bacteria isolated from animals and their environments [2], although the impact of this use on human health remains controversial [1]. The risk of spread of zoonotic bacteria from livestock to humans is still largely unknown, although most studies show limited overlap between extended-spectrum β-lactamase (ESBL) types and ESBL-carrying plasmids in humans and livestock [3]. Infection can affect humans directly through contact with or consumption of food of animal origin [4] or indirectly through environmental pollution by agricultural effluents [5]. Some studies have shown a high prevalence of ESBL-producing *Escherichia coli* in livestock, suggesting a risk of transfer between livestock and farm workers [6]. The high prevalence of ESBL-producers in human infectious diseases has forced clinicians to use carbapenems as empirical treatment for nosocomial pneumonia, which has led to increased resistance to these antibiotics as well [7]. A few publications have reported on carbapenemase-producing bacteria in animals. Fischer et al. reported strains of *E. coli* and *Salmonella* producing VIM-1 carbapenemase isolated from livestock farms in Germany [7]. In Egypt, high rates of ESBLs and carbapenemases in Enterobacteriaceae have been reported in retail chicken meat [8]. Recently in Algeria, Yousfi et al. reported the occurrence of *E. coli* isolates producing ESBLs (CTX-M-15, CTX-M-1, SHV-12 and TEM-1) and

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carbapenemases (OXA-48 and NDM-5) in the faeces of companion animals in Algeria [9,10].

In this study, molecular methods were used to assess the prevalence and diversity of ESBLs and carbapenemases in poultry from Algeria and Marseille (France).

2. Materials and methods

2.1. Study area and sample collection

Samples in this study consisted of faeces collected from industrial broiler chickens and chickens from slaughterhouses in Algeria and France. Field samples were collected in two stages. The first collection was conducted between August–December 2014 at breeding sites and slaughterhouses in eight regions in the north (El Tarf, Souk Ahras, Setif, Skikda and Jijel), centre (Algiers) and south (Biskra and Ouargla) of Algeria. The second collection was performed at three slaughterhouses in Marseille, France (Aubagne, Marché aux Puces and Noailles) in February 2015. All poultry in breeding farms in Algeria received locally manufactured feed, and the feed and drinking water were automatically distributed. Animals were apparently healthy and no symptoms were observed. The sites in Algeria were selected according to the extent of owner co-operation and the presence of >1000 chickens per breeding site.

Samples were collected directly from the cloaca of the chicken. Faeces from live chickens and breeding broilers was extracted directly from the cloaca using single-use gloves, whilst faecal samples from chickens in slaughterhouses were taken just after slaughter using the same method. Faeces were placed in single, sterile and identified tubes. Tubes containing the droppings were placed in a cooler and were transported directly to our laboratory (MEPHI, IHU Méditerranée Infection, Aix-Marseille Université, Marseille, France) to be stored at −80 °C until use in PCR assays.

2.2. Extraction and pooling of DNA

Chicken faeces was individually diluted in tubes, mixing 1 g of droppings with 200 μL of sterile distilled water. The suspension was subsequently immersed in a solution composed of 20 μL of proteinase K (QIAGEN, Hilden, Germany) and 180 μL of G2 buffer (QIAGEN, Hilden, Germany). All tubes were incubated in a dry bath overnight at 56 °C. DNA was then extracted using an automated BioRobot EZ1 (QIAGEN, Tokyo, Japan) with an EZ1 DNA Extraction Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions.

DNA from the extracted samples was tested by quantitative PCR (qPCR) targeting the *ompG* gene present in *E. coli* to control the extraction and DNA pool. qPCR was performed following DNA dilution to 1:10 and 1:100. The negative control corresponded to a single mix of qPCR with water (DNA/RNA-free). Conversely, the positive control consisted of filling the mix with DNA extracted from a colony of *E. coli* cultured in our laboratory. The results were considered positive if the cycle threshold (Ct) had a value of <35 obtained using a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, France).

2.3. Detection of extended-spectrum β-lactamase genes

The final qPCR reaction mixture consisted of 5 μL of DNA and 15 μL of mix from the Takyon qPCR Kit (QIAGEN, Hilden, Germany) [11]. Two negative controls were used in each qPCR and consisted of water and mixes. Positive controls included DNA extracted from a dilution of cultured strains of *Klebsiella pneumoniae* (for the detection of $bla_{CTX\text{-}M}$, bla_{TEM} and bla_{SHV}) [11]. Positive qPCR results were simultaneously tested by standard PCR. The purified PCR products were sequenced using specific primers and a BigDye® Terminator® v.1.1 Cycle Sequencing Ready Reaction Mix (Applied Biosystems, Foster City, CA).

Data were collected with an ABI Prism 3130xl Genetic Analyzer capillary sequencer (Applied Biosystems). Sequences were edited and assembled using Chromas Pro 1.34 (Technelysium Pty. Ltd., Tewantin, QLD, Australia).

2.4. Detection of carbapenems genes

The presence of carbapenemase-encoding genes was investigated by qPCR, including bla_{NDM}, bla_{VIM}, bla_{KPC}, $bla_{OXA\text{-}23}$, $bla_{OXA\text{-}24}$, $bla_{OXA\text{-}48}$ and $bla_{OXA\text{-}58}$.

3. Results

3.1. Study sites and collection of samples

During the period from August–December 2014, 503 droppings were collected from broilers in the eight regions of Algeria (Table 1). T he second collection, which was conducted in three slaughterhouses in Marseille (France), took place in February 2015 and provided 330 samples. DNA of all 833 droppings was extracted and pooled by twenty in 41 tubes.

3.2. Detection of extended-spectrum β-lactamase genes

Samples from Marseille were all negative for the various ESBL genes tested. In Algeria, 18/26 pools were positive for ESBL, including 9 pools that were positive for $bla_{CTX\text{-}M}$ (Souk Ahras, El Tarf and Ouargla), 10 pools that were positive for bla_{SHV} (Souk Ahras, El Tarf, Ouargla and Algiers) and 13 pools that were positive for bla_{TEM} (Souk Ahras, El Tarf, Ouargla, Biskra and Jijel). Overall, among 503 faecal samples collected in Algeria, 128 (25.4%) were positive for bla_{TEM}, 83 (16.5%) for bla_{SHV} and 46 (9.1%) for $bla_{CTX\text{-}M}$ (Fig. 1). The sequence results show the diversity of ESBL genes detected in faeces of chickens from Algeria (Fig. 2).

3.3. Detection of carbapenemase-encoding genes

None of the DNA pools tested were positive for bla_{NDM}, bla_{VIM}, bla_{KPC}, $bla_{OXA\text{-}23}$, $bla_{OXA\text{-}24}$ or $bla_{OXA\text{-}48}$ in France and Algeria. However, 19/26 DNA pools were positive for the $bla_{OXA\text{-}58}$ gene in Algeria. This corresponds to 132 (26.2%) of 503 faecal samples collected in five of the eight regions of Algeria (Fig. 3).

4. Discussion

Antimicrobials are widely used in animals and animal production as antibiotics to prevent and treat disease as well as probiotics

Table 1
Prevalence of extended-spectrum β-lactamase genes (bla_{SHV}, bla_{TEM} and $bla_{CTX\text{-}M}$) and the $bla_{OXA\text{-}58}$ carbapenemase gene in Algeria.

Site	No. of samples	No. (%) of samples positive for:			
		bla_{SHV}	bla_{TEM}	$bla_{CTX\text{-}M}$	$bla_{OXA\text{-}58}$
Souk Ahras	150	52 (34.7)	50 (33.3)	25 (16.7)	45 (30.0)
El Tarf	83	20 (24.1)	34 (41.0)	19 (22.9)	37 (44.6)
Ouargla	32	9 (28.1)	17 (53.1)	2 (6.3)	
Jijel	40		12 (30.0)		34 (85.0)
Biskra	35		15 (42.9)		
Algiers	101	2 (2.0)			12 (11.9)
Setif	37				4 (10.8)
Skikda	25				
Total	503	83 (16.5)	128 (25.4)	46 (9.1)	132 (26.2)

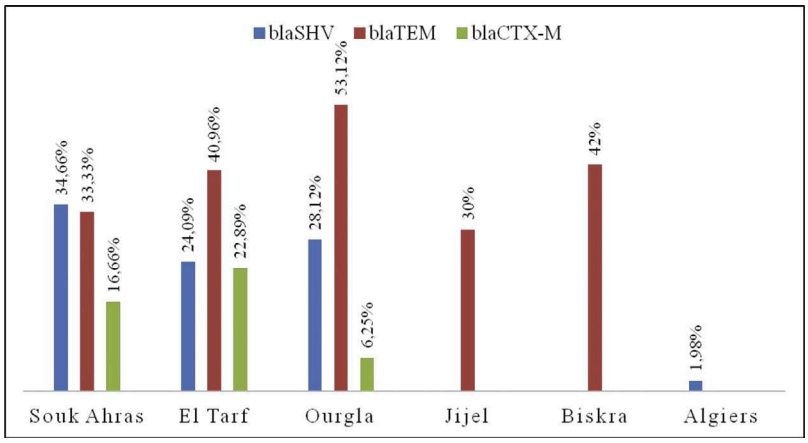

Fig. 1. Prevalence of extended-spectrum β-lactamases (ESBLs) in Algeria.

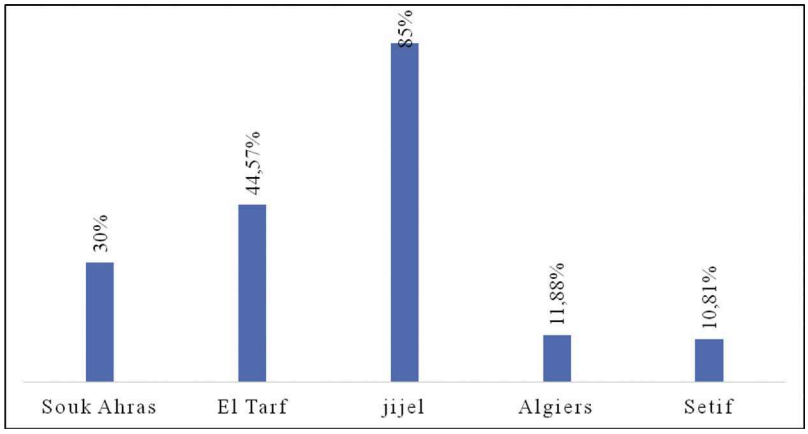

Fig. 2. Phylogenetic tree of the diversity of extended-spectrum β-lactamase (ESBL) genes detected in faeces of chickens from Algeria: (A) bla_{TEM}; (B) bla_{CTX}; (C) bla_{SHV}; and (D) bla_{OXA-58}.

to improve growth [12]. In addition, nearly all antimicrobial classes important for human medicine are used in animal production [4]. Antimicrobial resistance is a global health problem: over the last decade, many studies have reported the dissemination of ESBLs and carbapenemases, mainly from poultry and others animals, and there is extensive evidence available in the literature on monitoring antimicrobial-resistant bacteria in animals and food-producing animals [1,10]. In Algeria, very few studies have been published quantifying the prevalence of antimicrobial resistance in animals and describing the diversity of genes encoding resistance compared with developing countries.

The present study investigated the presence and prevalence of ESBLs and carbapenemases in poultry from Algeria and France. This work represents an important contribution towards a comparison between the diversity of genes encoding resistance and the use of antimicrobials in these two countries.

Using molecular approaches, the presence of bla_{TEM}, bla_{SHV}, $bla_{CTX-M-1}$, $bla_{CTX-M-15}$ and bla_{OXA-58} was detected in poultry faeces collected in eight regions of Algeria. Interestingly, no poultry tested positive in France. To the best of our knowledge, this is the first report in Algeria presenting direct evidence of these resistance-encoding genes in faeces from poultry in eight regions from the north, centre and south of Algeria. The robustness of the data reported is based on strict laboratory procedures and controls, including rigorous positive and negative controls to validate each test.

ESBL-producing Enterobacteriaceae were first reported in Europe in the 1980s and have since become a worldwide problem [13]. Infections caused by ESBL-producing organisms have been associated with high mortality [13]. In Algeria, little is known about the presence and prevalence of ESBLs, whilst Africa as a whole is a continent where antimicrobial resistance problems have not yet been adequately illustrated [13]. A high prevalence of bla_{TEM} was detected in the current study in poultry faecal samples collected from Algeria, where five of the eight regions tested were positive, with a prevalence ranging between 30.0–53.1%. bla_{TEM} was first reported in Algeria in 53/71 human isolates (from Sétif and Tizi-Ouzou hospitals) collected between 2010 and 2011 [14]. Recently, bla_{TEM} was detected in faecal isolates from healthy companion animals (dogs and cats) in Béjaïa, Algeria [9].

bla_{SHV} was also reported in this study in four of the eight regions in Algeria, with average ESBL faecal carriage rates of 16.5%. bla_{SHV} was first detected in Algeria in *Enterobacter cloacae* isolated from urine and pus of a patient (from Algiers) in 2006 [15].

bla_{CTX-M} was also detected in faecal samples from poultry in Algeria, with an average prevalence of 9.1%. $bla_{CTX-M-15}$ and $bla_{CTX-M-3}$ β-lactamases were first reported in Algeria in 2006 from clinical isolates of Enterobacteriaceae in Béjaïa [16]. bla_{CTX-M} was

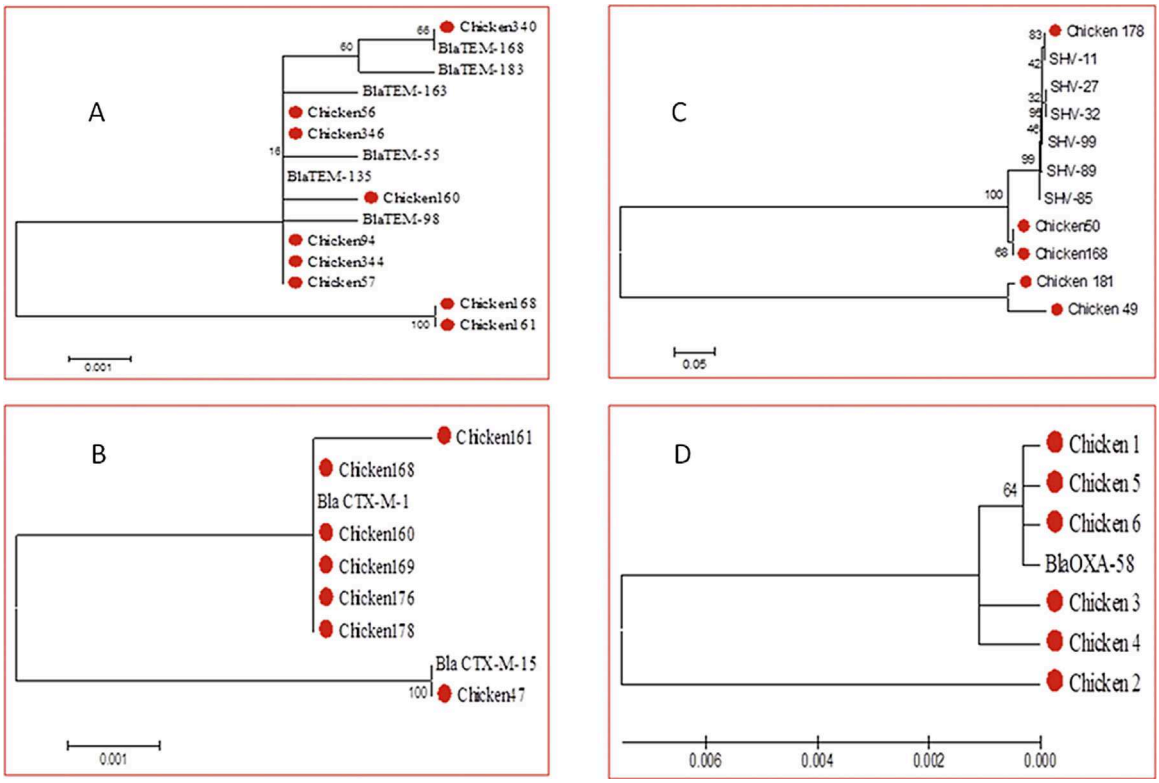

Fig. 3. Prevalence of the bla_{OXA-58} gene in Algeria.

previously detected in *E. coli* isolated from poultry organs, namely the spleen, liver, pericardium and ovary, in various regions of central Algeria (the provinces of Bouira, Béjaïa, Tizi Ouzou and Boumerdés) [17].

In the current study, 26.2% of samples (132/503) from Algeria were positive for bla_{OXA-58}. This gene was first detected in Algeria in 12 *Acinetobacter baumannii* clinical isolates in Tlemcen in 2008 [18] but has not yet been reported in poultry from Algeria.

In conclusion, here we report a wide diversity and a high prevalence of ESBL-producing bacteria as well as the presence of bla_{OXA-58} carbapenemase in poultry faeces from Algeria. In contrast, no chickens were found to be positive in France. We believe that such a high prevalence of antimicrobial resistance genes in poultry in Algeria is linked to the widespread and uncontrolled use of antibiotics in poultry, which promotes selection pressure and a heightened risk of zoonotic transmission. In addition, recent detection of the plasmid-mediated colistin resistance gene *mcr-1* in chickens from China and Algeria [19] confirms the major role of chickens as a reservoir for bacteria carrying antimicrobial resistance. Further studies are needed to monitor and survey the spread of ESBL- and carbapenemase-producing strains from these animals to humans, mainly to investigate the role of poultry as a reservoir for gene transmission by the study of plasmid vectors. We believe that the use of key antibiotics used in medicine should be avoided in animals.

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ARTICLE 4

Title: Prevalence and clonal relationship of ESBL-producing Salmonella strains from humans and poultry in northeastern Algeria.

Samia Djeffal, Sofiane Bakour, Bakir Mamache, Rachid Elgroud, Amir Agabou, **Selma Chabou**, Sana Hireche, Omar Bouaziz, Kheira Rahal and Jean Marc Rolain.

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DOI 10.1186/s12917-017-1050-3

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RESEARCH ARTICLE Open Access

Prevalence and clonal relationship of ESBL-producing *Salmonella* strains from humans and poultry in northeastern Algeria

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Abstract

Background: The aims of this study were to investigate *Salmonella* contamination in broiler chicken farms and slaughterhouses, to assess the antibiotic resistance profile in avian and human *Salmonella* isolates, and to evaluate the relationship between avian and human Extended Spectrum β-Lactamase (ESBL)-producing isolates. *Salmonella* was screened in different sample matrices collected at thirty-two chicken farms and five slaughterhouses. The human isolates were recovered from clinical specimens at the University Teaching Hospital of Constantine (UTH). All suspected colonies were confirmed by MALDI-TOF (Matrix Assisted Laser Desorption Ionization Time OF light) and serotyped. Susceptibility testing against 13 antibiotics including, amoxicillin/clavulanic acid, ticarcillin, cefoxitin, cefotaxime, aztreonam, imipenem, ertapenem, gentamicin, amikacin, ciprofloxacin, colistin, trimethoprim/sulfamethoxazole and fosfomycin, was performed using the disk diffusion method on Mueller-Hinton agar. ESBL-production was screened by the double-disk synergy test and confirmed by molecular characterization using PCR (polymerase chain reaction) amplification and sequencing of ESBL encoding genes. Clonality of the avian and human strains was performed using the Multi Locus Sequencing Typing method (MLST).

Results: Forty-five isolated avian *Salmonella* strains and 37 human collected ones were studied. Five *S. enterica* serotypes were found in avian isolates (mainly Kentucky) and 9 from human ones (essentially Infantis). 51.11% and 26.6% of the avian isolates were resistant to ciprofloxacin and cefotaxime, respectively, whereas human isolates were less resistant to these antibiotics (13.5% to ciprofloxacin and 16.2% to cefotaxime). Eighteen (12 avian and 6 human) strains were found to produce ESBLs, which were identified as $bla_{\text{CTX-M-1}}$ (n = 12), $bla_{\text{CTX-M-15}}$ (n = 5) and bla_{TEM} group (n = 8). Interestingly, seven of the ESBL-producing strains (5 avian and 2 human) were of the same ST (ST15) and clustered together, suggesting a common origin.

Conclusion: The results of the combined phenotypic and genotypic analysis found in this study suggest a close relationship between human and avian strains and support the hypothesis that poultry production may play a role in the spread of multidrug-resistant *Salmonella* in the human community within the study region.

Keywords: *Salmonella*, poultry, human, serotype, antimicrobial resistance, resistance genes, clonality

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Background

Salmonella infections are a major public health problem with a significant social and economic impact. Many animal species are potential reservoirs for this bacterium, especially chickens, pigeons and reptiles [1]. Humans can commonly acquire the infection through the food chain [2]. Young and immunocompromised patients are the most exposed to dangerous complications which are generally treated with fluoroquinolones and extended-spectrum cephalosporins that are largely used in veterinary medicine [3].

In Algeria, the poultry industry has grown remarkably since 1980. However, as a result of the deleterious hygienic conditions, many infectious diseases, such as Salmonellosis, were detected in broilers and constitute a risk to the human health [4]. Due to a lack of surveillance programs, information on the prevalence of *Salmonella* and other food pathogens is incomplete. For instance, a study by the Pasteur Institute of Algeria revealed that 11% of food poisoning cases were caused by *Salmonella* spp. in 2011 [5]. 47% of these cases were mainly related to the consumption of chicken meat, as reported by Mouffok et al., [5]. Eggs and ovoproducts are also among the major sources of human infections, which require a thorough assessment and control measures for *Salmonella spp.* in the poultry industry [6]. These measures are well detailed in the Official Journal of the Algerian Republic (No. 36 of June 8, 2003); but were only applied to the poultry industry facilities in the public sector as well as to private poultry production units and facilities [7].

Selective pressure due to the misuse of antibiotics in humans and domestic livestock is one of the many factors that has led to the emergence of antibiotic resistance in commensal and pathogenic bacteria; thus, multidrug-resistant (MDR) *Salmonella* have increasingly been isolated from various food products worldwide [8].

Drug resistance is growing and has affected critically important classes of antibiotics, such as the β-lactams, which are among the most significant bactericidal antibiotics used to treat bacterial infections in humans [9]. Extended spectrum β-lactamases (ESBLs) were identified following the introduction of extended-spectrum oxyimino-cephalosporins in the 1980s for the treatment of severe human infections [10]. In veterinary medicine, a variety of these drugs are currently authorized for use, resulting in the emergence of ESBL-producing Gram-negative bacteria [11, 12].

TEM, SHV and CTX-M are the most prevalent ESBL types. Over the last decade, rates of CTX-M producing bacteria have increased worldwide in comparison with TEM and SHV [13]. This situation is rendered more complicated as these enzymes confer co-resistance to other drug classes [13, 14].

In Algeria, the first ESBLs were detected in non-typhoidal *Salmonella* in 1994 from humans [15], but until now there has been no information on the magnitude of this problem in animals and humans. This situation drove us to identify the contamination status of *Salmonella* serotypes in chicken farms and slaughterhouses, to assess their sensitivity to antimicrobials and ESBL-production and, finally, to evaluate their clonality with human pathogenic strains.

Methods

Study area

The present study was carried out between December 2011 and May 2013. Thirty-two chicken farms and five chicken slaughterhouses located in the province of Skikda, Algeria, took part in this survey. The choice of this study area was motivated by the size of the poultry industry and the frequent occurrence of infectious gastrointestinal pathologies as reported by local veterinary practitioners [16].

Twenty-seven poultry houses had concrete walls and floors and corrugated metal sheet roofs while the remaining five houses had earth floors with walls and roofs made of straw and reeds covered with plastic foil. None of the farming sites was fenced, allowing free access for domestic and wild animals. Their rearing capacities vary from 3500 to 20,000 birds per house.

All slaughterhouses had concrete floors with earthenware walls. The slaughtering capacity ranges from 2000 to 7000 chickens per day and the broilers are brought from different poultry farms located in several neighboring provinces. The number of chickens and slaughterhouses is statistically representative of the study region and governed by the capacity of the laboratory processing the samples.

The human *Salmonella* strains were kindly provided by the University Teaching Hospital (UTH) of Constantine and included in the study.

Study design

For technical reasons, including access to the sampling sites, a total of 1194 samples were collected during two sampling periods (between December 2011 and September 2012 and between December 2012 and May 2013). The poultry houses were visited at two periods (when the birds were aged 15–30 days and 45–60 days).

From the chicken farms, a total of 320 samples of water were taken from drinking vessels, 160 samples of feed were taken from the feeding vessels, 330 cloacal swabs were taken and 320 droppings were collected and placed in sterile containers. 64 surface wipes (25 cm × 25 cm, AES Chemunex, Combourg, France) were also obtained from a height of 30 cm from the ground over a 400 cm^2 area of the four walls of the poultry house and placed into sterile Stomacher bags.

The five poultry slaughterhouses were visited once for sampling. Due to limited financial resources, in each poultry slaughterhouse, we pooled individual samples to minimize study costs. The samples were randomly taken from three organs from five chickens (5 g of 5 caeca, 5 g of 5 livers, 5 g of 5 neck skins), and from the environment (one sample of carcass rinsing water, one swab from a sticking knife and one wipe from the walls). The slaughtered animals were brought from several neighboring provinces.

All samples were transported to the laboratory, on ice packs within a period not exceeding two hours, to be treated on the same day or kept in the refrigerator overnight.

Salmonella isolation and identification

Bacteriological analyses were performed according to the EN/ISO 6579–2002/Amd1:2007 protocol for *Salmonella* detection in food and animal feedstuffs. 25 g of samples (droppings, feed, liver, caeca, neck skin) were individually pre-enriched with 225 mL of buffered peptone water broth (PWB) (Fluka, Sigma Aldrich, France). The swabs were individually placed in 10 mL PWB, while 100 mL of drinking and carcass rinsing water was individually mixed with 100 mL of double strength PWB for pre-enrichment according to NF U 47–101 Standard (2005) [17]. All samples were incubated at 37 °C for 18–20 h. From each pre-enrichment solution, 1 mL and 0.1 mL were respectively transferred into 10 mL of enrichment Muller-Kauffmann tetrathionate/novobiocin broth (AES Chemunex Combourg, France) and 10 mL of Rappaport Vassiliadis broth (Merck Darmstadt, Germany), incubated respectively at 37 °C and 42 °C for 24 h. Both enriched samples were then streaked on XLD (Fluka analytical Steinheim, Switzerland) and Hektoen agars (Pasteur Institute of Algeria) and incubated at 37 °C for 24 h [18]. Suspected colonies were first identified with the API 20E System (bioMérieux, France), then with MALDI-TOF (Bruker Daltonics GmbH, Germany) [19]. Confirmed *Salmonella* isolates were serotyped according to Kauffmann-White-Le Minor's scheme [20].

Human clinical strains

We selected 37 (non-repetitive) strains recovered from clinical specimens at the UTH of Constantine over a decade (2005–2015). These were isolated from stool samples collected from different wards and among which 26 strains derived from the diarrheic stools of infants admitted to the neonatology ward. The Main characteristics of patients are shown in Additional file 1.

The strains were confirmed using MALDI-TOF mass spectrometry for prescreening *Salmonella* species and sub-species and to identify epidemiologically important serovars which were further tested with conventional serotyping method.

Antimicrobial susceptibility testing and ESBL detection

All human and avian strains were submitted to susceptibility testing against antibiotics using the disk diffusion method on Mueller-Hinton (MH) agar, and the results were interpreted according to the European Comitee on Antimicrobial Suceptibility Testing (EUCAST) [21] (Additional file 2). Thirteen antibiotics (Bio-Rad, France) were tested: amoxicillin/clavulanic acid AMC (20/10 μg), ticarcillin TIC (75 μg), cefoxitin FOX (30 μg), cefotaxime CTX (5 μg), aztreonam ATM (30 μg), imipenem IPM (10 μg), ertapenem ETP (10 μg), gentamicin GEN (10 μg), amikacin AK (30 μg), ciprofloxacin CIP (5 μg), colistin CT (50 μg), trimethoprim/sulfamethoxazole SXT (1.25 μg/23.75 μg) and fosfomycin FF (50 μg).

Extended spectrum β-lactamase production was screened by the double-disc synergy test (DDST) [22].

PCR detection of ESBL genes

Total nucleic acids were extracted using a BioRobot EZ1 Advanced XL instrument (QIAGEN, Hilden, Germany) according to the manufacturer's instructions.

Detection of β -lactamase genes (including bla_{TEM}, bla_{SHV}, and bla_{CTX-M}) was carried out by polymerase chain reaction (PCR) using specific primers: $bla_{CTX-M-1}$ group [23], $bla_{CTX-M-9}$ group [24], bla_{TEM} group [25] and bla_{SHV} [26] and the master mix QuantiTect Probe PCR Kit (QIAGEN, Hilden, Germany).

Amplification products were detected by electrophoresis using agarose gels containing SYBR safe (Invitrogen, Leek, the Netherlands), along with a DNA molecular weight marker (BenchTop pGEM®DNA Marker, Promega, Madison, Wisconsin, USA). Visualization of gels was carried out using the BenchTop pGEM®DNA Marker (Promega, Madison, Wis- consin, USA) under ultraviolet illumination.

ESBL genes sequencing and multilocus sequence typing

ESBL-positive PCR products were purified using the NucleoFast 96 PCR plate (Machery-Nagel EURL, France) and sequenced using the BigDye terminator chemistry on an ABI3730 automated sequencer (Applied Biosystems, Foster City, California, USA). The obtained sequences were analyzed with the ARG-ANNOT database [27].

Multilocus sequence typing (MLST) is useful in assessing the role of specific STs in human and animal disease and assessing overlap between these hosts. It was carried out by PCR amplification and sequencing of seven housekeeping genes: *thrA* (aspartokinase + homoserine dehydrogenase), *purE* (phosphoribosylaminoimidazole carboxylase), *sucA* (alpha ketoglutarate dehydrogenase), *hisD* (histidinol dehydrogenase), *aroC* (chorismate synthase), *hemD* (uroporphyrinogen III cosynthase), and *dnaN* (DNA

polymerase III beta subunit), as described by Kidgell et al. 2002 [28].

Briefly, template DNA prepared from bacterial isolates was amplified by PCR with the use of an oligonucleotide sequence for seven housekeeping genes (available in the MLST database: http://mlst.warwick.ac.uk/mlst/dbs/Senterica). Sequencing with the same automated sequencer of the PCR product was carried out by the dideoxynucleotide chain termination method using the master mix QuantiTect Probe PCR Kit (QIAGEN, Hilden, Germany). Forward and reverse DNA sequences were assembled, trimmed, edited, and analyzed for each gene fragment using the ARG-ANNOT database [27]. Allelic profile and sequence type determinations were assigned according to the *Salmonella* MLST database: http://mlst.warwick.ac.uk/mlst/dbs/Senterica.

Clonality analysis

Protein mass profiles were obtained using a Microflex LT MALDI-TOF mass spectrometer (Bruker Daltonics, Germany), with Flex Control software (Bruker Daltonics). The spectrum profiles obtained were visualized with Flex analysis v.3.3 software and exported to ClinProTools software v.2.2 and MALDI-Biotyper v.3.0 (Bruker Daltonics, Germany) for data processing (smoothing, baseline subtraction and spectra selection) and evaluation with cluster analysis.

The phyloproteomic analysis of ESBL-positive *Salmonella* strains from human and poultry origins was assessed through construction and comparison of their characteristic reference spectra (main spectra) with the MALDI-Biotyper v.3.0 software (Bruker Daltonics, Germany). Cluster analysis was performed based on pairwise comparisons of specific main spectra (MSP: mean spectra projection dendrogram) of the different strains to generate a dendrogram of similarities among spectra profiles using the software default correlation function. A distance level of 560 was selected for clustering evaluation of the isolates.

Statistical analysis

Differences in contamination levels of poultry houses at two sampling periods (15–30 days versus 45–60 days), and the antimicrobial resistance patterns between avian and human *Salmonella* strains, were assessed by the Chi square test (at 95% CI and $p < 0.05$) or Fisher's exact test if N is less than 20 and one expected cell is less than or equal 5. All statistical analyses were performed using IBM SPSS Statistics version 24 software (2016).

Results

Frequency of isolation of *Salmonella* serotypes in broilers, slaughterhouses and human samples

Forty-five *Salmonella enterica* from slaughterhouses and poultry farms and 37 of human clinical origin were studied. 34.37% of the poultry farms and all slaughterhouses were contaminated with *Salmonella* and the isolation rate varied depending on the sampling matrix. The samples taken at the age of 15–30 days were more contaminated than those collected at 45–60 days; however, the difference was not significant *($p > 0.05$)*.

The isolated *Salmonella* strains belonged mainly to two serotypes: Kentucky and Heidelberg, and the remaining strains were Enteritidis, Virginia and Newport. There was an evident heterogeneous distribution of serotypes in poultry farms and slaughterhouses (Table 1).

Among the human *Salmonella* strains, Infantis was the most frequent serotype, followed by Senftenberg, Enteritidis, Kedougou, Tyhimurium, Heidelberg, Kentucky, Ohio and Arizona. Most of these strains were from infants and the others were from adult diarrheic stools (especially *Salmonella enterica* serotypes Enteritidis and Typhimurium).

Drug resistance patterns of the isolated *Salmonella* strains

A high frequency of resistance to ciprofloxacin (51.1%) was noted in *Salmonella* isolates from both chicken farms and slaughterhouses. These strains were resistant to cephalosporins (26.6% to cefotaxime), aztreonam (26.6%), ticarcillin (46.6%) and gentamicin (22.2%). ESBL-production was found in 26.6% of these avian isolates (11 *Salmonella* ser. Heidelberg and one *Salmonella* ser. Newport).

The human *Salmonella* isolates were highly resistant to ticarcillin (56.75%), amoxicillin (45.94%) and a less extent to trimethoprim/sulfamethoxazole (24.3%), gentamicin (24.3%), aztreonam (18.9%), cefotaxime (16.2%), ciprofloxacin (13.5%) and amikacin (10.8%). Six ESBL-positive strains were detected (Fig. 1).

The difference in resistance to antibiotics, between poultry strains and human ones was significant for: amoxicillin/clavulanic acid ($P < 0.05$), gentamicin ($P < 0.05$), ciprofloxacin ($P < 0.05$) and fosfomycin ($P < 0.05$). However, it was not significant for cefotaxime, ticarcillin, aztreonam and amikacin *($P > 0.05$)*.

The characteristics of the 18 ESBL-producing strains isolated from chicken farms/slaughterhouses and patients are shown in Table 2. Eleven of the *Salmonella* ser. Heidelberg strains harbored $bla_{\text{CTX-M-1}}$ genes. The bla_{TEM} group was identified in one avian *Salmonella* ser. Newport isolate as well as in S. Heidelberg, in which it was coupled with the $bla_{\text{CTX-M-1}}$ gene. Human strains harbored the $bla_{\text{CTX-M-15}}$ gene in association with $bla_{\text{TEM,}}$ except one strain which carried instead the $bla_{\text{CTX-M-1}}$. None of the strains were positive for $bla_{\text{CTX-M-9}}$ and bla_{SHV} genes.

The MLST analysis showed that the *salmonella* isolates belonged to six different sequence types (ST) including, ST14, ST15, ST16, ST32, ST38 and ST198 (Fig. 2). The results demonstrate that ST15 represents the predominant clone. Indeed, this ST was found in 13 *Salmonella* ser. Heidelberg strains (two human and 11 avian) (Fig. 2). In

Table 1 Frequency of isolation of *Salmonella enterica* subsp. *enterica* serotypes in different sample matrices from poultry farms and slaughterhouses

Serotype	Poultry farms and slaughterhouses		Samples	
	N° of positive (ID n°)	(%)	N° of positive samples	(%)
Kentucky	7 Poultry farms (F1, F6, F13, F14, F18, F20, F32)	21.87	5 cloacal swabs 6 droppings 2 wipes 3 water samples	1.51 1.87 3.12 0.93
	4 Slaughterhouses (S1, S2, S3, S4)	80	1 sticking knife 2 caeca 1 liver 1 wipe	20 8.0 4.0 20
Heidelberg	4 Poultry farms (F12, F13, F15, F27)	12.5	6 cloacal swabs 4 droppings 2 water samples 1 wipe	1.81 1.25 0.62 1.56
	(0) Slaughterhouse	0	-	-
Virginia	1 Poultry farm (F31)	3.12	1 water sample	0.31
	2 Slaughterhouses (S1,S4)	40	1 wipe 2 neck skins 1caeca	20 40 4.0
Enteritidis	3 Poultry farms (F18, F20, F27)	9.37	2 cloacal swabs 1 wipe	0.61 1.56
	1 Slaughterhouse (S3)	20	1 rinse water sample	
Newport	1 Poultry farm (F15)	3.12	1 wipe	1.56
	(0) Slaughterhouse	0	-	-
Total	11 Poultry farm (F1, F6, F12, F13, F14, F15, F18, F20, F27, F31, F32)	34.7	13 cloacal swabs 10 droppings 5 wipes 6 water samples	3.93 3.12 7.81 1.87
	5 Slaughterhouse (S1, S2, S3, S4, S5)	100	1 sticking knife 2 neck skins 1 rinse water sample 3 caeca 1 liver 2 wipe	20 8.0 4.0 12 4.0 40

F Farm, *S* Slaughterhouse

addition, the phylogenetic tree shows that seven *Salmonella* ser. Heidelberg strains (two human and five avian) clustered together and belonged to the same sequence type ST15 (Fig. 2), which suggests a possible crossing of this serotype, and particularly this ST between the poultry and the human community in northeastern Algeria.

Discussion

This study involved thirty-two poultry farms and five slaughter houses. It covered most of the districts in the Skikda province. The selection of the sites was based on the managers' willingness to cooperate with the study and to spend a significant amount of time and effort to perform and collect the various samples. This work provides epidemiological data on *Salmonella* serotype contamination in poultry farms and slaughterhouses in the region of Skikda, in order to investigate the molecular mechanisms of ß-lactam resistance in avian and human ESBL-producing *S. enterica* and to analyze the genetic relatedness of avian and human isolates.

The recorded prevalence rates (34.37% for poultry farms and 100% for slaughterhouses) are in accordance with those reported in Constantine and Batna provinces (northeastern Algeria): 36.6 and 60%, respectively [29, 30], but were higher than those reported in several European countries (Italy (9.2%), France (3.4%), Germany (2.7%), Spain (1.02%)) and Morocco (24%) [31–33]. This high prevalence can be attributed to the absence of a *Salmonella* infection-control plan (especially in healthy chicken flocks) [31] and to the poor hygienic state in poultry farms, where *Salmonella* can persist during several grow-outs [34]. Furthermore, our findings are in accordance with those of Gardel et al. (2003), who found that samples taken at the third week of the grow-out to be more contaminated by *Salmonella spp.* than those collected at advanced ages [35]. According to many surveys, contamination of poultry products with *Salmonella* may take place at different stages

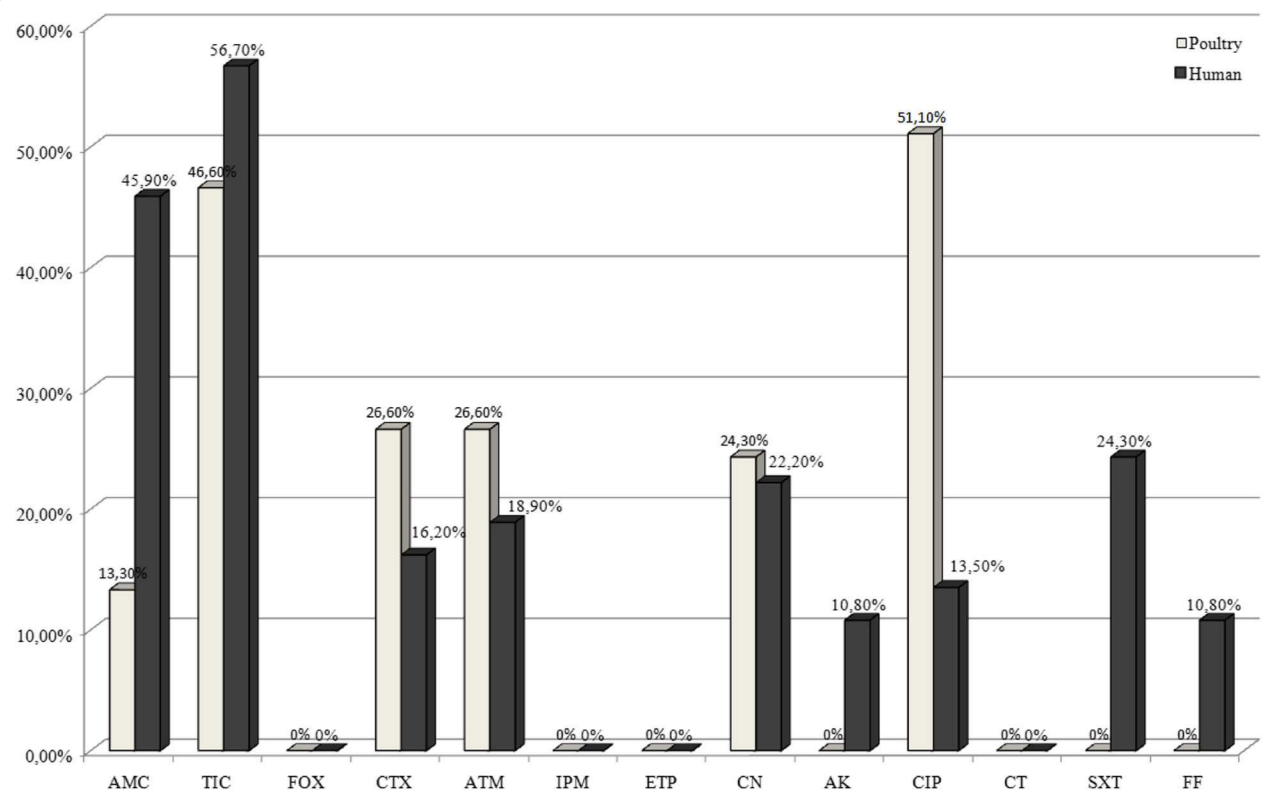

Fig. 1 Distribution of resistance to antibiotics among *Salmonella* isolates from poultry and humans

of the production process [36]. After contamination of the birds at the farm, bacteria colonize their intestines and can infect their carcasses at slaughter [37].

Elgroud et al. [29] found that 73.3% of the poultry slaughterhouses in Constantine were *Salmonella*-positive [29]. Among the avian serotypes isolated in our study, Kentucky was the most predominant. Currently, this serotype is distributed worldwide, especially its ST198 [3]. *Salmonella* ser. Heidelberg has been isolated from broiler carcasses collected at four provinces in the center of Algeria [38]. Despite the fact that we have isolated *Salmonella* ser. Enteritidis in only 9% of the samples, it is worth noting that this serotype is the most common in animal products, especially poultry [31]. Our *Salmonella* strains exhibited a high resistance rate to fluoroquinolones, and interestingly, a Kentucky ciprofloxacin-resistant serotype was isolated in France from a patient who had previously stayed in Algeria [39]. In the present study, we report the presence of ESBLs in *Salmonella* ser. Heidelberg, Senftenberg, Infantis and Newport of avian and human origins, with the CTX-M groups as the most prevalent. To our knowledge, this is the first report of $bla_{\text{CTX-M-1}}$ genes in avian *Salmonella* strains in Algeria. In fact, this group is the principal ESBL type in human *Salmonella* encountered in Europe [9]. CTX-M-15 was identified in all human *Salmonella* strains. This finding corroborates well with several studies performed in Algeria showing that different *Salmonella* serotypes (Heidelberg, Kedougou, Infantis and Enteritidis) isolated from humans harbored this gene [40–43].

The present study had demonstrated the presence of bla_{TEM} genes in one avian *Salmonella* ser. Newport, one avian *Salmonella* ser. Heidelberg strain and all human ESBL-positive strains. Olesen et al. (2004) reported that in Denmark that the major recorded ESBL was the TEM group [44].

In Algeria, cephalosporin use is uncommon in poultry production [22], and the fact that ESBL-positive *Salmonella* strains of avian origin were isolated suggest that these resistant strains may have been introduced into the poultry production chain from other sources, or resulted from the acquisition by avian *Salmonella* strains of ESBL resistance determinants that are generally carried on mobile genetic elements (such as plasmids) [45].

The fact that seven avian and human *Salmonella* ser. Heidelberg strains were of the same ST and clustered together suggests that this clone is circulating in the poultry production chain as well as in the human community. Chicken-to-human transmission of *Salmonella* during farming has been widely demonstrated. It may take place through the food chain [46], and also through occupational exposure from direct contact with live

Table 2 Antimicrobial resistance and resistant genes profiles of ESBLs producing *Salmonella enterica* strains isolated from poultry and humans

Strain ID N°	Origin	Antimicrobial Resistance Pattern	Serotype	B-Lactamase	ST
162	Poultry	TIC, CTX, ATM,	*Heidelberg*	CTX-M-1	15
163	Poultry	TIC, CTX, ATM,	*Heidelberg*	CTX-M-1	15
164	Poultry	TIC, CTX, ATM,	*Heidelberg*	CTX-M-1	15
165	Poultry	TIC, CTX, ATM,	*Heidelberg*	CTX-M-1	15
167	Poultry	TIC, CTX, ATM,	*Heidelberg*	CTX-M-1	15
169	Poultry	TIC, CTX, ATM,	*Newport*	TEM	198
170	Poultry	TIC, CTX, ATM,	*Heidelberg*	CTX-M-1	15
171	Poultry	TIC, CTX, ATM,	*Heidelberg*	CTX-M-1	15
172	Poultry	TIC, CTX, ATM,	*Heidelberg*	CTX-M-1, TEM	15
174	Poultry	TIC, CTX, ATM,	*Heidelberg*	CTX-M-1	15
177	Poultry	TIC, CTX, ATM,	*Heidelberg*	CTX-M-1	15
178	Poultry	TIC, CTX, ATM,	*Heidelberg*	CTX-M-1	15
305	Human	AMC,TIC,CTX, ATM, GEN,FF	*Senftenberg*	CTX-M-15	14
476	Human	AMC, TIC, CTX, ATM,GEN,AK,SXT	*Infantis*	CTX-M-15, TEM	38
883	Human	CTX, ATM, GEN, AK, SXT	*Heidelberg*	CTX-M-15, TEM	15
884	Human	AMC,TIC,CTX, ATM, GEN, AK, SXT	*Heidelberg*	CTX-M-15, TEM	15
YFA	Human	AMC, TIC, CTX,GEN	*Infantis*	CTX-M-1	32
1577	Human	AMC,TIC,CTX, GEN, AK, SXT	*Infantis*	CTX-M-15, TEM	16

AMC Amoxicillin/Clavulanic Acid, *TIC* Ticarcillin, *FOX* Cefoxitin, *CTX* Cefotaxime, *ATM* Aztreonam, *IPM* Imipenem, *ETP* Ertapenem, *GEN* Gentamicin, *AK* Amikacin, *CIP* Ciprofloxacin, *CT* Colistin, *SXT* Trimethoprim/Sulfamethoxazole, *FF* Fosfomycin

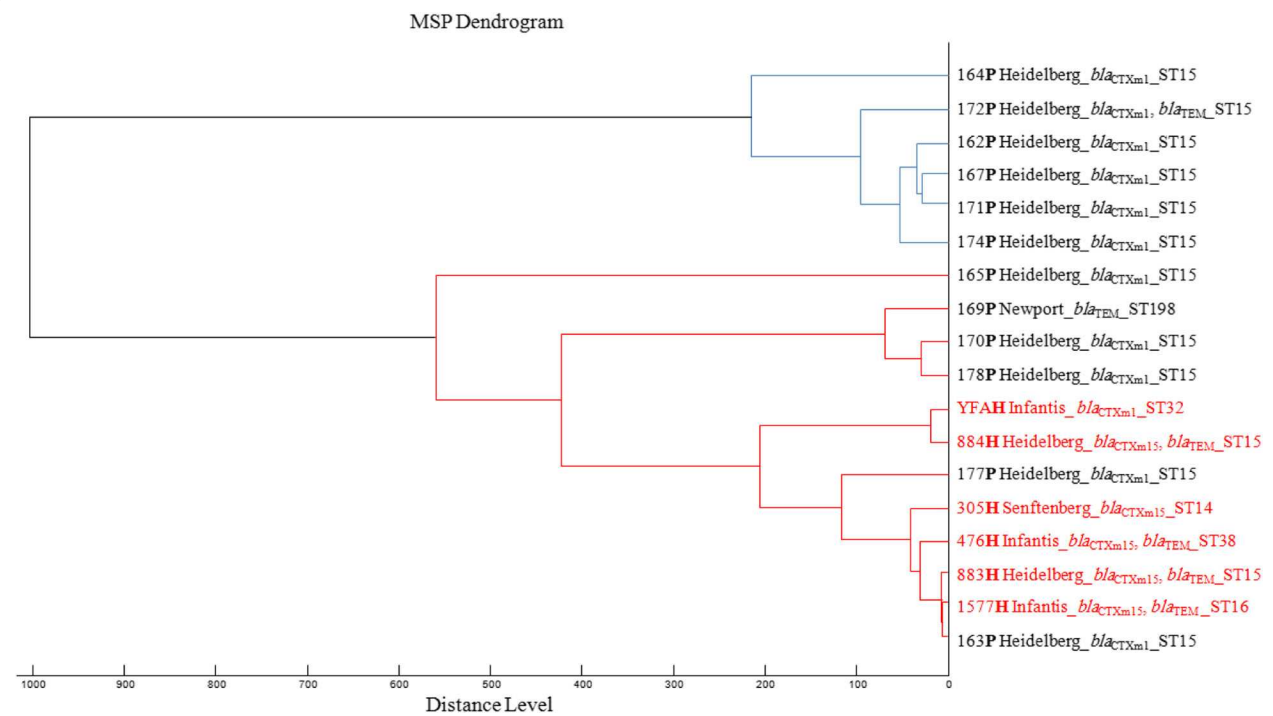

Fig. 2 MALDI-TOF MS-based phylogenetic tree of ESBL-positive *Salmonella enterica* strains from humans (H) and poultry (P)

animals and their environment in the broiler chicken industry [47].

Conclusion

The results of this study demonstrate that *Salmonella* contamination is highly prevalent in broiler poultry farms and slaughterhouses in the region of Skikda (northeastern Algeria), with an increasing resistance to medically important antibiotics. To the best of our knowledge, our results present for the first time, the emergence of ESBL-producing *S. enterica* isolates in poultry in this region. In addition, despite the different sampling times of avian and human *Salmonella* strains, their relatedness has been clearly demonstrated. The clonal relationship between human and avian strains indicates that the poultry industry may act as an important reservoir for ESBL-producing *Salmonella* that are transmitted to humans by direct contact or essentially through the food chain, but more discriminatory typing methods may be able to add more information as to the epidemiology of ESBL-producing *Salmonella* strains in Algeria. Infections caused by multidrug-resistant *Salmonella* species and therapeutic failures increase the risk of death. This is why surveillance programs, rational use of antibiotics and strict biosecurity measures have to be implemented in order to identify the sources, the exact routes of bacterial transmission and to limit the spread of these health-threatening bacteria in the local and national poultry industries.

Additional files

Additional file1: Main characteristics of patients with the corresponding serotype and *Salmonella* strain ID n°. (DOC 77 kb)

Additional file 2: Results of antibiotics sensitivity testing of poultry and human strains. (XLS 39 kb)

Abbreviations

DDST: Double-disc synergy test; ESBLs: Extended spectrum β-lactamases; MALDI-TOF: Matrix-assisted laser desorption/ionization time-of-flight,; MLST: Multilocus sequence typing; PCR: Polymerase chain reaction; PWB: Peptone water broth; ST: Sequence type; UTH: University Teaching Hospital

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Availability of data and materials

The dataset supporting the conclusions of this article is included in the [zenodo database] repository [https://zenodo.org/record/50346], DOI 10–5281/zenodo 50,346, Datasets Open access, License (for files):Creative Commons CCZero.

Authors' contributions

SD has actively worked on the isolation of avian *Salmonella* strains, identification of strains by mass spectrometry and their characterization (antibiotic-susceptibility testing, molecular typing of ESBL genes, sequencing, clonality assessment and Multilocus sequence typing MLST), data interpretation, drafting the paper and revising it. SB performed the data analysis, including sequence checking, Dendrogram of ESBL-positive *Salmonella* strains and revision of the article. BM, RE, and OB conceived and designed the study. AA participated actively in data interpretation, drafting the paper and critically revising it. SH contributed actively to the draft writing and revision of the article. SC participated in strains analysis (Identification by mass spectrometry and PCR characterization of human ESBL-positive *Salmonella* strains). KR contributed in part to the study design and data analysis (Serotyping and antibiotic- susceptibility testing of avian strains). JMR conceived and designed the study of ESBL-producing isolates, the clonality testing, antibiotic-susceptibility testing of the strains and contributed to the draft writing and the revision of the article. All the authors read and approved the article.

Competing of interest

The authors declare that they have no conflicts of interest.

Consent for publication

"Not applicable" in this section.

Ethics approval and consent to participate

Prior to sampling, poultry farmers and poultry slaughterhouse owners were contacted and informed about the purpose and the methods of the study to obtain their consent. All of them agreed to participate to this survey. Live bird swabbing methods were approved by thescientific council of the Institute of Veterinary Sciences (University Frère Mentouri, Constantine, Algeria) and comply with the guidelines for animal care and use in research andteaching.

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Conclusion du chapitre

Ce chapitre s'est intéressé à étudier la prévalence des bactéries productrices de carbapénèmases et de β-lactamases chez les animaux du bassin méditerranéen. Dans une première étude nous avons rapporté une forte prévalence des gènes codants pour les BLSE à travers plusieurs sites en Algérie. Nous pensons qu'une telle prévalence et grande diversité des gènes de résistance aux antimicrobiens chez les animaux est liée à l'utilisation incontrôlée des antibiotiques (**Broens et Geijlswijk., 2018**), ce qui favorise la pression de sélection sur les bactéries porteuses de ces gènes et augmente le risque d'une transmission zoonotique de ces bactéries à l'homme et une dissémination des gènes de résistance (**Scott et al., 2018**). Nous avons montré dans une deuxième étude la relation étroite qui existe entre les souches humaines et aviaires, cela soutient l'hypothèse selon laquelle les animaux en général sembleraient jouer un rôle dans la propagation et le transfert de gènes de résistance à la communauté humaine (**Weese et al., 2015**). D'autant plus, il existe des preuves solides que l'utilisation d'antimicrobiens chez les animaux peut favoriser la résistance aux antibiotiques chez certains agents pathogènes zoonotiques à titre d'exemple la détection de souches de Salmonella enterica serovar Heidelberg résistantes au ceftiofur (céphalosporines) dans la viande de poulet et chez des humains au Canada (**Dutil et al., 2010**).

La restriction d'usage des antibiotiques chez les animaux dans ces zones-là doit s'appliquer en urgence afin de réduire la prévalence des bactéries productrices de β-lactamases et de carbapénèmases chez l'homme et diminuer le risque de transfert de gènes de résistance de l'animal à l'homme (**Phillips et al., 2004**). Il est vrai que la restriction d'usage s'avère délicate en absence d'alternatives thérapeutiques efficaces. Nous pensons alors que la prévention de la survenue des maladies par l'amélioration des conditions d'élevage, la réduction de l'utilisation globale des antimicrobiens et l'amélioration de l'utilisation des antibiotiques chez les animaux

pourraient réduire considérablement les phénomènes d'antibiorésistance chez l'homme et chez l'animal (**Weese et al., 2015**).

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III–DETECTION DE LA RESISTANCE PLASMIDIQUE A LA COLISTINE CHEZ LES ANIMAUX EN ALGERIE (POULETS, BOVINS, OVINS ET CAPRINS).

La résistance aux antibiotiques est devenue une priorité mondiale en matière de santé publique et animale (**Mendelson et al., 2016**). Les polymyxines, une famille d'antibiotiques polypeptidiques cationiques, agissent comme une dernière ligne de défense contre les infections graves dues aux bactéries à Gram-négatif présentant une résistance médicamenteuse (**Mendelson et al., 2018**). Malheureusement, cet antibiotique de dernier recours a été remis en question par l'émergence et la propagation de plusieurs déterminants de la résistance à la colistine (mcr-2, mcr-3, mcr-4, mcr-5) (**Sun et al., 2018**). Étant donné que cela a suscité de nombreuses préoccupations dans le monde entier, nous nous sommes consacrés dans ce chapitre à la caractérisation de l'émergence et de l'épidémiologie de la dissémination de la résistance plasmidique à la colistine chez les animaux en Algérie, particulièrement chez les poulets, les bovins, les ovins et les caprins.

Nos travaux sur la résistance plasmidique à la colistine chez les animaux étaient motivés par les résultats de **Liu et al**., 2016. Simultanément, avec la première détection mondiale de la résistance plasmidique mcr-1 en Chine, et grâce à notre possession d'une collection de près de 1000 échantillons de fientes de poulets d'Algérie, collectés par nous même à partir du mois d'Aout 2014 sur différentes régions et étages bioclimatiques, nous avons décrit rapidement la première dissémination de mcr-1 en Afrique (Algérie). Dans cette étude (**Article N° 5)** nous avons rapporté la première description et dissémination en Algérie et en Afrique de ce gène qui code pour la résistance plasmidique à la colistine chez les poulets. Dans un premier temps nous avons utilisé dans notre travail les amorces de PCR standard publiées dans l'article de **Liu et al., 2016** sur une collection de souches d'E. coli résistantes à la colistine isolées des matières fécales d'humains du Laos, de Thaïlande, du Nigéria et de France ainsi que sur des souches isolées de fèces de porc du Laos et des fientes de poulets d'Algérie. Les résultats obtenus ont permis de détecter la présence de mcr-1 dans des souches E. coli d'humains du Laos et de Thaïlande ainsi que dans les E. coli de porcs du Laos et de poulets d'Algérie (**Article N° 5**).

Par la suite, nous avons jugé nécessaire de designer des systèmes de détection rapide, par PCR en temps réel. Nous avons rapporté dans une étude (**Article N° 6)** le développement de deux systèmes de PCR quantitative en temps réel avec des sondes TaqMan® pour détecter le gène de résistance à la colistine (mcr-1) à partir d'isolats bactériens et d'échantillons de fiente de poulets. La spécificité et la sensibilité des systèmes étaient testées sur des isolats bactériens incluant 18 isolats résistants à la colistine portant le gène mcr-1 (6 Klebsiella pneumoniae et 12 E. coli). Cinq des 833 échantillons de fèces provenant de poulets d'Algérie étaient positifs, parmi lesquelles trois souches d'E. coli ont été isolées et confirmées comme porteuses du gène mcr-1 par PCR standard et séquençage. L'ensemble de ces résultats a fait l'objet de ***l'article* N° 6**, et les systèmes de détection rapide mis en place dans cette étude sont utilisés actuellement en routine dans notre laboratoire que ce soit pour les échantillons d'origine animale ou humaine.

Nous avons ensuite utilisé ces systèmes de PCR en temps réel dans plusieurs études rétrospectives portant sur la détection de ce gène à partir d'isolats humains et animaux. En août 2016, 120 prélèvements de fientes de poulets ont été collectés dans trois régions d'Algérie : deux abattoirs à Alger (n = 79), un élevage de poulets à Blida (n = 22) et un autre élevage de poulets à Souk Ahras (n = 19). Au total, 25 des 120 prélèvements de poulets (20,8%) étaient positifs pour le gène mcr-1 (21/79 à Alger et 4/22 dans les fientes de poulets à Blida). L'ensemble de ces résultats a été soumis au journal Multi Drug Resistance (**Article N° 7)**.

Par la suite, nous nous sommes fixés comme objectif de tracer une carte qui résume la dissémination de mcr-1 en Algérie et d'identifier d'autres animaux réservoirs potentiels de bactéries porteuses de mcr-1. Dans cette dernière étude (**Article N° 8)** nous avons travaillé sur une collection de 20 fèces de bovins, 20 fèces d'ovins, 20 fèces de caprins et 20 échantillons d'écouvillonnage de surface d'une ferme d'élevage à El Tarf, Algérie, dont nous avons testé l'occurrence de mcr-1 dans ces échantillons. Parmi ces échantillons, 8 souches d'E. coli

porteuses du gène mcr-1 ont été isolées à partir de 4 fèces de caprins, 2 de bovins et 2 souches de l'écouvillonnage de la surface d'une étable ainsi que 2 souches Enterobacter cloacae à partir de la surface d'une étable.

ARTICLE 5

Title: Dissemination of the mcr-1 colistin resistance gene.

Abiola Olumuyiwa Olaitan, Selma Chabou, Liliane Okdah, Serge Morand and Jean Marc Rolain. Lancet Infect Dis. 2016 Feb;16 (2):147. doi: 10.1016/S1473-3099(15)00540-X.

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1 Liu Y-Y, Wang Y, Walsh RT et al. Emergence of plasmid-mediated colistin resistance mechanism MCR-1 in animals and human beings in China: a microbiological and molecular biological study. *Lancet Infect Dis* 2015. http://dx.doi.org/10.1016/S1473-3099(15)00424-7.
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4 Hu Y, Yang X, Qin J et al. Metagenome-wide analysis of antibiotic resistance genes in a large cohort of human gut microbiota. *Nat Commun* 2013; **4:** 2151.
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In their Comment on the Article by Yi-Yun Liu and colleagues about the emergence of plasmid-mediated colistin resistance involving the *mcr-1* gene from bacteria isolated in China,[1] David Paterson and Patrick Harris[2] referred to our finding of colistin resistance in two *Escherichia coli* isolates from a pig and a human being in Laos that were indistinguishable by pulsed-field gel electrophoresis.[3] Our results, suggested animal to human transmission for which no known chromosomally encoded colistin resistance mechanisms were identified, raising the question of a similar mechanism of colistin resistance to that identified by Liu and colleagues. We screened for the presence of the *mcr-1* gene in these isolates along with other colistin-resistant *E coli* isolates from several countries (Laos, Thailand, France, and Nigeria) from human beings and pigs (only in Laos)[4] and in poultry from Algeria using PCR and sequencing as described by Liu and colleagues (table).[1] We included as controls a colistin-susceptible revertant *E coli* strain (obtained at the 18th passage in colistin-free medium) from a pig[3] and 12 colistin-susceptible *E coli* strains isolated from the same stool samples from which the colistin-resistant *E coli* strains were isolated. 12 (63%) of 19 colistin-resistant *E coli* strains tested were positive for the *mcr-1* gene and sequences were 100% identical to that of *mcr-1* gene sequence reported by Liu and colleagues (table).[1] These include *E coli* strains from Laos (from asymptomatic people and pigs including the strain transferred from a pig to a farmer), Thailand (asymptomatic people), and from Algeria (chickens). None of the 12 colistin-susceptible *E coli* strains and the colistin-susceptible revertant strain (the parent resistant strain was positive for *mcr-1*) were positive for *mcr-1*. The susceptible revertant isolate has probably lost the resistance by loss of a plasmid that is under investigation. We clearly show that plasmid-mediated colistin resistance has spread beyond China to the neighbouring southeast Asian countries and even further to Africa where colistin is widely used in animal production. We anticipate that with the extensive use of colistin in animal production, including in Europe,[5] plasmid-mediated colistin resistance has already spread worldwide, and this calls for prompt international action to restrict or ban the use of colistin in agriculture to avoid further spread of resistance, as occurred with NDM-1 5 years ago.

We declare no competing interests.

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	Colistin minimum inhibitory concentration (mg/L)	Year	Source	Country	*mcr-1*
LH30	6	2012	Human	Laos	+
LH57	8	2012	Human	Laos	+
LH1	6	2012	Human	Laos	+
LH121	16	2012	Human	Laos	+
LH140	12	2012	Human	Laos	+
LH257	12	2012	Human	Laos	+
P10	6	2012	Pig	Laos	+
P6	6	2012	Pig	Laos	+
P17	4	2012	Pig	Laos	+
P7	4	2012	Pig	Laos	-
TH176	6	2012	Human	Thailand	-
TH214	6	2012	Human	Thailand	+
TH99	4	2012	Human	Thailand	+
FHM19*	12	2012	Human	France	-
FHA102†	12	2012	Human	France	-
FHA113‡	12	2012	Human	France	-
NH94§	12	2012	Human	Nigeria	-
235	4	2015	Chicken	Algeria	+
249	3	2015	Chicken	Algeria	-

Mutations in PmrB sensor kinase of the two-component system: *Pro7_Gln12del (deletion of 6 aminoacids); †Ala159Val; ‡Thr156Lys; and §Iso92 ins (insertion of isoleucine at position 92).

Table: **Colistin-resistant *Escherichia coli* with the associated *mcr-1* gene isolated from different sources**

3 Olaitan AO, Thongmalayvong B, Akkhavong K, et al. Clonal transmission of a colistin-resistant *Escherichia coli* from a domesticated pig to a human in Laos. *J Antimicrob Chemother* 2015; **70:** 3402–04.
4 Olaitan AO, Morand S, Rolain JM. Emergence of colistin-resistant bacteria in humans without colistin usage: a new worry and cause for vigilance. *Int J Antimicrob Agents* (in press).
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Plasmid-mediated transferable colistin resistance encoded by the *mcr-1* gene was described in *Escherichia coli* and *Klebsiella pneumoniae* isolates from pigs and chicken at a prevalence of around 20%, and in clinical isolates from human beings at a prevalence of around 1% in China.[1] The prevalence of the *mcr-1* gene in Enterobacteriaceae in other countries and in the community is unknown.

We did a prospective study of acquisition of fecal colonisation and carriage with extended-spectrum

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ARTICLE 6

Title: Real-time quantitative PCR assay with Taqman (®) probe for rapid detection of MCR-1 plasmid-mediated colistin resistance.

Selma Chabou, Thongpan Leangapichart, Liliane Okdah, Stéphanie Le Page, Linda Hadjadj and Jean Marc Rolain.

New Microbes New Infect. 2016 Jul 5;13:71-4. doi: 10.1016/j.nmni.2016.06.017. eCollection 2016 Sep.

Real-time quantitative PCR assay with Taqman® probe for rapid detection of MCR-1 plasmid-mediated colistin resistance

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Abstract

Here we report the development of two rapid real-time quantitative PCR assays with TaqMan® probes to detect the MCR-1 plasmid-mediated colistin resistance gene from bacterial isolates and faecal samples from chickens. Specificity and sensitivity of the assay were 100% on bacterial isolates including 18 colistin-resistant isolates carrying the *mcr-1* gene (six *Klebsiella pneumoniae* and 12 *Escherichia coli*) with a calibration curve that was linear from 10^1 to 10^8 DNA copies. Five out of 833 faecal samples from chickens from Algeria were positive, from which three *E. coli* strains were isolated and confirmed to harbour the *mcr-1* gene by standard PCR and sequencing.

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Keywords: Antibiotic resistance surveillance, colistin resistance, *mcr-1* gene, PCR detection, Taqman probe

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Introduction

The increasing prevalence of infections caused by multidrug-resistant Gram-negative bacteria combined with few antimicrobial agents being in development has led to a resurgence in interest in colistin as a last-line therapy with the inevitable risk of emerging resistance [1–3]. MCR-1 plasmid-mediated colistin resistance is a member of the phosphoethanolamine transferase enzyme family, with expression in *Escherichia coli* resulting in the addition of phosphoethanolamine to lipid A and resistance to colistin [4]. This plasmid-mediated colistin resistance is an emerging concern that has already spread worldwide [5] in *E. coli* and *Klebsiella pneumoniae* from pigs, chicken, retail meat (pork, chicken), humans [4]. In animal health, colistin is used to prevent infections from *E. coli* isolates that are known to cause serious adverse effects such as diarrhoea, sepsis and colibacillosis, which result in huge economic losses [6]. The extensive use of antibiotics in food-animal production has been shown to increase the risk of transferring resistant bacteria to humans [7].

There is a need to screen for colistin resistance even in patients without a history of colistin usage for the timely detection and isolation of patients harbouring such resistant strains to prevent clonal transmission [8]. For this reason the aim of this study was to develop rapid real-time quantitative PCR (qPCR) to detect the MCR-1 plasmid-mediated colistin resistance and to evaluate its sensitivity and specificity both from strains and stool samples.

Materials and methods

Specific primers and probes design

Primers and probes design. We designed specific primers and probes to develop two real-time qPCR assays (PE1 and PE2) for the detection of MCR-1-encoding gene (Table 1). Specificity of the primers and probes were verified *in silico* by BLASTN analysis on the National Center for Biotechnology Information (NCBI) database.

Sample collection

Bacterial strains. A total of 100 strains from humans and animals were used in this study including 18 colistin-resistant isolates carrying the *mcr-1* gene (six *K. pneumoniae* and 12 *E. coli*).

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TABLE 1. Primers and probe designed to target the plasmid-mediated colistin resistance (MCR-1)

Primer/probe name	Sequence	PCR product size (bp)	References
Real-time PCR			
PE_F1	GCAGCATACTTCTGTGTGGTAC	145	This study
PE_R1	ACAAAGCCGAGATTGTCCGCG		
PE_Probe 1	6 FAM –GACCGCGACCGCCAATCTTACC-TAMRA		
PE_F2	GGGTGTGCTACCAAGTTTGCTT		
PE_R3	TATGCACGCGAAAGAAACTGGC		
PE_Probe	6 FAM –GCGCTGATTTTACTGCCTGTGGTG-TAMRA		
Standard PCR			
PE_F1	GCAGCATACTTCTGTGTGGTAC	554	This study
PE_R3	TATGCACGCGAAAGAAACTGGC		
CLR5-F	5′-CGGTCAGTCCGTTTGTTC-3′		[4]
CLR5-R	5′-CTTGGTCGGTCTGTA GGG-3′		

TABLE 2. Presentation of strains of the study with the genes specificity

Species	Presence of gene MCR-1	COL MIC (mg/L)	Genes specificity	CT value with PE1 system	CT value with PE2 system	Origins	References
Escherichia coli (*n* = 25)	+ (*n* = 12) − (*n* = 13)	4–16 <1–16	None	18–25 0	19–25 0	Thailand, Laos, Algeria, France, Nigeria.	[5,7] unpublished data
Klebsiella pneumoniae (*n* = 33)	+ (*n* = 6) − (*n* = 27)	4–32 <1–32	*mgrB** (*n* = 2) pmrB*(*n* = 1) *mgrB** (12)	18–24 0	19–25 0	Thailand, Laos, France, Nigeria, Algeria	[9] unpublished data
Klebsiella oxytoca (*n* = 2)	–	6–12	*mgrB** (*n* = 1)	0	0		[9]
Salmonella enterica subsp. *enterica* (*n* = 5)	–	0.125–16	pmrB* (*n* = 2) $bla_{CTX-M-2}$ (*n* = 5)	0	0	France	[10]
Pseudomonas aeruginosa (*n* = 10)	–	<1	bla_{VIM-2} (*n* = 10)	0	0	Lebanon	[11]
Acinetobacter baumannii (*n* = 10)	–	<1	bla_{OXA23} (*n* = 2), bla_{OXA24} (*n* = 2), bla_{OXA58} (*n* = 1), bla_{VEB} (*n* = 1)	0	0	Algeria	[12–14]
Providencia rettgeri (*n* = 2)	–	>256	bla_{NDM-1} (*n* = 1)	0	0	Israel	[15]
Morganella morganii (*n* = 2)	–	>256	bla_{NDM-1} (*n* = 1)	0	0	Israel	[16]
Enterobacter cloacae (*n* = 5)	–	<1	None	0	0	Laos, Nigeria.	unpublished data
Proteus mirabilis (*n* = 2)	–	>256	None	0	0	Algeria	unpublished data
Proteus vulgaris (*n* = 2)	–	>256	None	0	0	Algeria	unpublished data
Serratia marcescens (*n* = 2)	–	>256	None	0	0	Algeria	unpublished data

* Mutation; +, positive; −, negative.
COL MIC, minimum inhibitory concentration of colistin.

Phenotypic and genotypic features of these strains are summarized in Table 2.

Chicken stool collection. A total of 833 faeces samples from broilers were collected between August and February 2015 from eight regions in Algeria (El Tarf, Souk Ahras, Skikda, Setif, Jijel, Algiers, Biskra and Ourgla; *n* = 503) and in three slaughterhouses in Marseille (*n* = 330). All the extracted DNA from the 833 faeces of broilers was tested using our qPCR assay and positive samples were inoculated on agar for isolation of positive *mcr-1* isolates.

Molecular analysis

Strategy for PCR amplification and sequencing. Standard PCR amplification and sequencing of the MCR-1-encoding gene was used as the gold standard and performed as previously described [4]. Quantification of the MCR-1-encoding gene using our two sets of primers and probes was performed using a quantitative CFX96™ Real Time system C1000™ Touch thermal cycler (Bio-Rad, Singapore). The qPCR conditions were as follows: the reaction mixtures were kept at 95°C for 15 min and subsequently put through 35 cycles of 95°C for 30 s and 60°C for 1 min.

Specificity and reproducibility of the new system of real-time PCR. The specificity of the primers and probes were verified *in vitro* using our local collection of 100 strains (Table 2). The sensitivity of our assays was determined using ten-fold serial dilutions (between 10^8 and 10^1 DNA copies) of *E. coli* strain P10 by triplicate amplification, the number of *mcr-1* in each sample was calculated based on the DNA copy numbers. The obtained Ct values were used to generate the calibration curves compared with the number of bacteria quantified by standard bacterial count on agar plates. The standard curve was

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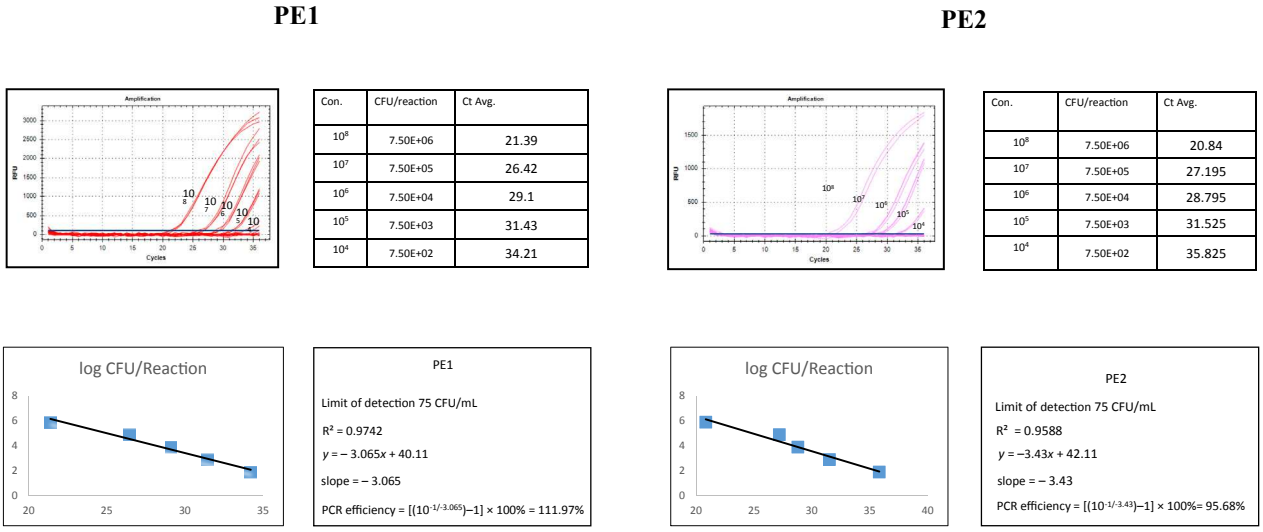

Con.	CFU/reaction	Ct Avg.
10^8	7.50E+06	21.39
10^7	7.50E+05	26.42
10^6	7.50E+04	29.1
10^5	7.50E+03	31.43
10^4	7.50E+02	34.21

Con.	CFU/reaction	Ct Avg.
10^8	7.50E+06	20.84
10^7	7.50E+05	27.195
10^6	7.50E+04	28.795
10^5	7.50E+03	31.525
10^4	7.50E+02	35.825

FIG. 1. Real-time PCR sensitivity test to detect -encoding plasmid-mediated colistin resistance (MCR-1) encoding gene from *Escherichia coli* strain P10. PE1 and PE2 are two quantitative PCR assays developed.

constructed on the basis of the concordance between Ct values and number of log CFU/mL. The limit of detection was based on the final dilution detected by PCR. Efficacy of qPCR was calculated from a standard curve according to Rutledge and Cote [17].

Results

Specificity and technical sensitivity of the qPCR

BLASTN analysis of the primers and probe designed for the development of the real-time PCR assay showed *in silico* a 100% homology with the MCR-1-encoding gene only. The sensitivity of the real-time PCR using the 18 *mcr-1*-positive strains using serial ten-fold dilutions of a calibrated inoculum was excellent with a calibration curve that was linear from 10^1–10^8 DNA copies corresponding to 35–21 Ct (Fig. 1). Regression formulae and PCR efficiency of the two real-time PCR assays are shown in Fig. 1. The reproducibility of the two qPCR assays was excellent, with a positive PCR at 21.4 ± 0.4 Ct for PE1 and 20.8 × 0.4 Ct for PE2 when testing one colony re-suspended in 200 μL of sterile water (Table 2). The specificity of the two qPCR assays *in vitro* against a panel of 82 clinically relevant bacteria negative for *mcr-1* gene was 100% (all real-time PCR were negative, Table 2).

Screening of faeces from broilers

Five of the 503 faecal samples from chickens from Algeria were positive, from which three *E. coli* strains were isolated and confirmed to harbour the *mcr-1* gene by standard PCR and sequencing. None of the 330 samples from France were positive.

Discussion

The recent description and emergence of MCR-1 plasmid-mediated resistance to colistin in humans and animals is a major concern worldwide [5]. In this study, two new qPCR assays using Taqman probes were developed that demonstrate high sensitivity and specificity for confirmation of the presence of this gene in colistin-resistant bacterial isolates as well as for screening directly from stool samples. Indeed both systems have the same performance to screen for the presence of MCR-1-containing isolates and in stools. We recommend the use of PE1 as a first set of primers for the rapid screening of *mcr-1* and PE2 system to confirm the positive results. Recently, Bontron *et al.* have reported a real-time PCR assay using SYBR green as fluorescent marker with similar sensitivity [18]. However it is well known that Taqman probes enhance specificity, which is a critical point when testing directly from biological samples. Our real-time PCR assays had advantages including sensitivity, specificity and the possibility of detecting MCR-1 plasmid-mediated colistin resistance very quickly (<2 h). We believe that these real-time PCR assays would be important and powerful tools that could be implemented easily in clinical microbiological laboratories that have molecular facilities, including at point of care, for identification of MCR-1 and implementation of healthcare policies.

Conflict of interest and financial disclosure

There is no potential conflict of interest or financial disclosure for all authors.

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ARTICLE 7

Title: Emergence of mcr-1 mediated colistin resistance in Escherichia coli isolates from chickens, Algeria.

Selma Chabou, Hamza Leulmi and Jean Marc Rolain.

Under review in Multi Drug Resistance Journal (MDR-2018-0091).

Microbial Drug Resistance
Mechanisms, Epidemiology, and Disease

Microbial Drug Resistance: http://mc.manuscriptcentral.com/mdr

Emergence of mcr-1 mediated colistin resistance in Escherichia coli isolates from chickens, Algeria.

Journal:	*Microbial Drug Resistance*
Manuscript ID	MDR-2018-0091
Manuscript Type:	Epidemiology
Date Submitted by the Author:	17-Mar-2018
Complete List of Authors:	Chabou, Selma; Aix-Marseille Univ., IRD, APHM, MEPHI, IHU Méditerranée Infection, Marseille, France Leulmi, Hamza; UMR VITROME, Aix-Marseille Université, IRD, Service de Santé des Armées, Assistance Publique-Hôpitaux de Marseille, IHU Méditerranée-Infection, 19-21 Boulevard Jean Moulin, Marseille, 13005, France. Rolain, Jean-Marc; URMITE CNRS-IRD-INSERM, Faculté de Pharmacie
Keyword:	E. Coli, Plasmid, Antimicrobial
Manuscript Keywords (Search Terms):	MCR 1, poultry, colistin, Algeria
Abstract:	The newly identified plasmid-mediated colistin resistance gene, mcr-1, triggered an avalanche of retrospective studies investigating the presence of this specific gene from various isolates including foods, animals and humans. In August 2016, 120 fecal samples were collected in three regions of Algeria: two slaughterhouses in Algiers (n=79), one broiler chicken farm in Blida (n=22), and one broiler chicken farm in Souk Ahras (n=19). Thanks to molecular biology techniques; qPCR, standard PCR, MLST and sequencing, we investigated the presence of the colistin resistance genes (mcr-1, mcr-2, mcr-3). Overall, 25 out of 120 (20.8%) poultry samples were PCR positive for the mcr-1 gene [(21/79 (26.6%) in Algiers and 4/22 (18.2%) in Blida], while mcr-2 and mcr-3 were not detected. The twenty-five mcr-1 positive samples were incubated in LBJMR for isolation of colistin resistant bacteria. Eight E. coli (7/79 from Algiers and 1/22 from Blida) out of twenty-five mcr-1 positive samples were isolated. They were confirmed to harbor mcr-1 gene by PCR and sequencing (sequences were 100% identical to that of the mcr-1 gene sequence reported by Liu and colleagues). Our results demonstrate that using colistin in poultry has led to dissemination of mcr-1colistin resistant gene in this country which could be later transmitted to humans.

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Title: Emergence of *mcr-1* mediated colistin resistance in *Escherichia coli* isolates from chickens, Algeria.

Author : Selma CHABOU[1], Hamza LEULMI [1,2] and Jean-Marc ROLAIN[1]*

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Abstract = 203

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Number of references = 30

Table = 1

Figure = 2

Keywords: *mcr-1*, poultry, colistin, Algeria.

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Abstract

The newly identified plasmid-mediated colistin resistance gene, *mcr-1*, triggered an avalanche of retrospective studies investigating the presence of this specific gene from various isolates including foods, animals and humans.

In August 2016, 120 fecal samples were collected in three regions of Algeria: two slaughterhouses in Algiers (n=79), one broiler chicken farm in Blida (n=22), and one broiler chicken farm in Souk Ahras (n=19). Thanks to molecular biology techniques; qPCR, standard PCR, MLST and sequencing, we investigated the presence of the colistin resistance genes (*mcr-1*, mcr-2, mcr-3).

Overall, 25 out of 120 (20.8%) poultry samples were PCR positive for the *mcr-1* gene [(21/79 (26.6%) in Algiers and 4/22 (18.2%) in Blida], while *mcr-2* and *mcr-3* were not detected. The twenty-five *mcr-1* positive samples were incubated in LBJMR for isolation of colistin resistant bacteria. Eight *E. coli* (7/79 from Algiers and 1/22 from Blida) out of twenty-five *mcr-1* positive samples were isolated. They were confirmed to harbor *mcr-1* gene by PCR and sequencing (sequences were 100% identical to that of the *mcr-1* gene sequence reported by Liu and colleagues). Our results demonstrate that using colistin in poultry has led to dissemination of *mcr-1*colistin resistant gene in this country which could be later transmitted to humans.

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Introduction

The polymyxin E (colistin) and polymyxin B are polycationic antimicrobial peptides. They are considered as the last-line antibiotic in the treatment of Gram-negative bacterial infections [1].

The increase and uncontrolled use of colistin especially in agriculture and animals has led to the emergence of Gram negative bacteria resistant to colistin [2]. Chromosomal resistance to colistin is one of the already known mechanisms. The newly identified plasmid-mediated colistin resistant gene (*mcr-1*) that has been reported worldwide in Gram negative bacteria is increasingly reported and is known to spread easily at least among Enterobacteriaceae [3]. This newly identified resistance gene, phospho-ethanolamine transferase gene, triggered an avalanche of retrospective studies investigating the presence of this specific gene from various isolates[4,5], including livestock animals [27] and humans [4,5]. Furthermore, this plasmid has been founded in food products [6], companion animals [7,8], and environment [9,10]. Recently, other *mcr* variants, including *mcr*-2/3/4/5 have been added to the list of the phospho-ethanolamine transferase genes causing resistance to colistin among Enterobacteriaceae [11,13]. The *mcr-2* gene that has 76,7% nucleotide sequence identity with *mcr-1* gene was reported firstly in pigs and bovines from Belgium [14]. The third mobile colistin resistance gene, *mcr-3* (45% nucleotide identity with *mcr-1*) was reported in *E. coli* isolate from pigs of Malaysia [13]. *Yin et al*. also identified similar elements in a human *Klebsiella pneumoniae* isolated from Thailand and a human *Salmonella enterica serovar Typhimurium* isolated from the United States [13]. Afterwards, the co-existence of two plasmid-mediated colistin resistance genes, *mcr*-1 and *mcr*-3.2 was detected in the same strain isolated from bovine samples in Spain [15].

The aim of the present study is to evaluate the prevalence and/or occurrence colistin resistance genes presence (*mcr-1, mcr-2, mcr-3*) in the fecal samples from chicken of Algeria, a country where colistin is widely used in animal breeding [6].

Materials and Methods

Study areas

In August 2016, fecal chicken samples were collected in various farms and slaughterhouses of Bilda, Souk Ahras and Algiers. Samples were harvested directly from the chicken cloacae and were placed in single sterile and identified tubes. All samples were conserved at -80° and sent to Marseille, France, prior to their phenotypic and genotypic analysis.

Molecular characterization of *mcr-1, mcr-2 and mcr-3*:

Once samples had been collected, DNA extraction was carried out using an automatic robot EZ1 (Qiagen BioRobot EZ1, Tokyo, Japan), using the extraction kit (EZ1 DNA, Qiagen, Hilden, Germany), following the manufacturer's instructions. The extracted DNA was used as a template for double quantitative real-time PCR assays, using the PE1 and PE2 systems targeting *mcr-1* encoding genes [16], and *mcr-2* and *mcr-3* as described [17]. The real time PCR reaction mixture consisted of 5 µl of DNA and 15µl of mix from the Takyon PCR Kit (Qiagen, Hilden, Germany). All real-time PCRs were performed using the CFX96^{Tm} Real Time system C1000^{Tm} Touch thermal cycler (Bio-Rad, Singapore). Results were deemed positive if the Cycle threshold (Ct) value obtained by CFX96^{Tm} was lower than 35. Standard PCR amplification and sequencing were used to confirm the presence of *mcr* genes. Positive PCR products were purified and sequenced using a Big Dye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA). The sequences obtained were assembled using ChromasPro 1.7 (Technelysium Pty Ltd., Tewantin, Australia) and compared with *mcr* sequences found in the GenBank database using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

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Strains isolation and identification

Regarding selection of bacteria carrying *mcr-1* gene, 1 gram of each PCR positive sample was plated on Tryptic Soy Broth (TSB) medium (BioMérieux, Marcy l'Étoile, France) and incubating for 24 h at 37 °C. After 24h of incubation, 100 µL of TSB were plated for 24 hours at 37°C on the LBJMR medium containing colistin [18].

Isolated colonies from each sample were identified by matrix-assisted laser desorption and ionization time-of-flight mass spectrometry (MALDI-TOF) using the Microflex LT spectrometer (Bruker Daltonics, Bremen, Germany). The spectra were analyzed by the MALDI Biotyper 2.0 (Bruker) program, using the standard configuration for bacteria identification, that compared the strains' spectrum to the database references.

Antimicrobial susceptible testing

Antibiotic susceptibility was determined on Mueller–Hinton agar using the standard disc diffusion procedure as recommended by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (www.eucast.org). The following 16 different antibiotics were tested: amoxillin, amoxillin clavulanic acid, piperacillin tazobactam, cefalotin, ceftriaxone, cefepime, ertapenem, imipenem, amikacin, gentamicin, ciprofloxacin, fosfomycin, nitrofurantoin, doxycycline, trimethoprim-sulfamethoxazole, and colistin. Minimum inhibitory concentrations (MICs) of colistin was determined by using the e-test and broth microdilution method. The microdilution plates were incubated at 37 °C for 24h. The results were interpreted according to EUCAST (www.eucast.org).

DNA sequencing and Multi-Locus Sequence Typing (MLST)

For each colistin-resistant isolate, the presence of *mcr*-1 was tested by qPCR and was confirmed by standard PCR , prior to their sequencing, as described previously [16].

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MLST was determined based on amplification and sequencing of seven housekeeping genes (adk, fumC, gyrB, icd, mdh, purA and recA) and ST was attributed according to MLST database (http://mlst.warwick.ac.uk/mlst/dbs/Ecoli).

Conjugation assay

Strains were tested for their ability to transfer resistance. Conjugation was tested with azide-resistant *E. coli J53*. Transconjugants were selected on MacConkey agar (Beckton Dickinson, Le Pont de Claix, France) supplemented with 120 µg/mL of sodium azide and 4 µg/mL of colistin, as described [19].

Quantification of colistin used in livestock animals from Algeria

The Oral solution of colistin used in livestock animals from Algeria was tested by MALDI-TOF. A solution of colistin has been prepared (water with 0.45% NaCl to solubilize the antibiotics with a concentration of 1 mg / ml). The solution was put on a MALDI-TOF target plate covered with 1 µl of matrix solution [(0.01 g of α-HCCA [alpha-cyano-4-hydroxycinnamic acid] in 1 ml d'acétone pure (100/%) with 1 µl of 100% pure trifluoroacetic acid)]. The spots were crystallized by air drying at room temperature for 15 minutes, then introduced in the mass spectrometer. The plate uses MTP AnchorChip™ 384 TF (Bruker Daltonik GmbH).

Results

In 2016, One hundred and twenty samples of poultry feces were collected in three regions of Algeria: two slaughterhouses in Algiers (n=79), one broiler chicken farm in Blida (n=22), and one broiler chicken farm in Souk Ahras (n=19). Using qPCR, 25 out of 120 (20.8%) poultry samples from Algeria were positive for the *mcr-1* gene [(21/79 (26.6%) in Algiers and 4/22 (18.2%) in Blida] by qPCR assay. We confirmed the 25 positive samples with a second qPCR system (**Table 1**). All fecal samples from Souk Ahras were negative. Neither *mcr*-2 nor *mcr*-3 was detected in those samples.

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The twenty-five positive samples were incubated in LBJMR. Eight *E. coli* (7/79 from Algiers and 1/22 from Blida) out of twenty-five *mcr*-1 positive samples were isolated. They were confirmed to harbor the *mcr-1* gene by PCR. The obtained sequences were 100% identical to the *mcr-1* gene sequence reported by Liu and colleagues [3].

Disk diffusion susceptibility tests showed that a total of eight *E. coli* strains were resistant to colistin and amoxicillin but also sensitive to all antibiotic tested. Minimum inhibitory concentrations (MICs) of colistin confirmed *E. coli* resistance with MIC ranging from 3 to 4 mg / l. The analysis of the phylogenetic showed that the eight *E.coli* isolates belong to the same genotype (ST 48) which has already been reported in Algeria[20]. The transconjugation results showed that the plasmid of these strains was not conjugative. Finally, the oral solution of colistin, often used in chickens and livestock in Algeria, was confirmed by Maldi TOF (**Fig 2**).

Discussion

Our results highlighted a high prevalence of *Escherichia coli* harboring *mcr-1* gene isolated from the poultry in Algeria. Recently, *El Garcha* et al. identified *mcr*-1-like gene in 68 (0.7%) *E. coli* and 2 (0.1%) *Salmonella* isolates[21]. These strains were isolated from food-producing animals at slaughterhouse (cattle, pig and chicken) from 11 European countries [21]. Commercial chicken meat in Brazil was also identified as a reservoir for isolates harboring *mcr-1* [22]. The most *E. coli isolated* carried *IncX4* plasmids, which were identified previously in human and animal isolates [22]. In China, studies have shown high levels of mcr-1 in animals [7,22]. They guessed that the use of colistin in animal as growth promoter has probably accelerated this emergence [24]. Subsequently, the Chinese government has implemented the revocation of colistin's use as a growth promoter in April 2017 [24]. Recently, *Yassin* et al. founded high prevalence of *mcr-1* in extra-intestinal *E. coli* from food-producing animals in China, and suggest that *mcr-1*-positive bacteria are associated with poultry and livestock [17].

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Few studies have studied colistin resistance in *E. coli* from animals in Algeria, therefore, few data is available on their colistin susceptibility, as well as the use of colistin in veterinary medicine. This study provides a comprehensive data on the prevalence of colistin resistance genes in fecal samples from poultry in Algeria. In this paper we clearly prove the emergence and the high prevalence (20.3%) of the *mcr-1* plasmid-mediated colistin resistant gene in *E. coli* isolates from poultry in Algeria. We have confirmed our previous results (the first detection of *mcr-1* in chicken feces from Skikda, East of Algeria) [25], when we have detected only one *E. coli* harboring *mcr-1* out of 2 colistin resistant *E. coli*. This plasmid was also detected in *E. coli* from human samples in two hospitals in Algeria [26,27]. In both studies, MLST indicated that the two *E. coli* isolates belonged to the same type of sequence, (ST405) **(Fig 1)**. Recently, *mcr-1* colistin resistant *E. coli* ST405 was also isolated from wildlife in Bejaia, Algeria [28]. This isolate also harbored the *mcr-1*, $bla_{CTX\text{-}M\text{-}15}$, $bla_{TEM\text{-}1}$, and *qnrB19* genes [28]. These results highlight the potential consequences of the *mcr-1* gene spreading from food production animals to humans, whereby the need for a better control of antimicrobial drug in animal production. Recently, in Vietnam, a study has demonstrated the relationship between colistin use on farms and the presence of the *mcr-1* gene in animals [29]. The increased and inappropriate use of colistin has led inexorably to the worldwide emergence of colistin-resistant bacteria. In Algeria, colistin is used in animals as a therapeutic agent and as prophylactic and growth promoter. Using MALDI-TOF, we confirmed the presence of the active ingredient, the colistin molecule, in the oral solution of colistin that was often used in chickens and livestock in Algeria (**Fig 2**). Most of the time, this solution is used as an additive in chicken feed from Algeria.

The current findings suggest that poultry can be used as a reservoir for colistin-resistant *E. coli*, adding another layer of complexity to the evolving epidemiology of plasmid-mediated colistin resistance in the community. The high prevalence of *mcr-1* in poultry from Algeria is

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likely to be due to a high consumption of colistin by animals in this country. Control policies of the use of colistin in animals are urgently needed in Algeria to avoid the spread of such strains to the human community.

CONFLICT OF INTEREST AND FINANCIAL DISCLOSURE

The authors report no conflicts of interest or issues requiring financial disclosure.

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Figure Legend:

FIG. 1. The distribution of plasmid-mediated mcr-1 colistin-resistant strains isolated from animals and humans in Algeria.

FIG. 2. The colistin use in livestock animals from Algeria

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Table 1. Results of positive MCR-1 stool

Samples	qPCR using PE1 system	qPCR using PE2 system	PCR standard	Sequencing	culture	COL MIC (mg/L)	qPCR using PE1 system	qPCR using PE2 system	MLST results
A27	25,37	25,49	+	+	*E. coli*	3	17,75	17,54	ST48
A26	25,63	26,03	+	+	*E. coli*	3	17,53	17,41	ST48
A24	27,16	27,40	+	+	*E. coli*	4	18,24	19,01	ST48
A16	27,57	28,18	+	+	*E. coli*	4	18,53	18,19	ST48
A21	28,20	28,82	+	+	*E. coli*	3	18,65	18,24	ST48
A34	28,95	28,91	+	+	*E. coli*	4	20,31	20,56	ST48
A15	29,45	29,68	+	+	*E. coli*	4	21,17	21,47	ST48
B5	30,16	30,47	+	+	*E. coli*	4	21,50	21,22	ST48
A28	30,90	31,10	+	+	-				
A30	29,23	30,37	+	+	-				
A32	29,34	30,40	+	+	-				
A25	30,19	31,07	+	+	-				
A33	30,48	31,03	+	+	-				
A14	31,42	31,02	+	+	-				
A23	31,59	32,01	+	+	-				
A31	32,25	32,20	+	+	-				
A36	30,59	31,95	+	+	-				
A29	31,57	32,50	+	+	-				
A22	31,63	32,53	+	+	-				
A37	31,80	32,18	+	+	-				

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A35	32,28	33,37	+	+	-
A38	32,72	35,18	+	+	-
B6	30,21	30,97	+	+	-
B7	31,32	31,94	+	+	-
B8	31,50	30,96	+	+	-

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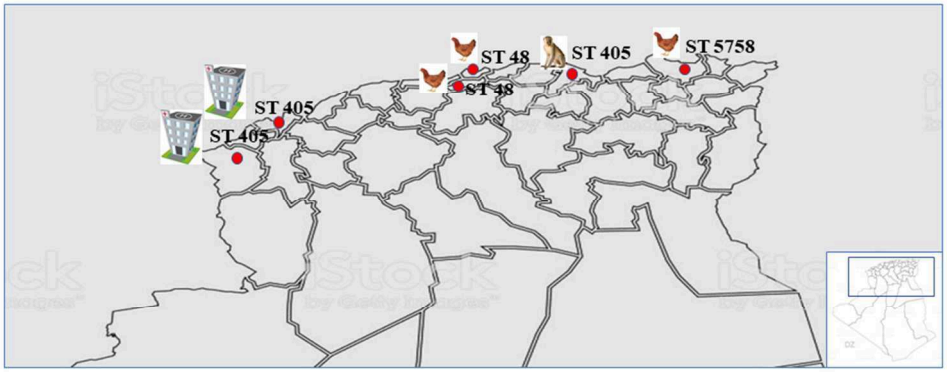

Fig. 1. The distribution of plasmid-mediated mcr-1 colistin-resistant strains isolated from animals and humans in Algeria .

Figure 1

254x190mm (96 x 96 DPI)

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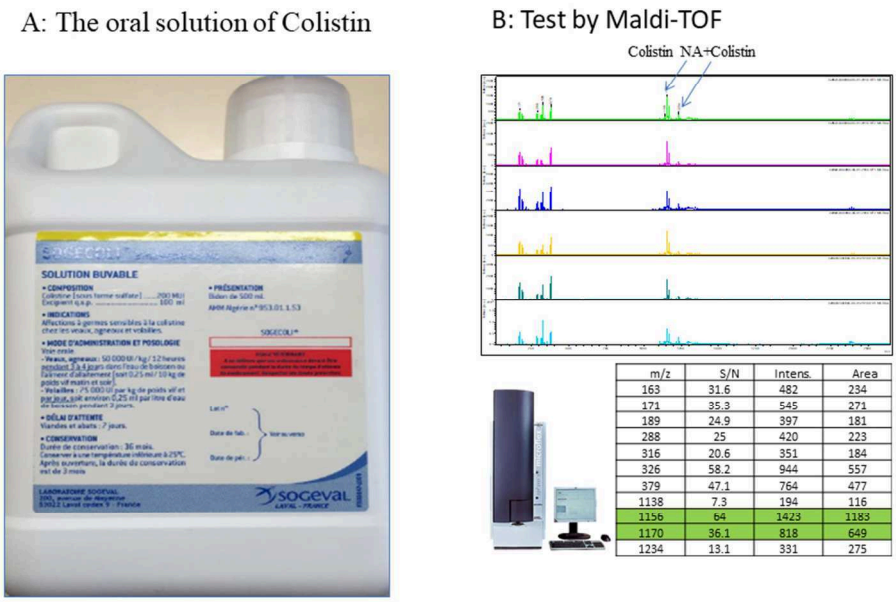

m/z	S/N	Intens.	Area
163	31.6	482	234
171	35.3	545	271
189	24.9	397	181
288	25	420	223
316	20.6	351	184
326	58.2	944	557
379	47.1	764	477
1138	7.3	194	116
1156	64	1423	1183
1170	36.1	818	649
1234	13.1	331	275

Fig. 2. The colistin use in livestock animals from Algeria

Figure 2

254x190mm (96 x 96 DPI)

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ARTICLE 8

Title: First report of the plasmid-mediated colistin resistance gene mcr-1 in Escherichia coli isolated from feces of livestock and farm surface, Algeria.

Selma Chabou, Hamza Leulmi, Tania Dagher Nawfal and Jean Marc Rolain.

To be submitted to Veterinary research journal

Title: First report of the plasmid-mediated colistin resistance gene mcr-1 in Escherichia coli isolated from feces of livestock and farm surface, Algeria.

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Keywords: mcr-1, livestock, E. coli, Algeria.

To editor

Colistin is extensively used as the last-line antibiotic for treating infections caused by multiresistant Gram-negative bacteria (1). Colistin resistance was previously linked only to chromosomal mutations on target genes and was not transferable (1). Recently, a novel plasmid-mediated colistin resistance gene (mcr-1) that encodes a phosphoethanolamine transferase enzyme was described (2), which was identified in Escherichia coli and Klebsiella pneumoniae strains isolated from animals and humans (2). Nowadays, several studies have revealed a wide geographically spread of mcr-1 gene in humans and animals worldwide (1).

It seems that the uncontrolled use of colistin in veterinary medicine is one of the main cause of the emergence and dissemination of this resistance worldwide. To determine the exact animal role as a possible mcr-1 gene reservoir, several investigations were concentrated on this item.

Therefore, this study was conducted to evaluate the occurrence of plasmid- mediated colistin resistance mcr-1 in livestock from Algeria.

In 2017, feces of cattle, goats and sheep were collected from El Tarf, eastern of Algeria. The feces was collected directly from animals using a rectal swabs, as well as, we have collected swabs from surfaces of a farm. The sampling was done following the permission of the animals' owners. The samples were kept at -80°C before being forwarded to the laboratory in Marseille, France. All samples were transferred to an enrichment broth Tryptic Soy Broth medium (TSB) (BioMérieux, Marcy l'Étoile, France) and incubated at 37°C. After that, 100 µL of this medium were cultivated on the selective medium LBJMR (3) that contains colistin for 24h at 37°C. The colonies were selected and cultured individually on Trypticase soy agar. All colonies were selected and identified by MALDI-TOF (matrix-assisted laser desorption and ionization time-of-flight mass spectrometry) (Microflex, Bruker Daltonics, Bremen, Germany).

Antibiotic susceptibility was determined on Mueller–Hinton agar using the standard disc diffusion procedure as recommended by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (www.eucast.org). The following Sixteen antimicrobial agents were tested: amoxillin, amoxillin clavulanic acid, piperacilline tazobactam, cefalotin, ceftriaxone, cefepime, ertapenem, imipenem, amikacin, gentamicin, ciprofloxacin, fosfomycin, nitrofurantoin, doxycycline, trimethropine sulphamethoxazole, and colistin (Bio-Rad, Marnes-la-Coquette, France). Minimum inhibitory concentrations (MICs) of colistin was determined by using the E-test and broth microdilution method. The microdilution plates were incubated at 37 °C for 24h. The results were interpreted according to the EUCAST guidelines (www.eucast.org).

All strains identified from each selective plate, were subjected to a real time PCR analysis for the detection of mcr variants genes (mcr-1, mcr-2, mcr-3, mcr-4 and mcr-5). DNA extraction was performed for all isolates using the DNA extraction kits EZ1 (Qiagen, Courtaboeuf, France). The primers and probes used in this study have been designed in our laboratory (data not published). Standard PCR and sequencing were performed to confirm the presence of mcr genes. The sequences obtained were assembled using ChromasPro 1.7 (Technelysium Pty Ltd., Tewantin, Australia) and compared with mcr-1 sequences found in the GenBank database using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The MLST was done based on allelic profiles to determine their genetic relationship among seven housekeeping genes (adk, fumC, gyrB, icd, mdh, purA and recA) and from the MLST database (http://mlst.warwick.ac.uk/mlst/dbs/Ecoli). Each single locus has different allele and a specific identification number that was given by adding the sequence types (ST) of the seven loci.

Strains were tested for their ability to transfer resistance. Conjugation was tested with azide-resistant E. coli J53. Trans-conjugants were selected on MacConkey agar (Beckton

Dickinson, Le Pont de Claix, France) added with 120 µg/mL sodium azide and 4 µg/mL colistin, as described (4). If conjugation assay failed, transformation assay was done (4).

A total of 80 samples was collected in the extrem East of Algeria (El Tarf), 20 fecal samples from goat, 20 fecal samples from sheep, 20 fecal samples from cattle and 20 samples from swab of the farm surface. Overall the 80 samples, 4 fecal samples from goats, 2 fecal samples from cattles and 3 samples from the farm surface have growth in the LBJMR medium. Eight E.coli and one E. cloacae were isolated and identified by MALDI-TOF MS, Microflex LT spectrometer (**Table1**).

Disk diffusion susceptibility test showed that a total of 10 strains (eight E.coli and one E. claocae) were resistant to colistin with a disc diameter between 9 and 13 mm (**table1**). Minimum inhibitory concentrations (MICs) of colistin confirmed resistance with MIC ranging from 4 to 8 mg/l of E. coli and >256 mg/l of E. cloacae. The antibiotic resistance profile of these isolates is shown in Table 1.

Overall strains, five E.coli out of eight (four E.coli from goat and one E.coli from surface farm) were harboring mcr-1 and were positive in qPCR. The results were confirmed by standard PCR and sequencing. The obtained sequences were 100% identical to the mcr-1 gene sequence reported by Liu and colleagues (2). The conjugation results showed that the strains plasmid was not conjugative. The analysis of the Multi Locus Sequence Typing showed that the four E. coli harboring mcr-1 gene isolates from the goat belonged to the same genotype that was a new sequence types (ST submitted). The E. coli harboring mcr-1 gene isolated from the farm surface as belonging to ST 48 which has already been reported in Algeria (Chabou et al., submitted in MDR). The two E. coli colistin resistant isolated from the cattle belonged to the same genotype (ST 164) which has already been reported in worldwide

livestock. This means that there is no relationship between the different strains isolated from the same farm. The E. cloacae isolates belonged to new sequence types (ST submitted).

The uncontrolled use of colistin in veterinary medicine has a highly important part in the global emergence of mcr-1 in animals. The World Health Organization has newly included polymyxins as critically important antibiotics (5). In France, the prevalence of mcr-1 was 5.9% in turkeys, 1.8% in broilers and did not exceed 0.5% in pigs (6). These findings support that mcr-1 gene has spread in French livestock (6). In Germany, 79.8% of colistin resistant isolates harbored the mcr-1 gene. The highest prevalence was detected in the turkey food chain (10.7%), followed by broilers (5.6%). A low prevalence was determined in pigs, veal calves and laying hens (7). The prevalence of mcr-1 was higher in compared with France (7). That confirm the European Summary Report for 2014 an EU-level prevalence of colistin resistance of 0.9% for E. coli from broilers and 7.4% in E. coli from turkeys was reported (8). In Algeria, the first mcr-1 was detected in E. coli from feces of chickens in 2016 (9). Recently, our team was confirmed the presence of mcr-1 gene in poultry from another region from Algeria (under rev). As well as, mcr-1 colistin resistant E. coli was also isolated from wildlife in Bejaia, Algeria (4). This isolate also carried the mcr-1, $bla_{CTX-M-15}$, bla_{TEM-1}, and qnrB19 genes (4).

The fact that mcr-1 gene is detected in poultry and also in livestock farm, confirm the rapid spread of the plasmid-mediated colistin resistance, not only in Europe but also in Algeria livestock. To the best of our knowledge, mcr-1 isolates from livestock have been previously reported in Africa country including Tunisia, Egypt and South Africa (1) but never in Algeria. Furthermore, this plasmid was detected in a clinical E. coli isolate from University Hospital from Algeria (1).

Emergence of mcr-1 gene in Algeria is probably related to the wide use of colistin in veterinary medicine (6) that should be banned in the future. This work shows that there is a

need for monitoring programs to avoid the spread of mcr-1 gene, as well as to implement colistin susceptibility testing for Gram negative bacteria in clinical microbiology laboratories.

CONFLICT OF INTEREST AND FINANCIAL DISCLOSURE

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1 **Table 1: Phenotypic and genotypic results of all strains.**

	AMC	AX	FEP	TPZ	CRO	CN	AK	CIP	KF	SXT	DO	F	FF	ETP	IMP	CT (mm)	CMI (mg/l)	Mcr-1	MLST
E. coli_ 29GT	**R**	**R**	**S**	**S**	**S**	**S**	**S**	**S**	**S**	**S**	**S**	**S**	**S**	**S**	**S**	**12**	**8**	**+**	**New**
E. coli_ 30GT	**S**	**R**	**S**	**S**	**S**	**S**	**S**	**S**	**S**	**S**	**S**	**S**	**S**	**S**	**S**	**13**	**4**	**+**	**New**
E. coli_ 32GT	**R**	**S**	**S**	**S**	**S**	**S**	**S**	**S**	**S**	**S**	**S**	**S**	**S**	**S**	**S**	**13**	**8**	**+**	**New**
E. coli_ 34GT	**S**	**S**	**S**	**S**	**S**	**S**	**S**	**S**	**S**	**S**	**S**	**S**	**S**	**S**	**S**	**13**	**4**	**+**	**New**
E. coli_ 11CT	**S**	**S**	**S**	**S**	**S**	**S**	**S**	**S**	**R**	**S**	**S**	**S**	**S**	**S**	**S**	**9**	**4**	**-**	**ST164**
E. coli_ 15CT	**R**	**R**	**S**	**S**	**S**	**S**	**S**	**S**	**S**	**S**	**R**	**S**	**S**	**S**	**S**	**9**	**8**	**-**	**ST164**
E. coli_ 10S	**R**	**R**	**S**	**S**	**S**	**S**	**S**	**S**	**R**	**S**	**R**	**S**	**S**	**S**	**S**	**11**	**8**	**-**	**ST48**
E. coli_ 9S	**R**	**R**	**S**	**S**	**S**	**S**	**S**	**R**	**R**	**R**	**R**	**S**	**S**	**S**	**S**	**13**	**4**	**+**	**ST48**
E. cloacae_ 12S	**R**	**R**	**S**	**S**	**S**	**S**	**S**	**S**	**R**	**S**	**S**	**S**	**S**	**S**	**S**	**6**	**>256**	**-**	**New**

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Conclusion du chapitre

A travers ce chapitre nous avons rapporté une émergence et dissémination de la résistance plasmidique à la colistine chez les animaux en Algérie. Nous avons également désigné des systèmes de détection rapide de cette résistance plasmidique, que nous l'avons appliqué sur plusieurs collections d'échantillons d'origine animale d'Algérie.

Il en ressort de cette étude que mcr-1 est présent en Algérie chez les animaux et qu'il a disséminé à travers plusieurs régions. Les méthodes de recherche développées dans ce travail notamment les systèmes de PCR mis en place, constituera sans doute une base utile dans la recherche afin de décrypter une peu plus ce mécanisme de résistance plasmidique à la colistine.

Le recours croissant aux antibiotiques de dernier recours, y compris la colistine, pour traiter le nombre croissant d'infections bactériennes multirésistantes chez les humains a attiré l'attention sur la manière dont les antibiotiques à utilisation partagée avec les animaux sont gérés et réglementés pour la santé humaine et animale (**Mandelson et al., 2018**). Récemment, l'émergence de la résistance à médiation plasmidique mcr-1 dans les isolats surtout d'origine animale (**Liu et al., 2016**) a suscité les recherches pour savoir le rôle des animaux dans le transfert des gènes de résistances aux humains.

Les discussions au niveau international et national se sont intensifiées (**Al-Tawfiq et al., 2017**) depuis l'identification de nouveaux gènes de résistance à la colistine en 2016, d'abord en Chine puis dans de nombreux autres pays (**Liu et al., 2016**). L'Afrique du Sud par exemple, a réagi à cette menace en procédant à une analyse de la situation et en examinant la législation en vigueur concernant l'utilisation de la colistine principalement chez les animaux, afin de déterminer la ligne de conduite à adopter (**Mandelson et al., 2018**). Les expériences partagées à travers plusieurs pays décrivent le processus, l'institution de la gouvernance avec un large engagement des parties prenantes, la surveillance et les interventions que certains pays ont prises pour

optimiser l'utilisation de la colistine surtout chez les animaux (**Mandelson et al., 2018**). Le principe du concept de « One Health » pour l'usage vétérinaire des antibiotiques au niveau des pays est un élément crucial de tout plan d'action pour combattre la résistance aux antibiotiques (**MC Ewen et Collignon., 2018**).

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IV-AUTRES VECTEURS ET/OU RESERVOIRS DE BACTERIES ZOONOTIQUES RESISTANTES A LA COLISTINE

Initialement la résistance à la colistine a été identifiée à partir de souches d'E. coli isolées de fientes de poulets en Chine (**Liu et al., 2016**). Après avoir discuté dans le chapitre précédent l'épidémiologie de l'émergence et de la dissémination de la résistance plasmidique à la colistine chez les animaux, nous avons voulu dans ce chapitre élargir le répertoire des réservoirs possibles capables (goélands et les tiques dures d'animaux) d'abriter des bactéries porteuses de ce gène de résistance à la colistine.

Un premier travail (**Article N° 9)**, s'est consacré à la recherche des gènes de résistance à la colistine chez des goélands au niveau de l'ile Frioul de Marseille. Nous avons collecté dans cette investigation 38 fientes de goélands. Trois souches résistantes à la colistine (EC3, EC35 et KP15) ont été isolées à partir du milieu LBJMR et identifiées par MALDI-TOF (E. coli, et K. pneumoniae) mais aucune souche mcr-1 n'a été identifiée.

Dans la même optique, une dernière étude menée dans une zone où mcr-1 a été détecté en Algérie sur des fientes de poulets, nous avons testé par PCR en temps réel la présence de mcr-1 à partir d'ADN de tiques collectées dans l'environnement immédiat de ces poulets (**Article N° 10)**. Nous avons pu détecter 17/228 (7.4%) tiques Ixodes ricinus positives pour mcr-1. Par biologie moléculaire nous avons identifié dans le microbiote des tiques Pseudomonas fluorescens comme étant la bactérie qui semble pouvoir porter mcr-1. D'autres études complémentaires seront réalisées afin d'isoler et d'identifier avec certitude la bactérie qui peut porter le gène de résistance.

ARTICLE 9

Title: First report of colistin resistance GNB in Yellow-legged Gull from Marseille. Selma Chabou, Tania Dagher Nawfal, Sophie Baron and Jean Marc Rolain.

To be submitted to J Glob Antimicrob Resist.

Title: First report of colistin-resistant bacteria in Yellow-legged Gulls from Marseille, France

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- Table: 1

Sir;

The increased global incidence of multidrug-resistant (MDR) bacterial pathogens, particularly carbapenem-resistant Gram-negative bacteria, has led to the reactivation of old and abandoned antibiotics, polymyxin (polymyxin B and colistin), for the treatment of severe MDR bacterial infections (Olaitan et al., 2014). It is currently used as a last-line drug against multidrug-resistant Gram negative bacteria and is an effective antibiotic to treat severe bacterial infections (Baron et al., 2016a). Recently, resistance to colistin has emerged among pathogens, but there is still a dearth of information on the comprehensive mechanisms of colistin resistance in Gram-negative bacteria (Baron et al., 2016b; Olaitan et al., 2014). Various modifications leading to colistin resistance have been described including modification of lipopolysaccharide (LPS). Loss of LPS production have been reported due to the mutations in the lpxACD genes or the addition of phospho-ethanolamine or 4-amino-4-arabinose mediated by mutations in the two-component systems pmrA/pmrB, phoP/phoQ (Baron et al., 2016a). More recently a new plasmid mediated colistin resistance mechanism due to mcr-1 gene that encodes a phospho-ethanolamine transferase has been described (Liu et al., 2016).

Migratory birds appear to be reservoirs of multi-resistant bacteria and could therefore play an important epidemiological role in the spread of resistance (Bouaziz et al., 2017). The objective of this study was to investigate the presence of colistin resistance Gram-Negative bacteria in fecal samples collected from a group of yellow-legged gulls (Larus michahellis) in Marseille, France.

In August 2017, feces from Yellow-legged gull (Larus michahellis) were collected in Frioul Iles Marseille. Samples were collected directly on site and placed in a single sterile, identified tube. For the selection of colistin resistant Gram-negative bacteria, samples were incubated on

a selective culture medium LBJMR for 24h à 37°C as described (Bardet et al., 2017). Isolated colonies from each sample were identified by MALDI-TOF. Antibiotic susceptibility testing was determined on Mueller–Hinton agar using the standard disc diffusion method against 16 antibiotics. Colistin susceptibility was determined by broth microdilution. DNA extraction was carried out subsequently in an automatic robot EZ1. Colistin resistance genes (pmrA, pmrB, phoP, phoQ, mgrB, mcr-1, mcr-2, mcr-3, mcr-4 and mcr-5) were screened by real time PCR and/ or standard PCR, and were then sequenced(Chabou et al., 2016; Yassin et al., 2017). Results of the sequenced genes were compared against the reference strain K. pneumoniae MGH 78578, and E. coli K-12 MG 1655 (NCBI GenBank accession no.CP000647). Translated amino acid sequences were analysed using npsa_clustalwan software (https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_clustalwan.htm.)PROVEAN software (http://provean.jcvi.org/seq_submit.php) was used to verify whether the mutation would affect the function of the proteins. Multi-locus Sequence Typing (MLST) was performed for genotyping using seven housekeeping genes.

Thirty-eight samples of feces from Yellow-legged gulls were collected in Iles Frioul, Marseille in August 2017. Three (7,8%) out of thirty-eight samples, colistin-resistant strains were isolated from LBJMR meduim and identified by MALDI-TOF, two E. coli (EC3, EC35) and one k. pneumonia (KP15). The three isolates were resistant to colistin and amoxicillin and sensitive to the other antibiotics tested. Minimum inhibitory concentrations (MICs) for colistin ranged from 8 to 16 mg/ l. To study the clonality of our isolates, a Multi Locus Sequence Typing (MLST) analysis was performed and revealed the same type of sequence for E. coli (ST 126). The MLST identified KP15 as belonging to ST 248. All qPCR for mcr genes (mcr-1, mcr-2, mcr-3, mcr-4and mcr-5) were negative. Results of sequence analysis of colistin-resistant genes are summarized in Table 1.

Analysis of E. coli (EC3) showed that the resistance was due to many mutations in the pmrB and phoQ genes) that have not previously been described. Olaitan et al showed that L140H (table 1) was similar to T140P found in the pmrB of K. pneumoniae (Olaitan et al., 2014). In addition, E. coli (EC35) had deletions and substitutions mutations in the pmrA and phoP/Q genes that have not been previously reported and were considered as deleterious using PROVEAN software (work in progress).In addition, K. pneumoniae was resistant due to large deletion in phoQ of 240 amino acid resulting in the formation of a truncated protein made of 113 amino acid.

The migratory birds play a potential role in the transport of resistant GNB. Most studies have previously shown the presence of ESBL-producing Gram-negative bacteria in wild birds (Bouaziz et al., 2017). However, the plasmid mediated colistin resistance gene mcr-1 has been identified in E. coli isolates from infected migratory Magellanic penguins (Spheniscus magellanicus) (Sellera et al., 2016).

Our results showed the potential for GNB to resist colistin transmission by yellow-legged gulls and could pose a public health risk. This requires wildlife and human studies to improve our knowledge of resistant bacterial transmission pathways and to determine the source of bacterial contamination. Despite the fact that mcr genes were not detected in our study, the presence of colistin resistant Enterobacteriaceae in urban gulls is worrisome since they are living in close contact with humans and could be a source of zoonotic transmission of multi drug resistant bacteria.

CONFLICT OF INTEREST AND FINANCIAL DISCLOSURE

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Table 1: Mutation of colistin resistance genes

Strains	**mgrB**	**pmrA**	**pmrB**	**phoP**	**phoQ**	Mcr genes
E. coli_3G	-	No mutation	V78G,L79S, T80P, L84Y, Q88P, A90G, V91T, R96A, P97R, L98W, A99R, E100S, L101C,L140H	No mutation	A22H, L28C, T129C, H157P, L218F	Negative
E. coli_15G	-	L11Q	No mutation	I44L, K171R, R185P, E186D, S187I, K200R, I210S	L26R, del_{30-40}, F44I, D45del, S116W, F119S, G441R, E451G, A470G	Negative
K. pneumonia_35G	No mutation	No mutation	T10P, E37D, L146X, G147X, V219I, L222P, Q223A, D224G, E225R, L226R, E227D, A228S, M229L, L230C, A231C, Q232R, R233X.	No mutation	A large deletion of 240 amino acid	Negative

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ARTICLE 10

Title: Possible transmission of mcr-1-harboring Pseudomonas fluorescens by ticks: first report of mcr-1 in Ixodes ricinus from Algeria.

Selma Chabou, Hamza Leulmi and Jean Marc Rolain.

To be submitted to to Emerging Infectious Diseases.

Title: Possible transmission of mcr-1-harboring Pseudomonas fluorescens by ticks : first report of mcr-1 in Ixodes ricinus from Algeria.

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Keywords: mcr-1, ticks, Algeria.

To the editor

Since the first description of the plasmid-mediated colistin resistance gene (mcr-1) in November 2015 in Gram-negative Enterobacteriaceae (Liu et al., 2016), multiple retrospective studies indicated a worldwide spread of this newly-identified resistance gene and is being increasingly recognized (Shen et al., 2016). The presence of this specific gene was also reported from various microorganisms Enterobacteriaceae that have been isolated from various sources including foods, food animals (Shen et al., 2016), human patients (Yu et al., 2016) and animals (Olaitan et al., 2016). It was evident that mcr-1 was prevalent in the animal (Olaitan et al., 2016) and human reservoir, however little is known about the potential reservoir and carriers.

Ixodes ricinustick is the most common tick species in Europe and is a vector of pathogens affecting human and animal health (Diuk-Wasser et al., 2016). It was also reported that Ixodes ticks can harbor several zoonotic agents and/or nonpathogenic micro-organisms and can acts as vector (Diuk-Wasser et al., 2016).

The aim of the present study was to investigate the presence of mcr-1 encoding gene in Ixodes ricinus ticks collected in Northern Algeria, and decipher the possible transmission of mcr-1 harboring bacteria by ticks.

In this work, we dispose DNA from ticks collected from our prior work on the detection of Borrelia garinii in Ixodes ricinus from Algeria (Benredjem et al., 2014). Ticks were free sampled by using the flag method in El Ghora (Bougous) El Tarf (36°39′34″N, 8°22′10″E) and in Seraidi(36°55′, 00″N - 7°40′, 00″E), Annaba (North East of Algeria). The collection of ectoparasites was performed once in Marchand April 2012 respectively. Ticks were identified to the genus and species level by using taxonomic morphologic keys. Each tick was rinsed twice for 5 minutes in sterile water and dried on filter paper. All experiments and handling of ticks were conducted in a

laminar flow biosafety hood. Total genomic DNA was then extracted using the QIAamp tissue kit (Qiagen, Hilden, Germany) and Qiagen-Robot EZ1 as described by the manufacturer. The extracted DNA was assesed for the presence of mcr-1 encoding genes by using double quantitative real-time PCR systems, (PE1 and PE2 systems)(Chabou et al., 2016). Results were considered positive if the Cycle threshold value obtained by CFX96^{Tm}qPCR thermocycler was less than 35. The DNA qPCR positive samples were subsequently sequenced as describer (Chabou et al., 2016). The success of the PCR amplification was verified by electrophoresis in 1.5% agarose gel stained with ethidium bromide at 130 V for 25 min and examined then under UV transillumination. The bands were visualized under ultraviolet illumination using BenchToppGEM®DNA Marker (Promega, Madison, Wisconsin, USA) to estimate the size of the products.The PCR products were then purified using the NucleoFast 96 PCR plate (Machery-Nagel EURL, France), as recommended by the manufacturer. The obtained amplicon was sequenced in the ABI 31000 automated sequencer (Applied Biosystems) by using the DNA sequencing BigDye Terminator Kit (Perkin- Elmer, Waltham, MA) as described previously. The sequences obtained were assembled using ChromasPro 1.7 (Technelysium Pty Ltd., Tewantin, Australia) and compared with mcr-1 sequences found in the GenBank database using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). In the second part of this work, identification of the possible Enterobacteria that carry mcr-1 was done. As we disposed only of DNA extracted from ticks, culture and isolation was not possible. mcr-1 encoding gene was known to be carried mostly by Escherichia coli and Klebsiella pneumoniae , for this reason qPCRs targeted E.coli and K. peneumaniae were done. Standard PCR and sequencing of the Rpob gene from all Enterobacteria was also done on positive mcr-1-ticks.

Two hundred and sixty-nine ticks were collected and belonged to two species: 121/162 (74.7%), which were collected in Seraïdi, were Ixodes ricinus; and 41/162 (25.3%) were Rhipicephalus

sanguineus. In El Ghora, Bougous we collected one hundred and seven ticks, all identified as Ixodes ricinus. Using qPCRs (PE1 and PE2 systems), 17/228 (7.4%) Ixodes ricinustick was positive for mcr-1. 12 of 17 (70.6%) mcr-1-positive ticks were collected in El Ghora and 5 out of 17 (29.4%) were sampled in Seraidi. Eight ticks (6 from El Ghora and 2 from Seraidi) have Ct value lower than 30 and were subsequently sequenced. DNA sequence analyses of the PCR products targeting the mcr-1 showed 100% similarity with Escherichia coli strain SHP45 plasmid pHNSHP45, complete sequence (GenBank accession no KP347127.1). qPCR targeting E. coli and K. pneumaniae were negative. DNA sequence analyses of the PCR products targeting the Rpob showed 100% similarity with Pseudomonas fluorescens strain M04 RpoB (rpoB) gene, partial cds (GenBank accession no KU963595.1).

In this study, we reported the first direct evidence of plasmid-mediated colistin resistance in Ixodes ricinus ticks most likely in P. fluorescens bacteria. To date mcr-1 is mostly present in E. coli, and Klebsiella pneumoniae but also reported in Salmonella, Shigella, Vibrio and Enterobacter (Irrgang et al., 2016). Poultry, Swine and companion animals were found also to harbor mcr-1 producing E. coli (Olaitan et al., 2016; Quesada et al., 2016; Zhang et al., 2016). In addition, P. fluorescens has also been associated with I. ricinus ticks in previous studies (Tveten and Sjastad, 2011), which consolidated our hypothesis of the carrying of mcr-1 by P. fluorescens in ticks.

Our findings suggest also that mcr-1–producing bacteria can colonize ticks and could be transmitted to animals and human through the possible contact that can occur between the ectoparasites and their hosts. In addition, our findings suggest that ectoparasites can also serve as a reservoir of colistin-resistant E. coli, adding another layer of complexity to the rapidly evolving epidemiology of plasmid-mediated colistin resistance in the community.

CONFLICT OF INTEREST AND FINANCIAL DISCLOSURE

No conflicts of interest or financial disclosure for all authors.

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All authors have read and approved the manuscript.

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Conclusion du chapitre

Ce chapitre traite des vecteurs/ réservoirs de la résistance plasmidique à la colistine et fait suite au chapitre précédent où nous avons détecté une émergence et une dissémination du gène mcr-1 à travers des isolats d'origine animale en Algérie. Récemment, le gène mcr-1 a été identifié dans des isolats d'E. coli producteurs de BLSE provenant de manchots Magellanic migrateurs infectés (Spheniscus magellanicus) (**Sellera, et al., 2016**). Très récemment, **Guenther et al., 2017** a trouvé une similitude entre des isolats cliniques européens et un isolat de la faune sauvage mongole, ce qui souligne le caractère zoonotique et la dimension environnementale de ces disséminateur de gènes de résistance à travers diffèrent vecteurs et réservoirs.

L'utilisation et la surutilisation d'antimicrobiens sont des facteurs importants qui provoquent les phénomènes de résistance (**Holmes et al., 2016**). Il existe d'autres facteurs qui interviennent dans le développement de la résistance aux antibiotiques et qui favorisent la propagation des bactéries résistantes et de leurs gènes localement et globalement. Ceci comprend un mauvais control d'une infection, les contaminations environnementales et surtout les mouvements des individus infectés que ce soit les humains ou les animaux (**Burow et Käsbohrer., 2017** ; **Marti et al., 2014**).

Partout où des antimicrobiens sont utilisés, il existe des réservoirs de résistance, notamment chez l'homme et les environnements locaux des hôpitaux et de la communauté, ainsi que chez les animaux et dans les environnements agricoles et aquacoles, mais aussi dans l'eau, le sol, la faune et bien d'autres niches écologiques, en raison de la pollution par les eaux usées, des déchets de l'industrie pharmaceutique et du ruissellement du fumier provenant des fermes (**Marti et al., 2014**, **Huijbers et al., 2015**). Les bactéries et leurs gènes se déplacent facilement à l'intérieur de ces écosystèmes (entre les humains, les animaux et l'environnement) (**MCEwen et Collignon., 2018**).

En conclusion, il semble important de développer des stratégies globales de restriction d'usage des antibiotiques, chez les animaux pour éviter la propagation de gènes de résistance au sein de ces écosystèmes avec le risque de transfert chez l'homme.

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V- EPIDEMIOLOGIE DES BACTERIES RESISTANTES A LA COLISTINE CHEZ LES HUMAINS EN FRANCE, AU LAOS ET AU LIBAN

Ce chapitre fait partie des travaux de collaboration et annexés à cette thèse. Il porte sur l'épidémiologie des bactéries résistantes à la colistine chez les humains en France, au Laos et au Liban. En raison de l'utilisation intensive d'antibiotiques, les bactéries résistantes aux antibiotiques sont largement répandues et deviennent une préoccupation mondiale (**Abat et al., 2018**). L'augmentation de la prévalence des bactéries résistantes à la colistine peut se produire à partir de plusieurs événements tels que l'abus d'usage d'antibiotiques chez les humains et les animaux, et l'acquisition de gènes de résistance aux antibiotiques à partir d'autres bactéries ou de l'environnement (**Olaitan et al., 2014 ; Olaitan, Morand, Rolain., 2016**).

Dans une première étude (**Article N°11**), nous avons testé par PCR en temps réel et confirmé par PCR standard et séquençage la présence du gène codant mcr-1 à partir de 32 isolats de K. pneumoniae résistants à la colistine précédemment isolée au Laos (11 isolats), en Thaïlande (14 isolats), en France (6 isolats), et au Nigeria (1 isolat). Nous avons trouvé que 6 isolats portaient le gène mcr-1 incluant 4 isolats humains au Laos ainsi que 2 isolats de France (un de Marseille et un d'Angers). Ce travail indique la propagation de mcr-1 chez les humains dans le monde entier, y compris en Europe. Tous ces isolats étaient sensibles à plusieurs classes d'antibiotiques, y compris aux β-lactamines. Cela pourrait être dû à l'utilisation intensive de la colistine dans les productions animales, y compris en Europe. En outre, la résistance à la colistine peut se produire par mutation de gènes chromosomiques associés à la résistance à la colistine. Ce point a été développé dans l'article 12.

L'**Article N° 12** a porté sur la prévalence et les mécanismes de résistance à la colistine et aux β-lactamines dans des isolats cliniques prélevés chez des patients hospitalisés dans le service de soins intensifs de l'hôpital Saint-Georges à Beyrouth, au Liban. Nous avons isolé de ces prélèvements

huit souches cliniques résistantes à la colistine sur vingt-trois écouvillons rectaux (5 E. coli, 2 Enterobacter cloacae et 1 K. pneumoniae). Parmi eux, 5 souches porteuses du gène BlaTEM-1 et une souche porteuse du gène BlaTEM-163. De plus, 4 E. coli portaient les gènes BlaCTX-M-A (2 BlaCTX-M-15, 1 BlaCTX-M-103 et 1 BlaCTX-M-189) et K. pneumoniae portait BlaSHV-1. La résistance à la colistine était due à une séquence d'insertion (IS1) dans le gène mgrB et à une mutation faux-sens dans le gène PmrB. Pour E. coli et E. cloacae, la résistance à la colistine a été liée à des mutations dans les séquences d'acides aminés des protéines PmrA, PmrB, PhoP, PhoQ et MgrB.

Ce travail rapporte la première détection de mutations dans le gène régulateur mgrB d'E. cloacae responsable de la résistance à la colistine et une propagation des bactéries à Gram-négatif résistantes à la colistine à l'hôpital Saint-Georges au Liban. Une stratégie urgente doit être adoptée pour éviter la propagation de telles bactéries.

ARTICLE 11

Title: Plasmid-Mediated mcr-1 Gene in Colistin-Resistant Clinical Isolates of Klebsiella pneumoniae in France and Laos.

Jean Marc Rolain, Marie Kempf, Thongpan Leangapichart, Selma Chabou, Abiola Olumuyiwa Olaitan, Stéphanie Le Page, Serge Morand and Didier Raoult.

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LETTER TO THE EDITOR

Plasmid-Mediated *mcr-1* Gene in Colistin-Resistant Clinical Isolates of *Klebsiella pneumoniae* in France and Laos

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Recently, Yi-Yun Liu et al. reported the emergence of plasmid-mediated colistin resistance involving the *mcr-1* gene from *Escherichia coli* and *Klebsiella pneumoniae* isolates from animals, food, and humans in China (1). Because of the plasmidic location of this new gene, which encodes a phosphoethanolamine transferase, it is transferable laterally between Gram-negative bacteria (1). We have recently reported the isolation of *mcr-1*-positive *E. coli* isolates from animals and humans from Laos (pigs and asymptomatic humans), Thailand (asymptomatic humans), and Algeria (chicken) (2). Although resistance to colistin in humans was believed to be linked only to colistin ingestion in humans, there is evidence of an independent emergence of colistin-resistant bacteria in humans without colistin usage, likely suggesting that such bacteria may preexist in the human gut and be selected upon colistin therapy (3, 4). We have tested by real-time PCR (5) and confirmed by standard PCR and sequencing the possible presence of the *mcr-1* gene in 32 colistin-resistant *K. pneumoniae* isolates previously isolated in Laos (11 isolates), Thailand (14 isolates), France (6 isolates), and Nigeria (1 isolate) (6). We found that 6 isolates, specifically, 4 isolates from humans in Laos and 2 isolates from France (1 from Marseille and 1 from Angers), harbor the *mcr-1* gene sequence (Table 1). MICs were determined by the Etest and interpreted with EUCAST guidelines. Interestingly, two of the four isolates from Laos also had either a stop codon (at Cys28 in strain KP LH131) or a mutation (A14S in strain KP LH61) in the *mgrB* gene sequence; the MICs for them were higher than those for the four other isolates that had only *mcr-1*, which is consistent with previous findings for colistin MICs for isolates with only *mcr-1* (1, 2). These strains were isolated from human stool samples collected in 2012 and 2013 either from healthy individuals (Laos) or from patients at the University Hospital La Timone (Marseille, 4-year-old girl) and the University Hospital Centre (Angers, 83-year-old male) who had never received colistin therapy. Interestingly, although our isolates are resistant to colistin, they remain susceptible to several classes of antibiotics, including cephalosporins and carbapenems (Table 2), but acquisition of additional antibiotic resistance genes within the human gut is a possible scenario (6). To the best of our knowledge, this is the first report of colistin-resistant *mcr-1*-positive *K. pneumoniae* clinical isolates in Marseille and Angers, France, and in Laos. Our report clearly demonstrates that plasmid-mediated colistin resistance has already spread all over the world, including Europe. This is probably due to the fact that colistin is extensively used in animal production, including in Europe (3, 4, 7).

Since colistin is now widely used in Europe (Greece and Italy, for example) to treat patients infected with *K. pneumoniae* carbapenemase producers and also in agriculture production, there is an urgent need to implement a screening strategy for the carriage of colistin-resistant Gram-negative bacteria in hospitalized humans, as has already been done for carbapenemase producers, and to ban the use of colistin in avian and pig production in order to avoid a frightening scenario of the colistin resistance gene spreading to humans, as NDM-1 did 5 years ago.

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TABLE 1 MICs for and genetic features of *Klebsiella pneumoniae* clinical isolates positive for *mcr-1* by PCR[a]

Country	Isolate	Colistin MIC (mg/liter)[b]	ST	*mgrB* feature
Laos	KP LH131	32	1319	Stop (at Cys28)
	KP LH17	12	37	Intact
	KP LH61	24	491	Sub (A14S)
	KP LH92	12	39	Intact
France	KP FHM128 (Marseille)	4	1310	Intact
	KP FHA60 (Angers)	8	8	Intact

[a] All strains were *mcr-1* positive. ST, sequence type; Intact, intact *mgrB* gene with no mutation; Stop, mutation leading to insertion of a stop codon; Sub, mutation leading to an amino acid substitution.
[b] MICs were determined by the Etest assay.

TABLE 2 Antibiotic susceptibility testing results for the 6 *mcr-1*-positive strains

Strain	Susceptibility profile for following antibiotic of the indicated class[a]:																					
	β-Lactams											Aminoglycosides			Quinolones		Cyclins			Sulfamide	Nitrofuran	
	AMX	AMC	TIM	TZP	CRO	CTX	FEP	ERT	MEM	IMP	ATM	GEN	TOB	AMK	NAL	CIP	DOX	MIN	TGC	SXT	NIT	FOF
KP LH17	**R**	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	**R**
KP LH92	**R**	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
KP LH61	**R**	S	**R**	S	S	S	S	S	S	S	S	S	S	S	S	S	**R**	S	S	**R**	S	S
KP LH131	**R**	**R**	**R**	S	S	S	S	S	S	S	S	S	S	S	S	S	**R**	**R**	S	S	S	S
KP FHA60	**R**	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	**R**	S	S	**R**	S	S
KP FHM128	**R**	S	S	S	S	S	S	S	S	S	S	**R**	**R**	S	S	S	**R**	**R**	S	**R**	S	**R**

[a] AMX, amoxicillin; AMC, amoxicillin-clavulanate; TIM, ticarcillin-clavulanate; TZP, piperacillin-tazobactam; CRO, ceftriaxone; CTX, cefotaxime; FEP, cefepime; ERT, ertapenem; MEM, meropenem; IMP, imipenem; ATM, aztreonam; GEN, gentamicin; TOB, tobramycin; AMK, amikacin; NAL, nalidixic acid; CIP, ciprofloxacin; DOX, doxycycline; MIN, minocycline; TGC, tigecycline; SXT, trimethoprim-sulfamethoxazole; NIT, nitrofurantoin; FOF, fosfomycin; R, resistant; S, susceptible.

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We declare that we have no potential conflict of interest or financial interests to report.

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ARTICLE 12

Title: Emergence of colistin resistant Gram-negative bacteria isolated from ICU patient at Saint Georges hospital in Lebanon.

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To be submited to Antimicrobial Agents and Chemotherapy journal.

Title: **First Detection of mutation in mgrB of colistin resistant Enterobacter cloacae and spread of colistin resistant gram-negative bacteria in Lebanon**

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Running Title: Colistin resistant gram-negative bacteria

Keywords: colistin-resistance, pmrA/B, phoP/Q, mgrB.

Abstract:

Background: Infections due to multidrug-resistant gram-negative bacteria (MDR-GNB), especially resistance to carbapenems, have become a major public health problem. This increase in resistance to antibiotics has led to the resuscitation of colistin, as a last-resort treatment option. The reintroduction of polymyxins E has been followed by an increase of colistin resistant gram-negative bacteria. This study investigated the prevalence and the mechanisms of colistin resistance in clinical isolates collected from ICU patients of Saint-George Hospital in Beirut, Lebanon.

Materials and methods: Eight colistin-resistant clinical strains out of twenty-three rectal swabs (5 Escherichia coli, 2 Enterobacter cloacae, and 1 Klebsiella pneumoniae) were isolated from LBJMR medium, which is selective for polymyxin-resistant bacteria. MALDI-TOF was used to identify bacteria. Antibiotic susceptibility testing was performed using disk diffusion method and E-test. Colistin susceptibility was determined using the broth microdilution method, and MICs were determined according to 2017 EUCAST guidlines. Genes encoding for carbapenemases, extended spectrum β-lactamases (ESBLs) and colistin resistance genes were investigated by RT-PCR and standard PCR, and were sequenced when positive. The epidemiological relatedness of the strains was studied using MLST.

Results: Real-time PCR results showed that 5strains harbored bla_{TEM-1} and one strain harbored $bla_{TEM-163}$. Moreover, 4 *E.coli* harbored the $bla_{CTX-M-A}$ gene (2 $bla_{CTX-M-15}$, 1 $bla_{CTX-M-103}$, and 1 $bla_{CTX-M-189}$), and *K.pneumoniae* harbored bla_{SHV-1}. Resistant to colistin was due to an insertion sequence (IS1) in the *mgrB* gene and a missense mutation in the *pmrB* gene. For *E.coli and* E. cloacae*,* the colistin resistant has been linked to mutations in the amino-acid sequences of proteins (pmrA, pmrB, phoP, phoQ, and mgrB). Here we report the first detection of mutation in mgrB of colistin resistant E. cloacae. Results of MLST revealed that the five *E.coli* belonged to five different sequence types including ST131, ST6174, ST405, ST162 and ST1451. *K. pneumoniae* had ST45, and the two *E.cloacae* had new sequence types ST924, and ST925. This result revealed that there was no relationship between the different types of strains isolated from the same hospital.

Conclusion: This study shows the spread of colistin-resistant gram-negative bacteria in Saint-George hospital in Lebanon, which is worrisome. An urgent strategy need to be adopted to avoid the spread of such bacteria.

1. Introduction

Resistance to antibiotics in Gram-negative bacteria such as Acinetobacter baumannii, Pseudomonas aeruginosa, and Enterobacteriaceae is becoming a field of interest over the last decade. This has led to a new fear of resistance to many antibiotics that seems to be not justifiable (1). Those bacteria develop a high resistance to most available antibiotics such as beta-lactams, aminoglycosides, carbapenems, and fluoroquinolones (2, 3). This growing infection caused by multidrug-resistant (MDR) Gram-negative bacteria has led to the renewal of colistin as a last-resort treatment option especially for patients in intensive care units (ICUs) (4, 5). Unfortunately, the resistance to polymyxin has been increasingly worldwide (2). Polymyxins were discovered in 1947, and are a group of natural polypeptide antibiotics that is composed of five different chemical compounds (polymyxins A-E). Only polymyxin E (colistin) and polymyxin B have been used in humans (6). Polymyxins are bactericidal toward Gram-negative bacteria except the bacteria that are intrinsically resistant to colistin such as Burkholderia, Edwardsiella, Proteus, Providencia, Morganella, and Serratia, (3). From 1959 colistin was available to treat infections caused by Gram-negative bacteria (7). Conversely, its utilization is limited due to the daily dose that cannot be increased because it will leads to nephrotoxicity in around 60% of patients (8).

Between the late 1990s and early 2000s, colistin was re-introduced into clinical medicine because of the emergence of resistance to beta-lactams, and carbapenems (2). This leads to the emergence of colistin resistant bacteria among patients treated with this compund (9). The bacterial cell membrane is the main target for the antimicrobial activity of colistin: colistin binds to the anionic lipid A moiety of lipopolysaccharide (LPS) and disrupts the cell membrane (6, 10). Gram-negative bacteria employ several strategies to protect themselves from polymyxin antibiotics (polymyxin B and colistin). The mainly mechanism of resistance occurs by modification of lipopolysaccharide (LPS) which is negatively charged, by the addition of phosphoethanolamine (PEtN) or 4-amino-4-deoxy-L-arabinose (L-Ara4N), to the lipid A moiety of the LPS. This can be done by specific mutations of the two-component systems (TCSs)

(pmrA/pmrB, phoP/phoQ), or its negative regulator mgrB. The modified LPS with this positive charge reduces its binding to polymyxins, and leads to this resistance. (2, 10, 11). Morover, it has been demonstrated that the resistance to colistin can also be due to the loss of LPS production due to mutations in the lpxACD genes (12), in addition to the use of efflux pumps, the formation of capsules and overexpression of the outer membrane protein OprH, which are all effectively regulated at the molecular level (2). Moreover, colistin resistance can be due to the presence of plasmid-mediated mcr-1 gene that encodes the enzyme phosphoethanolamine transferase that is able to modify the lipid A moiety of LPS with the addition of phosphoethanolamine (13, 14). In our country (Lebanon), colistin is regularly used to treat ICU patients. This expanded utilization of colistin as last-resort therapeutic medication to treat patients that were infected with multidrug-resistant (MDR) Gram-negative bacteria. The objective of the present study was to investigate the prevalence and the molecular mechanism of colistin resistance in gram negative clinical isolates collected from rectal swabs from ICU patients of Saint-George Hospital in Beirut, Lebanon.

2. Materials and methods

2.1 Bacterial isolates

Between October 2016 and February 2017, 23 rectal swabs were collected from ICU patients from Saint-George Hospital in Beirut and kept at -80°C before being transported to the laboratory in Marseille, France. They were then transferred to an enrichment Tryptic Soy Broth (TSB) medium and incubated overnight at 37°C. After incubation, 100 µL of the enrichment medium were cultivated for 24h at 37°C on the selective medium LBJMR that contains colistin and vancomycin (15). Species, intrinsically resistant to colistin (Proteus spp., Morganella morganii, Serratia spp and Providencia spp.), were excluded from this study. Bacterial identification at the species level was done using the matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF) method (Microflex; Bruker Daltonics) as described previously (17). Here we report the detection of eight colistin-resistant clinical Enterobacteriaceae isolates including Escherichia coli, Enterobacter cloacae, and Klebsiella pneumonia from those patients.

2.2. Antibiotic susceptibility testing

Antibiotic susceptibility testing of the strains was performed using the standard disk diffusion method on Mueller-Hinton agar as recommended by the European Committee of Antimicrobial Susceptibility Testing (EUCAST) 2017 (http://www.sfm-microbiologie.org/UserFiles/files/casfm/CASFM%20V2_0_Mai2017.pdf). The following 16 antibiotics were tested: amoxicillin, amoxicillin/clavulanic acid, piperacillin/tazobactam, cefalotin, ceftriaxone, cefepime, ertapenem, imipenem, amikacin, gentamicin, ciprofloxacin, fosfomycin, nitrofurantoin, doxycycline, trimethoprim sulfamethoxazole, and colistin.
The minimal inhibitory concentration (MIC) of colistin was determined using the broth microdilution method according to EUCAST 2017. Plates were incubated at 37 °C for 24h before determination of MICs. Results of MIC for colistin and disk diffusion diameters were interpreted according to susceptibility breakpoints of the European Committee on Antimicrobial Susceptibility Testing (EUCAST).Each strain that had an MIC >2 mg/L for colistin was considered as colistin-resistant.

2.3. DNA extraction

Extraction of the DNA of bacteria was done using the automatic robot EZ1 (Qiagen BioRobot EZ1-, Tokyo, Japan), using the extraction kit (EZ1 DNA, Qiagen, Hilden, Germany), following the manufacturer's instructions. The extracted DNA was eluted in 200 µL of elution buffer and stored at -20°C.

2.4 Screening of samples by real-time PCR

Real-time PCR assay was performed to detect the presence of carbapenemase encoding genes using specific primers for bla_{OXA-23}, bla_{OXA-24}, bla_{OXA-48}, bla_{OXA-58}, bla_{KPC}, bla_{NDM}, and bla_{VIM}. All colistin resistant bacteria were also screened for the presence of extended-spectrum β-lactamase (bla_{CTX-M}, bla_{TEM}, bla_{SHV}) genes and for the mcr1-2 gene. In each assay, negative and positive controls were used.

2.6 Multilocus sequence typing

Multilocus sequence typing (MLST) of the isolates was done to determine the genetic relationship among the clinical isolates using seven housekeeping genes for Escherichia coli, Klebsiella pneumoniae, and Enterobacter cloacae, as described on Institute Pasteur's MLST Web site (www.pasteur.fr/mlst).

2.5 Detection of antibiotic resistance genes and sequencing of colistin resistant genes.

PCR amplification products that were positive for the antibiotic resistance gene tested were sequenced using BigDye terminator chemistry on an automated ABI 3130 sequencer (PE Applied Biosystems, Foster City, CA).

Analysis of the sequenced genes was done using the ARG-ANNOT database (17) (http://en.mediterraneeinfection.com/article.php?laref=283&titre=arg-annot), and were compared to other genes using the BlastN and BlastP tool from the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov).

To determine the supposed gene(s) involved in colistin resistance, the genes (pmrA, pmrB, phoP and phoQ) of E.coli, and (mgrB, pmrA, pmrB, phoP and phoQ) of K.pneumoniae and E.cloacae were amplified and sequenced. Analysis of the sequenced genes was compared against the reference strain E.coli K-12 MG 1655, K. pneumoniae MGH 78578, and E.cloacae ATCC 13047 (NCBI GenBank accession no.CP000647). After that, analysis and comparison of the translated amino acid sequences was done using npsa_clustalwan software (https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_clustalwan.htm.) Next, we used PROVEAN (Protein Variation Effect Analyser) software (http://provean.jcvi.org/seq_submit.php), to verify whether the substitutions of the amino acids would affect the function of the proteins. In addition, the ISfinder database (https://isfinder.biotoul.fr/) was used to analyze the insertion sequences.

3. Results

3.1 Bacterial strains

For the selection of the bacteria, samples that were grown on LBJMR medium, were identified by MALDI-TOF. Eight colistin-resistant strains out of twenty-three rectal swabs, which were collected from ICU patients from Saint-George Hospital in Beirut, were isolated. Among these isolates, 5 strains were identified as Escherichia coli, 2 strains as Enterobacter cloacae, and 1 strain as Klebsiella pneumoniae. In addition, four bacteria that were intrinsically resistant to colistin were also isolated (1Proteus mirabilis and 3 Morganella morganii) that were excluded from this study.

3.2 Antimicrobial susceptibility testing

Antibiotic susceptibility testing results revealed resistance rates of all isolates to amoxicillin, amoxicillin/clavulanic acid, cefalotin, doxycycline, and colistin. All the isolates had MICs for

colistin that ranged from 8 to 32 mg/L (Table1). In addition, all the isolates were susceptible to ertapenem, imipenem. Resistant to piperacillin/tazobactam, trimethoprim sulfamethoxazole, ceftriaxone, ciprofloxacin, cefepime and fosfomycin, is shown in (Table1). None of the isolate was resistant to amikacin, gentamicin, and nitrofurantoin.

3.3 Molecular typing of clinical isolates

MLST analysis was done to investigate the clonal relationship of the isolates. Results showed that the five *E.coli* belonged to five different sequence types (STs) including ST131, ST6174, ST405, ST162 and ST1451, *K. pneumoniae* has ST45, and the two *E.cloacae* has new sequence types ST924, and ST925 according to Pasteur web site (Table 2).

3.4 Resistance-gene determination by RT-PCR

Real-time PCR results showed that *E.coli* (5, 10, and 23), *E.cloacae* (14A, and 14B), and *K. pneumoniae* (16), were positive for the extended spectrum ß-lactamases (ESBL) bla_{TEM} gene. All the sequences of the bla_{TEM} gene were identified as bla_{TEM-1} except E.coli (23) which was identified as $bla_{TEM-163}$. Moreover, four of five strains of *E.coli* (10, 12, 21, and 23) harbored the $bla_{CTX-M-A}$ gene. Two out of four harbored the $bla_{CTX-M-15}$, one isolate producing $bla_{CTX-M-103}$, and one isolate producing $bla_{CTX-M-189}$. In addition, *K.pneumoniae* (16) harbored bla_{SHV-1}. None of the strains contained the bla_{OXA-23}, bla_{OXA-24}, bla_{OXA-48}, bla_{OXA-58}, bla_{KPC}, bla_{NDM}, bla_{VIM}, $bla_{CTX-M-B}$, and mcr-1/2 genes (**Table 2).**

3.5 Mechanisms of Resistance to Colistin

Due to the absence of mcr-1/2 genes, *mgrB,* pmrA, pmrB, phoP and phoQ genes, that were responsible for colistin resistant, were amplified and sequenced. For the two-colistin resistance E.cloacae strains, sequences were compared to E.cloacae ATCC 13047 (NCBI GenBank accession no.CP000647). Results showed that E.cloacae (14) had mutations in *mgrB*, pmrA, and pmrB; and E.cloacae (14B) had mutations in *mgrB*, and phoP. These mutations in mgrB were detected for the first time and were considered as deleterious by the software PROVEAN. Moreover, the 5 E.coli strains sequences were compared against the reference strain E.coli K-12 MG 1655 (NCBI GenBank accession no.CP000647). The analysis revealed that the colistin resistance of E.coli (5) was due to different missense and deletion mutations in pmrA, and pmrB. For E.coli (10), there are missense mutations in phoP and phoQ; for E.coli (12), there are

missense, insertion and deletion mutations in pmrB, phoP and phoQ; for E.coli (21), there are missense mutations in pmrA, and phoP; and for E. coli (23) colistin resistant was due to mutation in pmrA, and phoPQ. All these mutations were verified as deleterious by the software PROVEAN. Finally, *K. pneumoniae* sequences were compared against the reference strain *K. pneumoniae* MGH 78578 (NCBI GenBank accession no.CP000647). Analysis revealed that there was two genetic changes linked to colistin resistance: first, an insertion sequence (IS) in the *mgrB* that belongs to the IS1 family; and second, a missense and deletion mutation in the *pmrB* that was identified as deleterious by the software PROVEAN (Table 3).

4. Discussion

The increase of multidrug resistance Gram-negative bacteria, especially carbapenem-resistant bacteria, is a worldwide clinical problem. This will leads to the re-introduction of colistin into clinical use to treat infections caused by MDR (12, 18). This study describes the prevalence and the mechanisms of colistin resistance in clinical isolates collected from rectal swab of Saint-George Hospital in Beirut, Lebanon. The high prevalence of colistin resistance strains may be due to the increasing use of colistin in this hospital. To the best of our knowledge, this is the first report that describe the mutation of mgrB in colistin resistant *E.cloacae.* Moreover in Lebanon, there is no report describing the mechanism of colistin resistance of Enterobacteriaceae clinical isolates except a study reported recently by one of our group Okdah et al. that describe three colistin-resistant K. pneumoniae isolates collected in 2015 from Sahel Hospital in Beirut. The resistant of those strains was due to a mutation of mgrB, pmrA, pmrB, and phoQ genes (14). Another study in Lebanese poultry was recently performed by Dandachi et al. where they first detected the presence of mcr-1 gene in colistin resistant E.coli strain collected in 2015 (19).

In our study, we founded 2 *E. coli*, 2 *E. cloacae*, and 1 *K. pneumoniae*, harbored bla_{TEM-1} gene, and 1 E.coli harbored $bla_{TEM-163}$. Moreover, two E.coli were positive for the $bla_{CTX-M-15}$, and two E.coli harbored $bla_{CTX-M-103}$, and $bla_{CTX-M-189}$ respectively (Table 2). In Lebanon, previous studies have revealed the presence of bla_{TEM-1}, and $bla_{CTX-M-15}$ (20, 21, and 22) whereas $bla_{TEM-163}$, $bla_{CTX-M-103}$, $bla_{CTX-M-189}$, and bla_{SHV-1} have not been previously reported.

MLST analysis revealed that the five *E. coli* belonged to five different sequence types (ST) including ST131, ST6174, ST405, ST162 and ST1451, *K. pneumonia* has ST45, and the two *E.cloacae* belonged to new sequence types ST924, and ST925.This finding suggests there were

no relationship between the different types of strains isolated from the same hospital (Table 2). It has been shown that the clone ST131 is a globally important pathogen among multidrug resistance E. coli and is linked to nosocomial and acquired infections (23). Moreover, the clones of the other Enterobacteriaceae ST405, ST162, ST1451 and ST45 have been reported in hospitalized patients in different counties such as Brazil, Spain, Sweden and Uruguay (24, 25, 26, 27), in addition ST405 was also reported in animals in Algeria (28).

In this study, we described colistin-resistant strains, isolated from patients admitted to colistin. To date, we were not detected the presence of mcr-1/2 that confer resistance to colistin in human in Lebanon. However, we found that the resistance of those strains was due to chromosomal mutations of the two-component systems (TCSs) pmrA/pmrB, phoP/phoQ, or its negative regulator mgrB. The results of the sequencing are presented in **Table 3**.

Sequencing suggests that the colistin resistant phenotype observed in E. coli (5) was mediated by a unique missense mutation of R81S in pmrA. This same mutation was found in E. coli (23). R81S has been detected in colistin resistant E. coli isolated from samples of animal origin through the Spanish Surveillance Network of Antimicrobial Resistance in Bacteria (29, 30). R81S is similar also to the missense mutation R81H that was found in the pmrA gene of S. Typhimurium (31, 32). In addition, a missense mutation, and two deletions in pmrB for E. coli (5) has been identified and has not been detected before. Using PROVEAN software, those mutations were considered as deleterious and it may contribute to resistance by affecting the activity of pmrA/B. No mutations was observed in phoP/Q system.

The colistin resistant E. coli (10) was due to unique missense mutation R163P in phoP, and different missense mutations in phoQ. No mutation was found in pmrB. In Taiwan, a similar mutation to the one that we found in phoQ, D150W has been reported in K. pneumoniae collected from patients of the Taipei Veterans General Hospital (33).

E. coli (12) was resistant to colistin due to a mutation in pmrB, and phoPQ. The major mutations were found for the first time and were considered as deleterious using PROVEAN software except T92P, and P94S in pmrB that were similar to T92A, P94Q described at pmrB of S. Typhimurium (12, 31). The polymorphism at this position (92T→P, 94P→S) does not exactly match the variants of the alleles detected previously (92T→A, 94P→Q), but it corresponds to a

conserved mutation at this position of the protein. L140H was similar to T140P at positions close to the one found in pmrB of K. pneumonia in a study done by olaitan et al (34).

The colistin resistant phenotype of E. coli (21) was due to mutation in pmrA, and phoP; and E. coli (23) was due to mutation in pmrA, and phoPQ. A single nucleotide polymorphism E220I in E. coli (21) was found in pmrA that has not been previously described. Different missense mutations in phoPQ of E. coli (21) has been found; D191Y was identified in the phoP gene in South Africa in clinical K. pneumoniae isolate (35). Olaitan et al. have also identified the mutation L96P in K. pneumoniae isolated from healthy individuals and patients in Lao PDR, Thailand, Nigeria and France (34) and that is similar to L96S found in the phoQ, which corresponds to a conserved mutation at this position of the protein. Another missense mutation in phoP and different mutations was found in phoQ in E. coli (23) that were not been previously reported.

Regarding the colistin-resistant K. pneumonia (16) isolates, results showed that the resistance was mediated by the inactivation of mgrB, and pmrB genes (Table 3). A disruption of the mgrB gene by an insertion sequence (IS) belonging to the IS1 family, have been observed. Okdah et al have already reported this type of mutation in Lebanon (14). Moreover, a different new gene mutations in the pmrB gene have been found and they were considered deleterious, affecting the protein function.

Finally, the E. cloacae (14) was resistant to colistin due to mutation of mgrB, and pmrAB genes, and the E. cloacae (14B) was resistant due to mutation of mgrB, and phoP. To date there were no results that describe the mutation of mgrB in E. cloacae that are likely to be responsible for colistin resistant such as K. pneumonia. It has been found by Jayol et al. that all the isolates of Enterobacter spp had no alterations in mgrB gene, leaving the mechanism of colistin resistance unexplained (36). In our study, an insertion of G between G37 and V38 leads to a frameshift mutation. Moreover, different missense mutations has been found; such as C39G, N42S, I45Y, W47V, and *48K, and they were similar to the mutation identified in the mgrB of K. pneumonia (30). In addition, the D8T mutation was similar to E8D found in pmrA of Acinetobacter baumannii, collected from French patient in Marseille (37) and the V277E mutation in pmrB was similar to Q277H at positions close to the one found in pmrB of Acinetobacter baumannii (12). For E. cloacae (14B), different missense mutation have been found in mgrB. W47S

48V are similar to W47R, and W47, and *478Y that were described by Poirel et al. (30). In addition different alterations have been described in phoP that were not been previously reported.

In conclusion, this study describes the first detection of mutation in mgrB of E. cloacae and the emergence of colistin resistance in Lebanon. The use of polymyxins to treat patients could significantly contribute to the emergence of colistin resistance strains. MLST showed that there was no relation between the circulating clones. Moreover, our data describe the genetic mechanism triggering the resistance to colistin in the different species. The TCS pmrA/pmrB, phoP/phoQ, or its negative regulator mgrB seemed to play a major role in the resistance regulation. An urgent strategy need to be adopted to control the spread of such resistant microorganisms among patients.

Conflict of Interest

No conflicts of interest for all authors to declare.

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TABLE 1: Antimicrobial susceptibility of the colistin resistant strains isolated from Saint Georges hospital in Lebanon

Sample	Bacterial Species	Beta-Lactamines								Aminoglycosides								Colistin MIC (µg/ml)
		AMX	AMC	TZP	CF	CRO	FEP	ERT	IPM	AK	GEN	CIP	FF	F	DO	SXT	CS	
5	*E.coli*	R	R	S	R	S	S	S	S	S	S	S	S	S	R	S	R	**16**
10	*E.coli*	R	R	S	R	R	R	S	S	S	S	R	S	S	R	S	R	**16**
12	*E.coli*	R	R	S	R	S	R	S	S	S	S	S	S	S	R	S	R	**16**
21	*E.coli*	R	R	R	R	R	R	S	S	S	S	R	S	S	R	S	R	**16**
23	*E.coli*	R	R	S	R	S	R	S	S	S	S	R	S	S	R	R	R	**8**
14	*E.cloacae*	R	R	S	R	S	S	S	S	S	S	S	R	S	R	S	R	**8**
14B	*E.cloacae*	R	R	S	R	S	S	S	S	S	S	S	R	S	R	S	R	**16**
16	*K.pneumoniae*	R	R	R	R	R	R	S	S	S	S	R	S	S	R	R	R	**32**

R_resistant strains; **S** _sensitive strains.

For the phenotypic profile, the antibiotics tested were: amoxicillin (**AMX**), amoxicillin clavulanic acid (**AMC**), piperacillin tazobactam (**TZP**), Cefalotin (**CF**), ceftriaxone (**CRO**), cefepime (**FEP**), ertapenem (**ERT**), imipenem (**IPM**), amikacin (**AK**), gentamicin (**GEN**), ciprofloxacin (**CIP**), fosfomycin (**FF**), nitrofurantoin (**F**), doxycycline (**DO**), trimethoprim sulfamethoxazole (**SXT**), colistin (**CS**).

TABLE 2: Genotypic features of nine colistin resistant strains Isolated from Saint-Georges hospital in Lebanon

Strain #	Strain Type	*bla*SHV	*bla*TEM	*bla*CTX-M-A	mcr1-2	ST
5	***E.coli***	-	TEM1	-	-	1451
10	***E.coli***	-	TEM1	CTX-M-15	-	131
12	***E.coli***	-	-	CTX-M-189	-	6174
21	***E.coli***	-	-	CTX-M-15	-	405
23	***E.coli***	-	TEM163	CTX-M-103	-	162
14A	***E.cloacae***	-	TEM1	-	-	924
14B	***E. cloacae***	-	TEM1	-	-	925
16	***K. pneumoniae***	SHV1	TEM1	-	-	45

TABLE 3: Mutation of the colistin Resistant gene

Sample	Enterobacter	mgrB	pmrA	pmrB	phoP	phoQ
5	***E.coli***		L11Q R81S	S205F, $del_{206-211}$, D216T, V217S, $del_{221-227}$	No mutation	No mutation
10	***E.coli***		No mutation	No mutation	R163P	I88T, L95P, P111T, W113R, L114S, S116W, R145W, D150W, H157P, L218F
12	***E.coli***		No mutation	L79S, T80P, T80_L84insDA, C84Y, V88P, V88_A90insG, I91T, T92P, R93H, P94S, L95P, L98W, Q99R, E101C, L102K, L140H, E143A, R144G	Q113P, K171R $del_{188-199}$, K200R, I201P, Q202L, $del_{205-222}$	W192R, L231R, N235I
21	***E.coli***		G15E R81S D82del D86R	No mutation	K171R, R185P, S187I, D191Y, K200R, I210S, V213G, R214S, G215R, Q216P,	No mutation
23	***E.coli***		R81S	No mutation	N119I	R53H, T61H, $del_{139-149}$
16	***K. pneumoniae***	IS1	No mutation	D150N, $del_{205-212}$, L214R, $del_{215-232}$	No mutation	No mutation
14	***E. cloacae***	G37_V38insG V38S	M1del, K2del, L3del,	E33S, Q34del, I35del, ,	No mutation	No mutation

Conclusion du chapitre

Via ce chapitre, nous avons rapporté la dissémination de la résistance plasmidique et chromosomique à la colistine chez les humains en France, au Laos et au Liban. La propagation de la résistance à la colistine dans ces régions et dans le monde entier semblerait s'expliquer par l'utilisation intensive de la colistine dans les productions animales. Il a été démontré que les gènes de résistance présents dans les isolats d'origine animale peuvent se transférer à l'homme (**Olaitan AO, Morand S, Rolain J-M. 2016**). Récemment, le phénomène de la résistance aux antibiotiques notamment à la colistine est devenu une préoccupation mondiale (**Abat et al., 2018**). Nous estimons que les enquêtes épidémiologiques chez les humains à travers des techniques de détection rapide en routine pourraient nous permettre de surveiller cette dissémination et l'émergence de la résistance aux antibiotiques et à la colistine en particulier. Cela permettra de préserver un arsenal thérapeutique efficace contre les maladies infectieuses.

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VI- ANNEXES

Au cours de ma thèse, j'ai pu participer à des travaux de collaboration avec d'autres doctorants, d'une part (**Article N° 13**) sur la résistance aux antibiotiques en particulier sur la détection des BLSE et la caractérisation des bactéries multirésistantes isolées à partir des oiseaux urbains de la ville de Marseille notamment à partir des pigeons et des goélands. Dans cette étude nous avons rapporté la présence de gènes de résistance aux antibiotiques chez les oiseaux urbains de Marseille dont nous pensons qu'ils n'ont à priori aucun contact avec les antibiotiques. Cela démontre dans ce cas que les oiseaux urbains peuvent constituer un réservoir de gènes de BLSE et cela soit parce qu'ils sont en contact étroit avec les activités humaines, ou soit parce qu'ils sont porteurs de ces gènes.

Une deuxième étude collaborative (**Article N° 14**) a porté sur la détection et la description de souches d'Enterococcus faecium résistantes à la vancomycine (VREfm) isolées à partir d'échantillons cliniques au Liban. Cette étude démontre la présence de souches VREfm chez des patients au Liban, ce qui constitue un problème de santé publique majeur. En outre, nous rapportons des souches d'entérocoques résistantes à la vancomycine, isolées chez des patients qui n'ont jamais reçu de vancomycine. Des mesures de surveillance et de contrôle doivent être adoptées pour éviter la propagation de telles bactéries dans les hôpitaux.

Par le biais d'une dernière approche de taxonogénomique, nous avons contribué à la description d'une nouvelle espèce, (**Article N° 15**), Clostridium niameyense sp. Nov., bactérie qui a été isolée à partir d'un échantillon fécal recueilli chez un patient souffrant d'anorexie et de marasme vivant au Nigeria.

ARTICLE 13

Title: Molecular investigation of Beta-lactamases-producing Gram-negative bacteria and new ST857 E. coli isolated from birds in Marseille city, France.

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(en correction).

Molecular investigation of Beta-lactamases-producing Gram-negative bacteria and new ST857 E. coli isolated from birds in Marseille city, France

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Keywords: Pigeon, Chicken, yellow-legged gull, antibiotic resistance; Extended-spectrum beta-lactamases (ESBL), Parks, Beaches**.**

Abstract

Background: There is a need to identify the reservoirs of antibiotic resistance genes including hospital and non-hospital settings. Therefore, the aim of this study was to characterize the multiresistant bacteria isolated from urban birds in the city of Marseille, France.

Methodologies: Between July-September 2016, one hundred and thirty-six fresh stool samples from pigeons; yellow-legged gulls and Chicken were collected in the parks and beaches of Marseille. Strains were isolated on MacConkey agar supplemented with ceftriaxone and LBJMR medium and identified by MALDI-TOF. Resistance genes including bla_{CTX-M}, bla_{SHV}, bla_{TEM-1}, and mcr-1-2 were screening for by PCR followed sequencing when positive**.**

Results: Eight Gram-negative bacterial producing extended-spectrum beta-lactamases (ESBL) were identified and characterized on MacConkey. Escherichia coli producing-ESBL was isolates in predominant. Hafnia alvei and Klesbiella oxytoca isolates of LBJMR medium have MICs to colistin ≥2µg/ml. bla_{CTX-M} (n=4) and bla_{TEM} (n=4) genes were detected in bacterial strains isolated from five yellow-legged gulls, two chickens and one pigeons. Neither mcr-1 or mcr-2 gene was detected in colistin resistant isolates. Multilocus sequence typing performed for E. coli strains revealed 4 different STs already described in humans and animals and one new ST857.

Conclusion: Our study reveals the presence of antibiotic resistance genes in urban birds that are believed to have no contact with antibiotics. We have showed that urban birds are a reservoir of ESBL genes either because these birds are in close contact with human activities or because they are carriers. This lead, to the question of limitation of proliferation of these birds to avoid the diffusion of antibiotic resistance genes in the community.

Introduction

Extended-spectrum beta-lactamases (ESBL) are mainly plasmid-encoded enzymes that induce third-generation cephalosporin resistance. They are usually acquired by the horizontal gene transfer (HGT) mobilized in the environment [1]. This ESBL enzymes including Class A β-lactamases, such as TEM, SHV type, which confer resistance to penicillin class and CTX-M type conferring resistance to cephalosporin class such as ceftriaxone, cefotaxime, Ceftazidime. ESBL-producing Enterobacteriaceae caused infections in both humans and animal via water, food contamination [2] [3]–[5]. They have been also detected in birds from different countries [6]. However, CTX-M β-lactamase enzymes are the most emerged and reported in the worldwide [1]. Emergence of the pandemic bla_{CTX-M} gene during the new century and outbreak of CTX-M-15-producing Enterobacter cloacae were described in France [1], [4], [7]. In recent years, divers reports have been indicated the presence of ESBL-producing bacteria among different species of urban and wild birds with no apparent exposure to antimicrobials [8]. In fact, pigeons and gulls are considered migratory birds and reservoirs of resistant bacteria [9], [10]. Especially gulls are free-living aquatic birds and can serve as reservoirs, vectors for antibiotic-resistant bacteria. Due to their frequent mobility in landfills, wastewater and the ease with which they pick up foods from various media, they can induce fecal contaminants in natural water tanks or food that they are in contact with [6], [11]. However, E. coli producing $bla_{CTX-M-1}$ and bla_{TEM-1} isolates have been detected in healthy chickens in Tunisia [12], Germany [13], Algeria [14] and in France [4]. In 2011, Abulreesh et al. described pigeons as an environmental reservoir of antimicrobial-resistant enteric bacterial pathogens in Saudi Arabia [15].

Here, we aim to investigate and to characterize the phenotypes and genotypes of bacterial strains isolated from urban birds in the city of Marseille, France.

Materials and Methods

Sample collection: Between July and September 2016, six Parks located respectively in four different districts of Marseille city and one beach located in the 16th district of Marseille were investigated (**fig. 1**). A total of 136 fresh fecal samples from 71 pigeons (Columba livia), 28 chickens (Gallus gallus domesticus), and 37 yellow-legged gulls (Larus michahellis) were collected in these parks and beaches (**fig. 2**). Each fresh fecal sample was collected on the ground using sterile forceps and transferred into sterile 1.5ml Eppendorf tubes. Samples were placed immediately into ice during transportation and microbiological processes that followed the days of collection.

DNA Extraction and ESBL detection: Genomic DNA from fecal sample was prepared from an overnight incubation at 56°C and extracted with the EZ1 DNeasy Blood Tissue kit (Qiagen GmbH, Hilden, Germany) using the protocol of the manufacturer. The presence of genes encoding CTX-M, TEM, and SHV was investigated by Real-Time PCR with the specific primers. Briefly, 5µl genomic DNA was added on 15 µl of Mix (Quantitec, primers, probe and water) according to protocol by using thermo cycler CFX Bio. The program parameters for the Real-Time PCR consisted of a hold at 50°C for 2 min; 35 cycles of 95°C for 15 min (denaturation), 95°C for 1s (annealing), 60°C for 35 s (extension) with a slope of 2; and a final extension at 45°C for 30s.

Isolation and detection of strains: After homogenizing of all positive PCR detected stool samples with 1ml sterile water, 100µl were enriched in Tryptic Soy Broth (BBL™ Trypticase™ Soy Broth, Becton, Dickinson and Company Sparks, MD 21152 USA) and incubated overnight at 37°C for preliminary analysis. For further investigation, these samples suspected for ESBL producing strains were cultured on MacConkey agar supplemented with ceftriaxone (2µg/ml) and LBJMR medium [16] which constituted of colistin (4µg/ml) and

vancomycin (50µg/ml) and incubated at 37°C during 24 h. One to eight colonies showing different morphology were taken from each medium MacConkey with ceftriaxone and LBJMR medium and re-isolated on Trypticase Soy Agar (TSA, BioMerieux SA). Each of these colonies was subcultured 3 additional times to obtain a pure bacterial culture. Isolates were identified by matrix assisted laser desorption/ionization, time of flight mass spectrometry (MALDI-TOF MS), as described previously (Biotyper Microflex, Bruker Daltonik GmbH, Bremen, Germany)(Cox, Saichek et al. 2014) .

Antimicrobial susceptibility testing: Antibiotic susceptibility testing was performed using the agar disk diffusion method as described by the Antibiogram Committee of the French Society for Microbiology (CAS-SFM) (www.sfm-microbiologie.org). Bacterial strains of each sample were suspended in saline solution to a density of 0.5 on the McFarland scale. The surface of Mueller-Hinton agar plates (Merck) was inoculated and antibiotic disk were placed. After incubation, aerobically at 37°C for 18-22h, bacterial resistance was determined by measuring the inhibition zone according to the CA-SFM guidelines. The following Antimicrobic agents were tested (µg/disk): amoxicillin (25), amoxicillin-clavulanic acid (30), cefepime (30), piperacillin-tazobactam (85), céfalotin (30), ceftriaxone (30), ertapenem (10), imipenem (10), fosfomycin (50), nitrofurantoin (300), trimethoprim -sulfaméthoxazol (25), amikacin (30), ciprofloxacin (5), doxycycline (30), colistin (50), gentamicin (15).The growth inhibition by antibiotic associated to clavulanic acid, a ß-lactamase inhibitor indicates the presence of an ESBL enzyme. For bacteria isolated on LBJMR medium which have not natural resistance to colistin, the MICs by broth microdilution method were performed from 256µg/ml colistin using E. coli AY236073 as strain positive control and interpreted according to the coloration of plaque.

Gene amplification: DNA of ESBL-positive isolates was extracted with the DNeasy Blood &Tissue kit (Qiagen, Hilden, Germany) using the protocol of the manufacturer. These DNA extracted from bacterial cultures were used for determination and confirmation of the presence of resistance genes. The resistance genes encoding for ESBL genes including bla_{SHV}, bla_{CTX}, bla_{TEM}, bla_{PER}, bla_{GES}, and bla_{VEB} were analyzed by Real-time PCR and confirmed by conventional PCR. PCR reactions were carried out in a 25µl final volume using 6.5µl sterile water, 1.25mL Master Mix (2X) Quantitec, forward primer (25mM), reverse primer (25mM) and 5µl DNA isolated from bacterial strains ESBL-positive. PCR reactions were carried out in an automated thermocycler with the PCR cycling conditions of initial cycle at 94°C for 15 min (hold); 35 cycles of 94°C for 1 min (denaturation), 55°C for 50 s (annealing), 72°C for 1 min (extension) with a slope of 2; and a final extension at 72°C for 7min. Primers used for PCR are presented in **Table S1**. To confirm PCR amplification, 5µl of each PCR products with 2µl of charge blue (Blue Juice™, Invitrogen) were separated in 1.5% agarose and 0.5%TBE gel with SYBR Safe (1µg/mL) using the Electrophoretic (Mupid-One, Japon). After electrophoresis done for 20 min with a voltage of 135V, DNA profiles were visualized by ultraviolet (UV) light on a UV trans illuminator.

Sequencing and MLST: The nature of the ESBL was determined by sequencing of the PCR amplicons, which were purified with a PCR purification kit according to the manufacturer's instructions. Sequencing was performed by using the BigDye Terminator Cycle Sequencing kit and an automated fluorescent DNA sequencer ABI 3730xl (Applied Biosystems, Foster City, USA) in accordance with the manufacturer's instructions. The obtained nucleotide sequences were compared with those from the GenBank database (www.ncbi.nlm.nih.gov) by BlastN analysis. Multilocus sequence typing (MLST) was performed for E. coli strains by using the genes from both the Warwick (adk, fumC, gyrB, icd,

mdh, purA, and recA) (http://mlst.warwick.ac.uk/mlst/dbs/Ecoli) and Pasteur Institute (icd, pabB, polB, putP, trpA, and trpB) (http://www.pasteur.fr/recherche/genopole/PF8/mlst/) schemes. The ST not found in the database was submitted to the Pasteur MLST database in order to define the new profile.

Results

Isolation and identification of bacterial strains and susceptibility antimicrobial

Out of 136 birds stool samples tested, 28 Gram-negative bacterial strains were isolated on MacConkey and 23 Gram-negative bacterial on LBJMR medium. The bacterial colonies identification by MALDI-TOF MS reveals the presence of Escherichia coli, Cronobacter sakazakii, Pseudomonas aeruginosa, Acinetobacter baumannii, Acinetobacter junii, Enterobacter cloacae, Acinetobacter pittii, Stenotrophomonas maltophilia, Morganella morganii, Hafnia alvei, Klesbiella oxytoca, Proteus mirabilis, Proteus hauseri, Panteoa ananatis, Providencia alcalifaciens and Serratia marcescens isolates. Only 9 isolates showed a reduce susceptibility to third-generation cephalosporin including E. coli (n=6), M. morganii (n=1), C. sakazakii (n=1) and P. aeruginosa (n=1). These isolates exhibit a resistance phenotype to amoxicillin-clavulanic acid, ceftriaxone and cefotaxime. Combined resistance to these three antibiotics in addition to ceftazidime resistance occurred overall in 7 of the bacterial isolates and was particularly frequent in E. coli (n=6) from yellow-legged gulls (n=4); chicken (n=1) and pigeon (n=1). Resistance phenotype to aminoglycosides (gentamicin and doxycyclin) was observed in one E. coli isolate from chicken. Resistance to ertapenem was observed in C. sakazakii and P. aeruginosa isolates from yellow-legged gulls and chicken respectively (**Table 1**). All E. coli isolates were susceptible to imipenem and ertapenem. Apart from naturally resistant strains to colistin (M. morganii, S. maltophilia, Proteus mirabilis, Proteus hauseri, Panteoa ananatis, Providencia alcalifaciens and Serratia

marcescens), the MICs by microdilution method were 16µg/ml and 32µg / ml respective in one Hafnia alvei and one Klesbiella oxytoca resistant to colistin. Others Hafnia alvei isolates have a MIC of 4µg/ml as also E. coli (AY236073) strain positive control (**Table2**).

None strain of positive RT-PCR samples for SHV and TEM genes in pigeons and chickens were isolated on MacConkey with ceftriaxone.

Molecular detection of antibiotic resistance-encoding genes

Real-time PCRs performed directly on fecal specimens showed 15 positive SHV genes in pigeons (n = 5), chicken (n = 5) and yellow-legged gull. But unfortunately, the bacteria of these samples did not push on the culture medium to confirm the positivity of the SHV genes in standard PCR.

For 9 strains, phenotypically identified as resistant to third-generation of cephalosporins, the standard PCR and sequencing revealed the presence of one of the ESBL genes including bla_{CTX-M} and bla_{TEM} in 8 strains (**fig. 3**). Genes encoding CTX-M-type were detected in four isolates (**Table 1**). The $bla_{CTX-M-15}$ gene was found in three E. coli isolates and $bla_{CTX-M-1}$ was only found in one E. coli isolate. The bla_{TEM-1} β-lactamase gene was detected in four of these ESBL-positive isolates: E. coli (n=2), C. sakazakii (n=1), P. aeruginosa (n=1). One of the E. coli isolate, which carried the bla_{TEM-1} gene, was resistant to gentamicin and doxycyclin. None of the seven different aminoglycosides resistance genes (aad, ant, aph, aac(6')-Ib, aac(3'), armA, and aac(6')-Ib-cr) was detected in this E. coli strain, resistant to gentamicin and doxycyclin. The blaCTX-M-15 gene was also detected in three E. coli isolates in two yellow-legged gulls and one pigeon. Simultaneous presence of $bla_{CTX-M-1}$ and bla_{TEM-1} was detected in one E. coli. As well, blaTEM-1 were detected in one E. coli, C.sakazakii and P.aeruginosa

isolates (chicken and yellow-legged gulls) respectively (**Table 1**). bla_{VEB}, bla_{GES}, bla_{PER}, bla_{SHV}, mrc-1, and mcr-2 genes have not been detected in any of isolates.

MLST analysis performed of E. coli isolates revealed five different sequence types (STs) including ST10 complex, ST34 (ST10 complex), ST38 complex, ST1652 carrying CTX-M type β-lactamases and a new ST who has been defined as ST857. The E. coli ST10 complex and ST38 complex carried the $bla_{CTX\text{-}M\text{-}15}$ detected in yellow-legged gull. E. coli ST34 belonging to the ST10 complex isolated in Pigeon harbored a $bla_{CTX\text{-}M\text{-}15}$ gene. Finally, E. coli ST1652 carried $bla_{TEM\text{-}1}$ gene detected in chicken and the E. coli ST-new carried $bla_{CTX\text{-}M\text{-}1}$ detected in yellow-legged gull. E. coli sequence type ST131 associated with CTX-M-15 extended-spectrum β-lactamase (ESBL), who emerged as the leading cause of community-acquired urinary tract infections and bacteremia worldwide was not detected among our isolates.

Discussion

Emergence and wide dissemination of ESBL, mainly of CTX-M class, among E. coli isolates have been widely reported in recent years in community patients and have also been found in food producing animals, pets, as well as the environment [17] [2]. A more recent study in Brazil showed wild birds and urban pigeons as reservoirs of E. coli responsible for severe diarrhea in humans [15]. In the current study, we have isolated E. coli produced ESBLs with CTX-M and TEM type enzymes. All of them were identified as CTX-M-15 or CTX-M-1 or as combinations of CTX-M-1 with TEM-1 (n=1) or CTX-M-15 (n=3) or even TEM-1 (n=3). $bla_{TEM\text{-}1}$ gene was the predominant enzyme in yellow-legged gulls (n=2) and chickens (n=2), followed by CTX-M-15 in yellow-legged gull (n=2) and pigeon (n=1) and final one CTX-M-1 isolated in yellow-legged gull.

In recent years, the bla_{TEM-1} gene has been widely described in poultry in Brazil [18], Tunisia [19] , Romania [2] and Germany [20]. TEM-1-type beta-lactamases- producing K.pneumoniae isolates were detected from companion animals in Italia [21] and France [22]. TEM-1-beta-lactamase-producing Klebsiella pneumoniae was isolated from Barbary macaques and wild boars in Algeria [23]. bla_{TEM-1} gene was reported in pig [24], gulls [25], birds [22] and human respective in Portugal, Spain, France and Germany [26].

The $bla_{CTX-M-1}$ gene was the most frequent ESBL gene in poultry isolates in Brazil [27], Romania [2], Portugal [28], Germany [29], Tunisia [19], [30], and Algeria [14]. Recently, Tiago et al. [4] was showed the high prevalence of $bla_{CTX-M-1}$ type ESBLs in retail chicken meat in France. CTX-M-1-type beta-lactamases- producing E. coli isolates were detected in wild birds in Spain [31], companion animals in Italia [21], and Germany [32]. Recently, Haenni and colleagues have reported the high prevalence of $bla_{CTX-M-1}$ in healthy urban dogs in France [33] . Seagulls were identified as reservoirs of CTX-M-1 gene in Sweden [34], Spain [31] and Portugal [35]. CTX-M-1- and CTX-M-15-type beta-lactamases in clinical E. coli isolates were detected in Romania [2], Germany [26] and Algeria [36]. CTX-M-1 and CTX-M-15-producing E. coli were screened in dog faeces from public gardens in Denmark [37].

Anterior studies have described the $bla_{CTX-M-15}$ gene spread pandemically in humans [3] [5] [26] but this gene was only detected incidentally in seagulls [8] [25], pigeon [38][39], poultry [13],[40], horse and companion animals [32], pig and cattle [40]. High rates of CTX-M-15-producing Escherichia coli and Klebsiella pneumoniae were observed in wild boars, in Barbary macaques and wild fish in Algeria [41][23]. In France, $bla_{CTX-M-15}$ gene was detected from E. cloacae and K. pneumoniae in birds [42], companion animals [22], horse, water [43] and human [7].

ESBL-producing-E. coli and K. pneumoniae isolates carrying the blaCTX-M and blaTEM-1 genes and belonging to a sequence type clonal complex ST10 and ST38 have been recovered from human [3] and wild birds [8]. Major STs, ST38 (n = 15, 11.5 %) and ST10 (n = 8, 6.2 %, 8) were among the main clones of E. coli producing CTX-M in Canada [44] and among ill companion animals in Japan [45]. The ST10 clonal complexes was revealed in ESBL-producing E. coli isolated from piglets with diarrhea in 16 swine farms located in Taiwan [46]. More recently, the ST10 complex has described the contamination of surface waters by E. coli strains belonging to clinically [47]. In general, the clonal phylogenetic group A complex (ST10 / 167/617) represents a successful clonal line, which can be found in humans, livestock, as well as in companion animals [5]. The sequence types (ST) 38 and ST131 are typically observed in humans [48], but the frequent presence of ST38 isolates was observed in wild birds [31], in animals and human [49]. More recently, the team of Linxian Yi declared the spread of mcr-1 in pigs at slaughter in China associated with clonal dissemination of S. typhimurium ST34 [50]. S. aureus sequence type 34 (ST34) was described by Jonathan et al as ST34 has spread to multiple continents and can cause community-associated skin and soft tissue infections in human [51] [52].

We have observed that the STs isolated in our study are completely different from the STs found in gulls with yellow legs in south of France. These STs isolated from urban birds are clones that circulate in humans, animals and the environment, especially waters. Therefore, our study supports previous studies that stated that human and animal isolates shared mainly identical ST, suggesting transmission [13]. In particular, pets such as dogs and cats are considered as potential reservoirs of antibiotic-resistant bacteria due to their close contact with humans and the intensive use of antimicrobial agents in humans and the domestic animals. Indeed, the spread of resistance can occur through direct or indirect contact, through

feeding, water and animal waste disposal in farm fields. It can be by the horizontal transfer of genetic elements. It should be noted that parks are open to dogs that are suspected of being a reservoir of resistance genes; then this supports the study that described the beaches as reservoirs of antibiotic resistance bacteria.

The rapid emergence of CTX-M-15-producing-E. coli in worldwide is thought to be attributable to the spread of mobile genetic elements, as well as to the spread of specific clones, mainly the international ST131 clone [56]. The ST131 clone producing CTX-M-15 is a global pandemic clone, causing mainly infections in the community. In 2008, its pandemic spread was identified in CTX-M-15 producing E. coli isolates from three continents but was not detected in our study. It should be noted that DNAs of ESBL-producing bacteria such as SHV are present, but the bacteria are not cultured, they are dead because of the preservation of the samples.

Conclusion

We have identified the urban birds as vectors for propagation and emergence of ESBLs genes among human populations and natural environments. In addition, we have signaled the role of parks in the horizontal transfer of antibiotic resistance genes. The three different types sequences (ST10cplx, ST34 and ST38) found here have already been detected in humans and animals. This study describes also a ST857, new type sequence in E. coli and for the first time the emergence of Gram-negative bacterial bacteria isolated from birds in parks and beach of Marseille. This is probably due to close contact with human activities.

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Conflict of interest and financial disclosure

No conflicts of interest or financial disclosure for all authors.

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All authors have read and approved the manuscript.

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Figure 1. Map depicting sites of sample collection in Marseille. The sampling sites are located in different districts of the city of Marseille. The pigeon's site, Longchamp Park and the site of yellow-legged gull Palais Longchamp are both located in the 4th district. Parc chanot, site of pigeons and Parc Borely, site of yellow-legged gull is in the 8th district. The Parc 26eme Centenaire site of Pigeons and Chicken is located in the 10th arrondissement. Finally, the Park Mistral (Espace Mistral) site of pigeons and Beach of Estaque (Plages de Corbière) site of yellow-legged gull are located every 2 in the 16th arrondissement.

Table S1: Primers used for Extended-spectrum beta-lactamases (ESBLs) genes detection.

Genes name	Type of PCR	Primer name	Primer sequence (5'->3')	Amplicon size (bp)	Reference
blaTEM	Standard PCR	ALLTEM_STD_F	ATGAGTATTCAACATTTCCGTG	861	Kruger et al., 2004
		ALLTEM_STD_R	TTACCAATGCTTAATCAGTGAG		
	Real-time PCR	ALLTEM_RT_F	TTCTGCTATGTGGTGCGGTA	213	JM_lab
		ALLTEM_RT_R	GTCCTCCGATCGTTGTCAGA		
		ALLTEM_RT_Probe	AACTCGGTCGCCGCATACACTATTCTCAGA		
blaSHV	Standard PCR	SHV_STD_F	ATTTGTCGCTTCTTTACTCGC	1051	Yagi et al., 2000
		SHV_STD_R	TTTATGGCGTTACCTTTGACC		
	Real-time PCR	SHV_RT_F	TCCCATGATGAGCACCTTTAAA	105	Roschanski et al., 2014
		SHV_RT_R	TCCTGCTGGCGATAGTGGAT		
		SHV_RT_Probe	TGCCGGTGACGAACAGCTGGAG		
blaCTX-M	Standard PCR	CTX-M_group1_STD_F	CCCATGGTTAAAAAATCACTGC	944	Roschanski et al., 2014
		CTX-M_group1_STD_R	CAGCGCTTTTGCCGTCTAAG		
		CTX-M_ALL_STD_F	TTTGCGATGTGCAGTACCAGTAA	544	Edelstein, et al., 2003
		CTX-M_ALL_STD_R	CGATATCGTTGGTGGTGCCATA		
	Real-time PCR	CTX-M_groupA_RT_F	CGGGCRATGGCGCARAC	105	Roschanski et al., 2014
		CTX-M_groupA_RT_R	TGCRCCGGTSGTATTGCC		
		CTX-M_groupA_RT_Probe	CCARCGGGCGCAGYTGGTGAC		

Table 1: ESBL producing Gram-negative bacteria isolated on MacConkey medium with cefotaxime.

ID	Isolates	Sources	Sites	Beta-Lactams										Aminoglycosides					*Beta-lactamase* genes		MLST
				AX	AMC	FEP	TPZ	KF	CRO	ETP	IMP	FF	F	SXT	CIP	DO	CT	GEN	CTX-M	TEM	
B11	*E. coli*	yellow-legged gull	Palais Longchamp	R	R	R	S	R	R	S	S	S	S	R	S	R	S	S	CTX-M-15		10 complex10
B32	*E. coli*	Chicken	Parc 26e centenaire	R	R	R	S	R	R	S	S	S	S	R	S	R	S	R		TEM-1	1652
B60	*E. coli*	yellow-legged gull	Plage Estaque	R	R	R	S	R	R	S	S	S	S	R	S	S	S	S	CTX-M-15		38 complex
A61	*E. coli*	Pigeon	Parc 26e centenaire	R	S	R	S	R	R	S	S	S	S	S	S	S	S	S	CTX-M-15		34 complex10
B62	*E. coli*	yellow-legged gull	Plage Estaque	R	R	S	S	R	R	S	S	S	S	S	S	S	S	S	CTX-M-1	TEM-1	857
B62	*C. sakazakii*	yellow-legged gull	Plage Estaque	R	R	R	S	R	R	S	S	R	S	S	S	S	S	S		TEM-1	
B34	*P. aeruginosa*	Chicken	Parc 26e centenaire	R	S	S	S	R	R	R	S	S	R	R	S	R	S	S		TEM-1	

AX: amoxicillin; AMC: amoxicillin-clavulanic acid; FEP: cefepime; TZP: piperacillin-tazobactam; KF: céfalotin; CRO: ceftriaxone; ERT: ertapenem; IMP: imipenem; FF: fosfomycin; F: nitrofurantoin; SXT: trimethoprim -sulfaméthoxazole; CIP: ciprofloxacin; DO: doxycyclin; CT: colistin; CN: gentamycin and ST: sequence type.

Table 2: Enterobacteria isolated on LBJMR medium

ID	STRAIN	AX	AMC	FEP	TPZ	KF	CRO	ETP	IMP	FF	F	SXT	AK	CIP	DO	CT	GN15	MIC colistin (µg/ml)
A42	*K. oxytoca*	R	S	S	S	S	S	S	S	R	R	S	S	S	S	R	S	32
B42	*Hafnia alvei*	R	R	S	S	R	S	S	S	S	S	S	S	S	S	S	S	16
B02	*Hafnia alvei*	R	R	S	S	R	S	S	S	S	S	S	S	S	S	S	S	4
B04	*Hafnia alvei*	S	S	S	S	R	S	S	S	S	S	S	S	S	S	S	S	4
B11	*Hafnia alvei*	R	R	S	S	R	S	S	S	S	S	S	S	S	S	S	S	4
B21	*Hafnia alvei*	S	S	S	S	R	S	S	S	S	S	S	S	S	S	S	S	4
B47	*Hafnia alvei*	R	R	S	S	R	S	S	S	S	S	S	S	S	S	S	S	4
B59	*Hafnia alvei*	R	R	S	S	R	S	S	S	R	R	S	S	S	S	S	S	4
A63	*Hafnia alvei*	R	R	S	S	R	S	S	S	R	R	S	S	S	S	S	S	4
A61	*Panteoa ananatis*	R	R	S	S	R	S	S	S	R	R	S	S	S	S	R	S	>256
B05	*Proteus hauseri*	S	S	S	S	S	S	S	S	S	R	S	S	S	S	R	S	>256
B22	*Serratia marsescens*	S	S	S	S	S	S	S	S	S	R	S	S	S	S	R	S	>256
B36	*Providencia alcalifaciens*	S	S	S	S	S	S	S	S	S	R	S	S	S	S	R	S	>256

ARTICLE 14

Title: Emergence of Vancomycin-Resistant Enterococcus faecium Isolated from Clinical Samples in Lebanon.

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Marseille, 20/03/2018

Journal of Global Antimicrobial Resistance

Dear Editor,

Please find enclosed our manuscript entitled « **Emergence of Vancomycin-Resistant Enterococcus faecium Isolated from Clinical Samples in Lebanon».**

We report in this study the detection of vancomycin-resistant Enterococcus faecium isolated from rectal swabs from patients in University Hospital in Lebanon. The strains harbored the vancomycin resistance gene vanA and have new sequence type. To the best of our knowledge, this is the first detection of VREfm strains in Lebanon.

We believe that this work will be of interest for your readers. I hope it could be published in "**Journal of Global Antimicrobial resistance"** as a **"letter"** to the Editor.

All co-authors have seen and approved the manuscript and have complied with the stipulations in the Instructions to authors. No conflict of interest.

As potential qualified referees, we propose: "Dr.Marie Kempf, Dr.Rafael Canton, Dr.Giuseppe Cornaglia, Dr Max Maurin".

Sincerely yours.
Pr ROLAIN Jean-Marc

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Emergence of Vancomycin-Resistant Enterococcus faecium Isolated from Clinical Samples in Lebanon

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Running Title: Vancomycin-Resistant Enterococcus faecium

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Sir

Infections due to vancomycin-resistant enterococci (VRE) have been recently reported in many countries. There is a high incidence of nosocomial infections that is due to the presence of VRE in intensive care units [1]. In 1988, the first vancomycin-resistant E. faecium (VREfm) was isolated in UK [2]. In Europe, according to the 2012 report of the European Antimicrobial Resistance Surveillance Network (EARSNet), the prevalence of VRE infections ranged from 0% in the Netherlands and Sweden to 44% in Ireland [2]. In the North of France, outbreaks of VRE infections have occurred between 2004 and 2008, meanwhile in the South of France no VRE had ever been detected till now [2]. In a study done in the Middle East and Africa between 2004 and 2011, 32 vancomycin-resistant E. faecium isolates were reported in Israel, Oman, Pakistan and Saudi Arabia [3].

The aim of the present study was to investigate the molecular epidemiology and the prevalence of vancomycin resistance genes in E. faecium collected from rectal swabs in Lebanon. To the best of our knowledge, there is no report describing VREfm clinical isolates in Lebanon.

In our study, 23 rectal swabs were collected from ICU patients between October 2016 and February 2017 from Saint-George Hospital in Beirut. Rectal swabs were shipped to Marseille. Once they arrived, they were transferred to an enrichment broth, Tryptic Soy Broth medium (TSB), and incubated at 37°C. After an overnight incubation, 100 µL of the enrichment medium was cultivated for 24h at 37°C on the selective medium LBJMR that contains colistin and vancomycin [4]. Colonies growing on this medium were identified using the matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometer~~s~~ as described previously [2]. Antimicrobial susceptibility testing was performed using the disk diffusion method on Mueller-Hinton agar as recommended by the 2017 European Committee of Antimicrobial Susceptibility Testing (EUCAST). E-test was

51 performed to validate the resistance phenotype of VRE [2]. Bacterial DNA was extracted
52 using the automatic robot EZ1 (Qiagen BioRobot EZ1-, Tokyo, Japan) with the extraction kit
53 (EZ1 DNA, Qiagen, Hilden, Germany), following the manufacturer's instructions. Molecular
54 detection of vancomycin resistance genes vanA and vanB was performed using PCR
55 amplification. Positive PCR products of the vanA gene were identified by gel electrophoresis
56 and sequenced. To determine the genetic relationship among the clinical isolates, MLST was
57 done by using the seven housekeeping genes adk, atpA, ddl, gdh, gyd, pstS and purK,
58 according to MLST Pasteur.
59 Samples that grow on LBJMR medium were identified by MALDI-TOF. A total of four E.
60 faecium were obtained. Antibiotic susceptibility testing revealed resistance of all isolates to
61 vancomycin, teicoplanin, oxacillin, erythromycin, gentamycin, ciprofloxacin, doxycycline,
62 rifampicin, amoxicillin, daptomycin, and nitrofurantoin. Nearly all the isolates were sensitive
63 to pristinamycin and linezolid. Two out of four strains showed sensitivity to fosfomycin
64 (Table 1). Vancomycin disc diffusion tests showed high resistance levels. The results of the
65 agar dilution were confirmed by E-tests that showed that the MIC of vancomycin was greater
66 than 256 µg/ml. All the isolates harbored the vancomycin resistance gene vanA and none of
67 them carried vanB gene (Table 1). Result of MLST analysis and the Pasteur Institute showed
68 that the four E. faecium isolates belonged to new sequence types (ST1327, ST1328, ST1329
69 and ST1330). This means that there was no relationship between the strains isolated from the
70 same hospital.
71
72 The MDR enterococci have become a major public health issue due to an increase in VRE. In
73 the Middle East and Africa, 32 VREfm isolates were collected and had MIC greater than 64
74 only [3]. In Lebanon, there was no detection of vancomycin-resistant isolates except for one
75 E. gallinarum (vanC phenotype resistance) that had a MIC of 16 mg/l and was recovered

from an infected catheter [5] . In contrast, the Middle Eastern countries presented a wide range of enterococcal glycopeptide resistance rates (0.8–75%) [5]. Throughout the world, E. faecium and E. faecalis carrying the gene vanA are the dominant VRE [1]. Moreover, bacteria harboring vanA phenotype are characterized as having a high resistance level to vancomycin, while the vanB phenotype has various levels of resistance to vancomycin [1]. Furthermore, our strains were highly resistant to teicoplanin in addition to vancomycin. It has been shown by Faron et al that the strains harboring the vanA gene conferred resistance to the glycopeptide teicoplanin (Te) in addition to vancomycin. This is due to the presence of an additional vanZ gene that is present on the vanA operon, conferring this resistance by an unknown mechanism [1]. In conclusion, this is the first study showing the presence of VREfm strains in patients from Saint-George hospital in Lebanon, which is a major public health issue. Moreover, we report vancomycin-resistant enterococcus strains, isolated from patients without previous use of vancomycin. Monitoring and control measures need to be adopted to avoid the spread of such bacteria in this hospital.

Funding source:

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Competing interests: None declared

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Isolate no.	Bacterial species	Source	M / F	B-Lactamines			Glycopeptides		Macrolides									VAN	VanA	VanB	ST
				OXA	AMX	CRO	VA	TEC	DA	E	PT	GEN 500	LNZ	FF	F	DO	RA	MIC (µg/ml)			
5	**E. faecium**	Rectal swab	M	R	R	R	R	R	R	R	S	R	S	R	R	R	R	>256	+	-	1327
7	**E. faecium**	Rectal swab	F	R	R	R	R	R	R	R	S	R	S	S	R	R	R	>256	+	-	1328
8	**E. faecium**	Rectal swab	M	R	R	R	R	R	R	R	S	R	S	R	R	R	R	>256	+	-	1329
15	**E.**	Rectal	M	R	R	R	R	R	R	R	S	R	S	S	R	R	R	>256	+	-	1330

Table 1 Phenotypic and genotypic features of the four vancomycin-resistant E. faecium clinical isolates

ARTICLE 15

Title: Genome sequence and description of Clostridium niameyense sp. Nov. isolated from human with marasmus in Nigeria.

Selma Chabou, Tidjani Alou M, Khelaifia S, Lagier JC, Labas N, Cimmino T, Diene S, Raoult D and Rolain JM. DOI: https://doi.org/10.1016/j.nmni.2018.03.00. In Press Accepted Manuscript Published online: March 14, 2018 in New microbes and new infection journal.

Genome sequence and description of *Clostridium niameyense* sp. nov., isolated from a human with marasmus in Nigeria

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Abstract

Clostridium niameyense sp. nov. strain MT5 is the type strain of *C. niameyense* sp. nov., a new species within the genus *Clostridia*. *C. niameyense* is a Gram-positive, anaerobic bacillus. The strain MT5 (= CSUR P1468 = DSMZ 100441), whose genome is described here, was isolated from a faecal sample collected from a patient with anorexia and marasmus living in Nigeria. The genome is 2 542 841 bp long with 27.44% G + C content and consists of six scaffolds.
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Keywords: *Clostridium niameyense*, culturomics, genome, marasmus and anorexia, taxonogenomics
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Introduction

Clostridium niameyense strain MT5 (= CSUR P1468 = DSMZ 100441) is the type strain of *C. niameyense* sp. nov. This bacterium is a Gram-positive, anaerobic, motile bacterium that was isolated from the stool of an anorexic patient with marasmus living in Nigeria as part of a culturomics study aiming at cultivating all species individually within human faeces [1,2].

The usual parameters used to delineate a bacterial species include 16S rDNA sequence identity and phylogeny [3], genomic G + C content diversity and DNA-DNA hybridization [3]. Recently a new approach has been proposed, taxonogenomics, which includes genomic data in a polyphasic taxonomy to describe new bacterial species [4]. This strategy is based on a combination of genomic and phenotypic characteristics, including matrix-assisted desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) as well as spectrum and genomic analyses [5].

Clostridium is a genus of Gram-positive bacteria which includes several significant human pathogens and consists of obligate anaerobic bacteria [6]. These bacteria are the cause of some human diseases such as botulism (*C. botulinum*), tetanus (*C. tetani*), pseudomembranous colitis (*C. difficile*) and food poisoning–gas gangrene (*C. perfringens*) [7]. The pathogenic clostridial species have evolved several mechanisms to survive inside and outside a number of hosts, as evidenced by various diseases often linked to their protein toxins and spores [8].

Here we present a summary classification and set of features for *C. niameyense* sp. nov. strain MT5 together with the description of the complete genome sequence and annotation. These characteristics support the circumscription of the species *C. niameyense*.

Phenotypic and biochemical characterization

Clostridium niameyense sp. nov. strain MT5 was isolated from a faecal sample collected from an anorexic patient with marasmus living in Nigeria. The faecal specimen was preserved at −80 °C after collection and sent to Marseille. The MT5 strain was cultivated on 5% sheep's blood–enriched Columbia agar (bioMérieux, Marcy l'Etoile, France) after 5 days' preincubation in a blood culture bottle with rumen fluid.

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For the growth of *C. niameyense,* we tested different temperatures (25, 30, 37 °C). However, optimal growth occurred at 37 °C, 24 hours after inoculation. Colonies were opaque with a smooth aspect, irregular and 1 mm in diameter on blood-enriched Columbia agar. Growth was observed only under anaerobic conditions, and no growth occurred under aerobic or microaerophilic conditions. Gram staining revealed a Gram-positive nonsporulating bacillus; the motility test was positive (Fig. 1).

The obtained colonies were identified using a Microflex spectrometer (Bruker Daltonics, Bremen, Germany). MALDI-TOF MS identification, measurement and analysis were performed as previously described [1]. No significant MALDI-TOF MS score was obtained for strain MT5 against the Bruker database, suggesting that our isolate was not a member of a known species. We added the spectrum from strain MT5 to our database (Fig. 2).

Biochemical features of the strains were determined using API50-CHL and the API ZYM (bioMérieux). Oxydase (Becton Dickinson, Pont-de-Claix, France) and catalase assays (bioMérieux) were performed separately. API ZYM presented positive reactions for α-glucosidase (maltase) and β-glucosidase (cellulase). The differential phenotypic characteristics with other *Clostridium* species are summarized in Table 1.

Antibiotic susceptibility of our isolate was assessed using the disc diffusion method on Muller-Hinton agar plates supplemented with 5% blood (bioMérieux) using the standard disc diffusion procedure as recommended by the European Committee on Antimicrobial Susceptibility Testing (EUCAST; http://www.eucast.org/). *C. niameyense* was likely resistant to penicillin (14 mm), amoxicillin (13 mm), trimethoprim/sulfamethoxazole (6 mm), colistin (6 mm) and metronidazole (6 mm) but susceptible to vancomycin (22 mm), ertapenem (20 mm), fosfomycin (27 mm), teicoplanin (19 mm), oxacillin (22 mm), cefotaxime (20 mm), rifampicin (25 mm), gentamycin (27 mm), doxycycline (25 mm), pristinamycin (22 mm), erythromycin (26 mm), lincomycin (27 mm), imipenem (22 mm), tazocillin (24 mm), ofloxacin (25 mm), meropenem (20 mm) and ciprofloxacin (19 mm).

16S rRNA gene sequencing and phylogenetic analyses

The organism was selected for sequencing on the basis of its phylogenetic position and 16S rRNA similarity to members of the genus *Clostridium* [9] and is part of a study of the human digestive flora aiming to isolate all bacterial species in human faeces [1]. A phylogenetic tree was constructed using the maximum-likelihood method in the MEGA6 software package (https://www.megasoftware.net/). Phylogenetic analysis of the complete sequence of the 16S rRNA gene of *C. niameyense* strain MT5 (= CSUR P1468 = DSMZ 100441) exhibited a 98.3% nucleotide sequence similarity with *Clostridium botulinum* A (L37585). This value was lower than the 98.7% 16S rRNA gene sequence threshold recommended by Stackebrandt and Ebers [10] to delineate a new species without carrying out DNA-DNA hybridization (Fig. 3).

Genome properties

The genome is 2 542 841 bp long with 27.44% G+C content (Table 2). It is composed of six scaffolds (composed of seven contigs). Of the 2489 predicted genes, 2410 were protein-coding genes and 79 were RNAs (five genes are 5S rRNA, one gene is 16S rRNA, two genes are 23S rRNA and 71 genes are tRNA genes). A total of 1790 genes (74.27%) were assigned

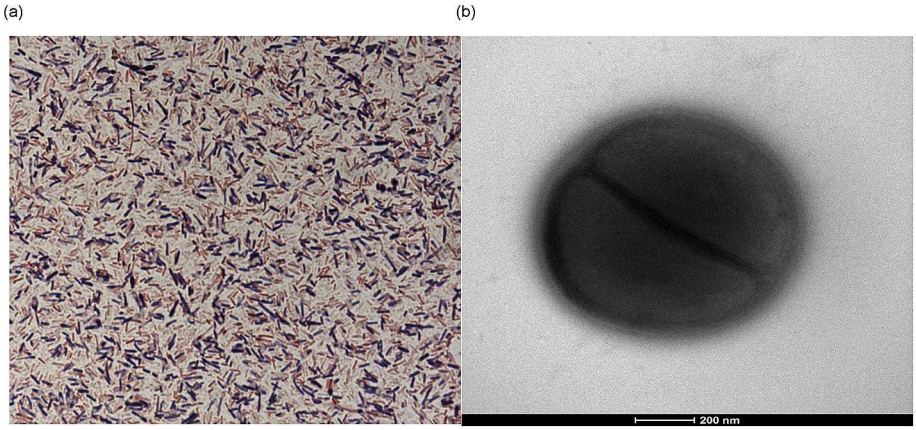

FIG. 1. (a) Gram staining of *Clostridium niameyense* strain MT sp. (b) Transmission electron microscopy of *Clostridium niameyense* sp. nov. strain MT5 using TechnaiG^2 Cryo device (FEI Company, Limeil-Brévannes, France) at operating voltage of 200 keV. Scale bar = 200 nm.

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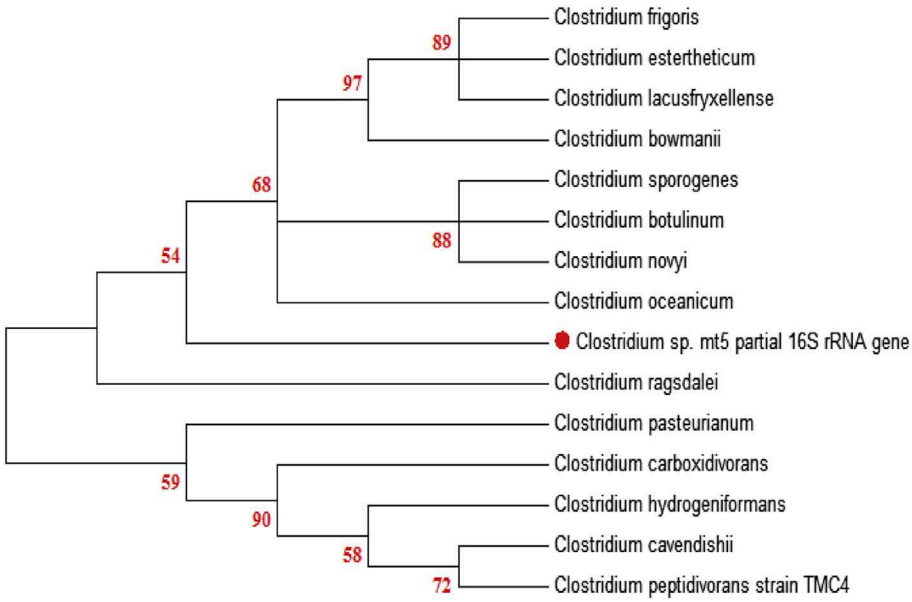

FIG. 2. Phylogenetic tree highlighting position of *Clostridium niameyense* sp. nov. strain MT5 relative to other type strains within *Clostridium* genus. GenBank accession numbers are indicated in parentheses. Sequences were aligned using CLUSTALW and phylogenetic inferences obtained using maximum-likelihood method within MEGA software.

a putative function by Clusters of Orthologous Groups database (COGs) or by NR BLAST. Fifty-nine genes were identified as ORFans (2.45%). The remaining genes were annotated as hypothetical proteins (401 genes, >16.64%). The properties of the genome are summarized in Table 2. A genomic comparison of *C. niameyense* with seven other *Clostridium* species is provided in Table 3. The distribution of genes into COGs functional categories is presented in Table 4 (Fig. 4). The genome was assembled and annotated by Xegen (http://www.xegen.fr/).

Genome annotation

The ARG-ANNOT database for acquired antibiotic resistance genes (ARGs) was used for a BLAST search using the Bio-Edit interface. The assembled sequences were searched against the ARG database under moderately stringent conditions (*E* value of 10^{-5}) for the *in silico* ARG prediction [11]. The results obtained by ARG-ANNOT demonstrated that there are no known antibiotic-resistance genes in the genome of *C. niameyense* strain MT5.

TABLE 1. Differential characteristics of *Clostridium niameyense, C. beijerinckii* strain NCIMB 8052, *C. disporicum* NCIB12424, *C. carboxidivorans* strain P7, *C. dakarense* strain FF1, *C. difficile* strain B1 and *C. saudii* JCCT

Property	*C. niameyense*	*C. beijerinckii*	*C. disporicum*	*C. carboxidivorans*	*C. dakarense*	*C. difficile*	*C. saudii*
Cell diameter (μm)	1	1.7	1.5	1.5	1.2	3.0	1.0
Oxygen requirement	Strictly anaerobic	Strictly anaerobic	Strictly anaerobic	Strictly anaerobic	Strictly anaerobic	Strictly anaerobic	Strictly anaerobic
Gram strain	positive	Variable	Positive	Positive	Positive	Positive	Positive
Motility	Motile	Motile	NA	Motile	Motile	Motile	Motile
Endospore training	NA	+	NA	+	+	+	+
Indole	−	NA	−	−	+	NA	−
Production of:							
Alkaline phosphatase	−	NA	NA	NA	+	NA	−
Catalase	−	−	−	−	−	NA	−
Oxidase	−	NA	NA	−	−	NA	−
Nitrate reduction	−	−	NA	−	−	−	−
Urease	−	−	NA	−	−	NA	−
β-Galactosidase	+	NA	NA	NA	−	NA	−
N-Acetyl-glucosamine	−	NA	NA	NA		NA	−
Acid from:							
L-Arabinose	−	+	NA	+	−	−	−
Ribose	−	−	+	+	−	−	−
Mannose	+	+	+	+	−	+	−
Mannitol	+	+	+	+	−	+	−
Sucrose	−	+	+	+	−	+	−
D-Glucose	+	+	+	+	+	NA	−
D-Fructose	+	+	+	+	−	+	−
D-Maltose	−	+	+	+	+	−	−
D-Lactose	−	+	+	+	−	−	−
G+C satisfied (%)	27.44	28	29	31	27.98	28	28
Habitat	Human gut	Human gut	Rat gut	Environment	Human gut	Human gut	Human gut

+, positive result; −, negative result; NA, data not available.

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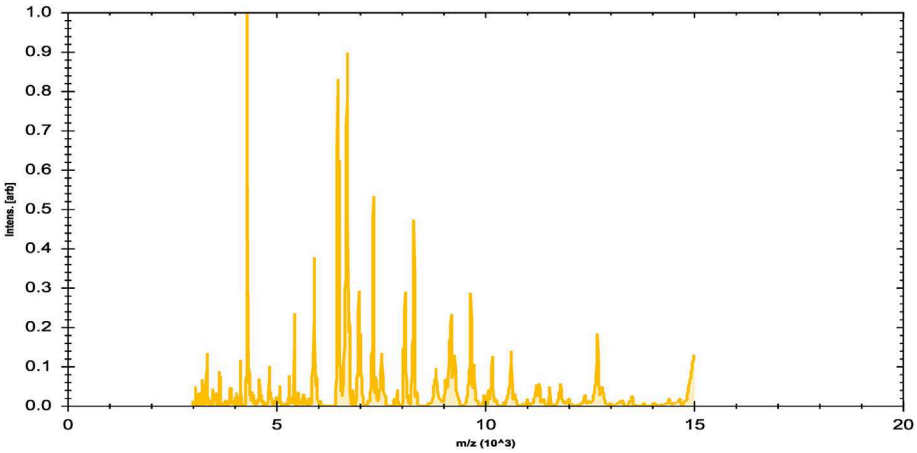

FIG. 3. Reference mass spectrum from *C. Clostridium niameyense* sp. nov. strain MT5. Spectra from 12 individual colonies were compared and reference spectrum generated.

TABLE 2. Nucleotide content and gene count levels of genome

Attribute	Value	% of total
Genome size (bp)	2 542 841	100
No. of G+C (bp)	697 772	27.44
Genes	2489	100
Protein genes	2410	96.82
RNA genes	79	3.17
tRNA genes	71	2.85
RNA (5S, 16S, 23S) genes	8	0.32
Coding sequence size	2 232 886	87.81
Coding sequence gene protein size	2 219 514	87.28
Coding sequence tRNA gene size	5558	0.21
Coding sequence (5S, 16S, 23S) genes size	7814	0.307
Protein-coding gene	2410	100

These sequences were submitted to Rapid Annotation Using Subsystem Technology (RAST) [12]. An exhaustive search of the bacteriocin database available in our laboratories (Bacteriocins of the URMITE, BUR; http://drissifatima.wix.com/bacteriocins) was performed by collecting all currently available sequences from the databases and from the National Center for Biotechnology Information. Protein sequences from this database allowed putative bacteriocins from human gut microbiota to be identified using BLASTp methodology [13].

TABLE 3. Genomic comparison of *C. niameyense* with seven other *Clostridium* species

Species	Strain	Genome accession number	Genome size (Mb)	G+C content
C. niameyense	MT5	CVPI00000000	2.54	27.4
C. perfringens	ATCC 13124	ATCC 13124	3.26	28.4
C. difficile	B1	NC_017179	4.46	28.4
C. dakarense	FF1	CBTZ010000000	3.73	27.9
C. leptum	DSM753	ABCB02000000	3.27	50.2
C. botulinum	ATCC 3502	NC_009495	3.90	28.2
C. beijerinckii	NCIMB 8052	NC_009617	6.0	29.0
C. senegalense	DSM 25507	CAEV01000001	3.89	26.8

The analysis of the resistome did not identify antibiotic resistance genes, and the absence of bacteriocin was confirmed.

Analysis of the presence of polyketide synthases and nonribosomal peptide synthetases was performed by discriminating the genes with large sizes using a database realized in our laboratory; predicted proteins were compared against the nonredundant GenBank database using BLASTp and finally examined using antiSMASH [14].

Analysis revealed five clusters of clusters of differentiation (CDs) (11 642 bp) predicted to code for enzymes involved in the nonribosomal synthesis of peptides (Fig. 5), which in turn

TABLE 4. Number of genes associated with 25 general COGs functional categories

Code	Value	% of total	Description
J	152	6.3070545	Translation
A	0	0	RNA processing and modification
K	138	5.726141	Transcription
L	99	4.107884	Replication, recombination and repair
B	1	0.041493777	Chromatin structure and dynamics
D	27	1.120332	Cell cycle control, mitosis and meiosis
Y	0	0	Nuclear structure
V	53	2.19917	Defense mechanisms
T	97	4.0248966	Signal transduction mechanisms
M	81	3.360996	Cell wall/membrane biogenesis
N	60	2.4896266	Cell motility
Z	0	0	Cytoskeleton
W	0	0	Extracellular structures
U	37	1.5352697	Intracellular trafficking and secretion
O	56	2.3236516	Posttranslational modification, protein turnover, chaperones
C	95	3.9419088	Energy production and conversion
G	83	3.4439836	Carbohydrate transport and metabolism
E	138	5.726141	Amino acid transport and metabolism
F	60	2.4896266	Nucleotide transport and metabolism
H	75	3.1120331	Coenzyme transport and metabolism
I	44	1.8257263	Lipid transport and metabolism
P	87	3.6099586	Inorganic ion transport and metabolism
Q	24	0.9958507	Secondary metabolites biosynthesis, transport and catabolism
R	208	8.630706	General function prediction only
S	160	6.6390047	Function unknown
—	812	33.692947	Not in COGs

COGs, Clusters of Orthologous Groups database.

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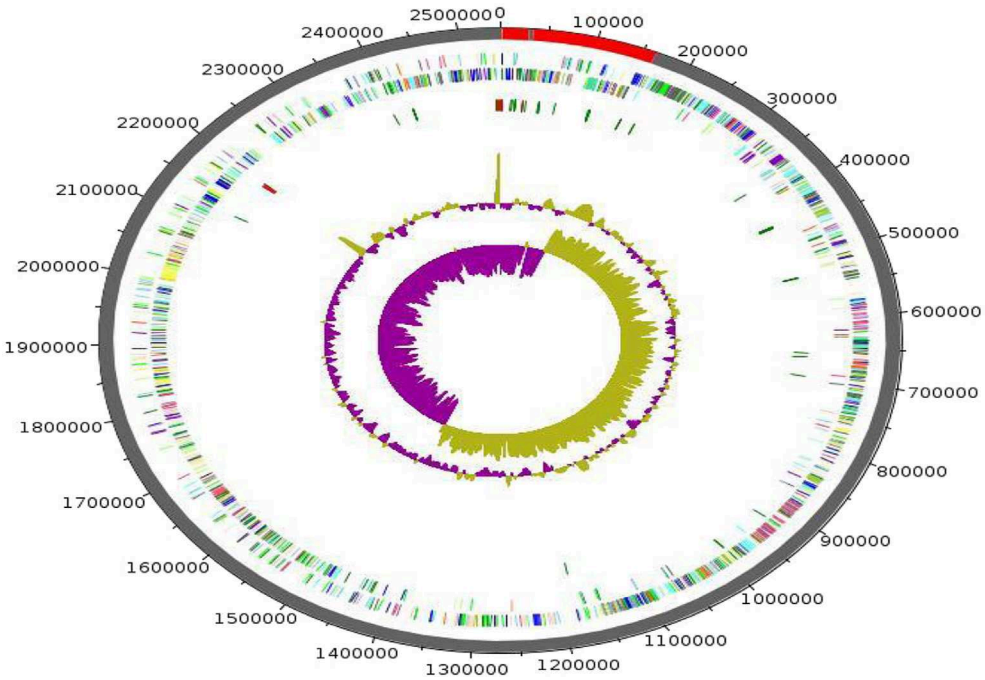

FIG. 4. Graphical circular map of chromosome. From outside to centre: genes on forward strand coloured by COGs categories (only genes assigned to COGs), genes on reverse strand coloured by COGs categories (only gene assigned to COGs), RNA genes (tRNAs green, rRNAs red), G+C content and G+C skew. COGs, Clusters of Orthologous Groups database.

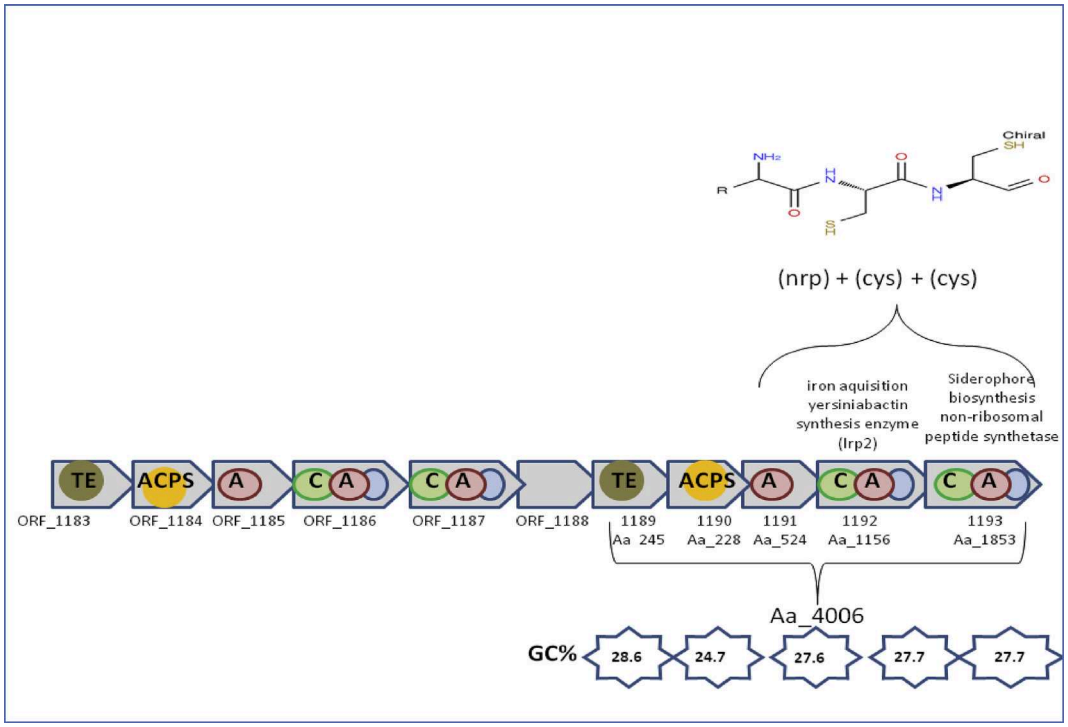

FIG. 5. CDS clusters predicted to encode siderophore biosynthesis nonribosomal peptide synthetase. A, C, ACPS and TE are domains of non-ribosomal peptide synthetases. A, AMP binding, adenylation domain; C, heterocyclization; TE, thioesterase domain.

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were predicted to catalyze the synthesis of a siderophore (Fig. 5), which could be used under iron-deficient growth conditions.

This mechanism is already known in *Clostridium* spp., particularly in in *C. kluyveri* DSM555. The alignment of non-ribosomal synthesis in *C. kluyveri* DSM555 (WP_012101903.1) and *C. niameyense* MT5 (WP_050607297.1) showed a 31% similarity with 99% coverage.

Description of *Clostridium niameyense* sp. nov. strain MT5

Clostridium niameyense sp. nov. strain MT5 is the type strain of *C. niameyense* sp. nov., a new species within the genus *Clostridia.* The strain MT5 (= CSUR P1468 = DSMZ 100441), whose genome is described here, was isolated from a faecal sample collected from an anorexic patient with marasmus living in Nigeria. Optimal growth occurred at 37 °C, 24 hours after inoculation. The colonies were 0.1 to 0.3 mm in diameter on blood-enriched agar. *C. niameyense* is a Gram-positive, obligate anaerobic bacterium with a mean diameter of 1.3 μm.

C. niameyense has positive reactions for glycerol, erythritol, D-glucose, D-fructose, D-mannitol, D-saccharose, D-trehalose D-maltose, D-melezitose, amidone D-turanose and potassium 5-cétogluconate. Also, API ZYM presented positive reactions for α-glucosidase (maltase) and 17-β-glucosidase (cellulase).

C. niameyense is resistant to penicillin (14 mm), amoxicillin (13 mm), trimethoprim/sulfamethoxazole (6 mm), colistin (6 mm) and metronidazole (6 mm) but susceptible for another antibiotic.

The G+C content of the genome is 27.44%. The 16S rDNA and genome sequences are deposited in GenBank under accession numbers LN827532 and CVPI00000000, respectively. The type strain is MT5 (= CSUR P1468 = DSMZ 100441).

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Conflict of interest

None declared.

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V-CONCLUSION GENERALE ET PERSPECTIVES

A travers ce parcours de thèse, qui s'est consacré à l'étude approfondie de la résistance des bactéries d'origine animale aux antibiotiques notamment à la colistine, aux β-lactamines et aux carbapénèmes, dans le bassin méditerranéen particulièrement en Algérie, nous nous sommes fixés comme objectif d'étudier le rôle des animaux comme réservoirs de bactéries résistantes aux antibiotiques et compléter les données existantes sur le risque qu'ils peuvent exercer dans le transfert des gènes de résistance aux antibiotiques à l'homme.

A travers nos résultats nous pouvons conclure qu'il existe au moins une dissémination locale (Algérie) et mondiale des gènes de résistance à travers les animaux et qui s'est étendue à l'homme. Cela implique la mise en place de mesures d'urgence de contrôle et de bonne utilisation des antibiotiques, notamment chez les animaux.

A travers les résultats de cette thèse et de nos recherches bibliographiques, il en ressort que le problème de l'antibiorésistance en Algérie est un phénomène complexe (**Laxminarayan et al., 2013**). Il se caractérise par l'accroissement des taux de résistances parmi les bactéries pathogènes pour l'homme et pour l'animal suite à la mauvaise utilisation des antibiotiques soit par surconsommation ou par le recours limité aux moyens de diagnostic permettant de mieux cibler les traitements (**MCEwen et Collignon., 2018**). Cela engendre la raréfaction de l'arsenal thérapeutique efficace pour faire face aux infections, et par la suite une atteinte de la santé humaine et animale avec des répercussions économiques défavorables (**O'Neill., 2016**).

L'antibiorésistance est un problème universel qui nécessite une action coordonnée associant santé humaine, animale et environnement au niveau mondial pour faire face à ce problème. C'est le principe de « One Health ».Les expériences récentes ont montré que seule une politique mondiale peut amortir et faire face à ce problème (**MCEwen et Collignon., 2018**). L'exemple le plus concret est celui de l'augmentation de la prévalence d'Enterococcus faecium résistantes à la vancomycine (ERV) dans l'union européenne qui a conduit à l'interdiction de l'avoparcine,

un antibiotique chimiquement apparenté à la vancomycine. Dans les années qui ont suivi l'interdiction, les données de la surveillance des taux d'ERV dans les hôpitaux en Europe n'ont montré aucune réduction évidente (**Kahn., 2017**). Les États-Unis n'ont jamais approuvé l'avoparcine, mais l'ERV a été un énorme problème dans ses hôpitaux. Les données de séquençage du génome entier suggèrent que l'ERV pourrait avoir évolué à partir de souche d'E. faecium résistante à l'ampicilline chez les chiens. Les animaux de compagnie ont été complètement ignorés dans le débat sur la résistance aux antimicrobiens ce qui montre que le problème de l'antibiorésistance est un problème universel nécessitant une action associant une politique mondiale avec la santé humaine, animale et environnement (**Kahn., 2017**). Par ailleurs, L'Organisation Mondiale de la Santé a adopté en mai 2015 un plan d'action global, recommandant aux Etats membres d'élaborer un plan national sous le concept d'une seule santé, « One Health » (**MCEwen et Collignon., 2018**).

Autres stratégies que nous proposons à travers ces résultats de thèse pour **lutter contre l'antibiorésistance** à l'échelle locale (en Algérie) et mondiale, est de mettre en place des mesures de **surveillance de l'antibiorésistance**, cela par le **renforcement des réseaux de surveillance** existants et la création d'autres réseaux de surveillance en s'appuyant sur des structures mondiales existantes notamment l'organisation mondiale de la santé (OMS) et l'organisation mondiale de la santé animale (OIE) (**WHO., 2017**). Il faut admettre, que la surveillance de la résistance chez les animaux, voire en milieu rural n'a pas les mêmes facilités que la surveillance de l'antibiorésistance chez les humains, mais la recommandation **de l'amélioration des paramètres d'élevage** avec la **mise en place d'une politique et le financement de projets de recherche** pourraient améliorer la situation. Un autre point important est **le contrôle de la consommation d'antibiotiques** surtout chez les animaux (**MCEwen et Collignon., 2018**). Nous avons montré à travers cette thèse le pouvoir de transfert des gènes de résistance entre les humains et les animaux. De ce fait, une règlementation de

l'usage des antibiotiques, voire une interdiction d'utilisation de certains antibiotiques, doit être mise en place par les gouvernements. Le problème qui se pose en cas de restriction d'usage de certains antibiotiques chez les animaux est l'alternative thérapeutique en cas d'infections qui peut s'accompagner de conséquences économiques importantes. Pour pallier à ce problème nous pensons qu'il faut mettre en place des **formations de qualité aux professionnels de santé et un renforcement de l'encadrement de la prescription des anti-infectieux** en médecine humaine et vétérinaire. Un autre point très important est le renforcement de **la surveillance des marchés noirs** et de l'automédication et la médication par les non professionnels. D'autres méthodes **alternatives à l'antibiothérapie** peuvent aussi s'appliquer à savoir le développement des mesures de prévention, en particulier **la vaccination** chez les animaux d'élevage. Un dernier axe de lutte contre l'antibiorésistance peut se résumer dans la recherche **d'outils innovants** en matière de maîtrise de l'antibiorésistanceet de traitements alternatifs notamment l'utilisation de la **phago-thérapie** pour faire face à la dissémination de l'antibiorésistance à travers le monde.

En conclusion, notre travail de thèse a contribué à une meilleure connaissance de l'épidémiologie et des facteurs de risque d'acquisition de bactéries multirésistantes chez les animaux, notamment en Algérie. Ainsi notre travail s'inscrit dans le concept récent de « One Health » qui met en évidence l'importance d'une intervention globale et le développement de mesures adaptées pour éviter la dissémination de l'antibiorésistance aussi bien chez les animaux et dans l'environnement que chez l'homme.

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27th ECCMID EUROPEAN CONGRESS OF CLINICAL MICROBIOLOGY AND INFECTIOUS DISEASES

05 May 2017

To whom it may concern:

CONFIRMATION OF PRESENTATION AT ECCMID 2017

We hereby confirm that the following abstract has been submitted, accepted and presented at the 27th ECCMID, the European Congress of Clinical Microbiology and Infectious Diseases, which took place in Vienna, Austria, 22 – 25 April, 2017.

Title: Emergence of mcr-1-mediated colistin resistance in Escherichia coli isolates from poultry in Algeria

Abstract Authors: S. Chabou, H. Leulmi, S. Diene, J. Rolain

Presenter: Selma Chabou

Session Title: The mcr genes around the world

Presentation Type: Paper poster session

Presentation Number: 5219

Yours sincerely,

Winfried V. Kern
ECCMID 2017 Programme Director

ESCMID European Society of Clinical Microbiology and Infectious Diseases

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April, 20, 2016

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PRESENTATION CONFIRMATION AT ECCMID 2016

We hereby confirm that the following abstract has been submitted, accepted and presented at the 26th ECCMID Congress, the European Congress of Clinical Microbiology and Infectious Diseases, which took place in Amsterdam, Netherlands April 9 – 12, 2016.

Title: Prevalence of extended-spectrum beta-lactamase and Carbapenemases in feces of poultry from Algeria

Abstract Authors: S. Chabou, H. Leulmi, B. Davoust, A. Aouadi, D. Raoult, J. Rolain

Presenter: Selma Chabou (Marseille CEDEX 05/ France)

Session Title: Antibacterial resistance: the animal and food production part of one health

Presentation Type: Poster Presentation

Presentation Number: P1510

Yours sincerely,

Carla Seiler

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MÉDITERRANÉE INFECTION

Marseille, le 10 juillet 2017

ATTESTATION DE PRESENCE

A qui de droit,

Je soussignée, Melle Micheline Pitaccolo, Assistante de Gestion de la Fondation Méditerranée Infection atteste que Mme CHABOU Selma a participé à la journée d'information de l'Infectiopôle Sud – Méditerranée Infection le 7 juillet 2017.

Mme CHABOU Selma a présenté un poster.

Cordialement,

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Marseille, le 4 juillet 2016

ATTESTATION DE PRESENCE

A qui de droit,

Je soussignée, Melle Micheline Pitaccolo, Assistante de Gestion de la Fondation Méditerranée Infection atteste que Mme CHABOU Selma a participé à la journée d'information de l'Infectiopôle Sud – Méditerranée Infection le 1er juillet 2016.

Mme CHABOU Selma a présenté un poster.

Cordialement,

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