Journal of Global Antimicrobial Resistance 22 (2020) 538–545

Contents lists available at ScienceDirect

Journal of Global Antimicrobial Resistance

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

Co-occurrence of *mcr-1* mediated colistin resistance and β-lactamase-encoding genes in multidrug-resistant *Escherichia coli* from broiler chickens with colibacillosis in Tunisia

Sana Dhaouadi[a,b], Leila Soufi[a], Amani Hamza[a], Didier Fedida[c], Chtioui Zied[c], Emna Awadhi[d], Mohamed Mtibaa[d], Bilel Hassen[e], Ameur Cherif[a], Carmen Torres[f], Mohamed Salah Abbassi[e], Ramzi Boubaker Landolsi[a,*]

[a] *Manouba University, ISBST, BVBGR-LR11ES31, Biotechpole Sidi Thabet, Ariana, Tunisia*
[b] *University of Tunis El Manar, Farhat Hached El Manar Campus – B.P. 94, Rommana City, Tunis, Tunisia*
[c] *Ceva Animal Health, Tunis, Tunisia*
[d] *Taissir Agricol Society, Tunis, Tunisia*
[e] *University of Tunis El Manar, Institute of Veterinary Research of Tunisia-Laboratory of Bacteriological Research, Tunis, Tunisia*
[f] *Área de Bioquímica y Biología Molecular, Universidad de La Rioja, Logroño, Spain*

ARTICLE INFO

Article history:
Received 1 November 2019
Received in revised form 1 January 2020
Accepted 17 March 2020
Available online 3 April 2020

Keywords:
MDR
E. coli
Broiler chickens
Colibacillosis
mcr-1
ESBL
B2 phylogroup

ABSTRACT

Objectives: Colibacillosis caused by avian pathogenic *Escherichia coli* (APEC) is considered a major hindrance in poultry farming worldwide. This study aimed to characterize the genetic content and the relatedness between multidrug-resistant *E. coli* isolates from broiler chickens died due to colibacillosis from three farms from Tunisia.
Methods: One hundred samples were collected from chickens' fresh carcasses from three poultry farms in Tunisia. *E. coli* isolation and identification were performed. Then, antimicrobial susceptibility regarding antibiotics, the ability to produce β-lactamases and minimum inhibitory concentration for colistin were determined according to Clinical and Laboratory Standards Institute guidelines. β-Lactam and non-β-lactam antimicrobial resistance genes, integrons, virulence genes, and phylogenetic groups were investigated using polymerase chain reaction. The genetic relatedness of the *E. coli* isolates was analysed by pulsed field gel electrophoresis (PFGE) and multilocus sequence typing (MLST).
Results: A high infection rate of *E. coli* (50%) in infected organs of chickens was observed. The majority of *E. coli* isolates were multidrug resistant (96%); among them, 24% were colistin resistant and 30% were ESBL producing. Seven of 12 colistin-resistant isolates harboured the *mcr-1* gene; among them, 10 were ESBL producing and carried $bla_{\text{CTX-M-1}}$, bla_{TEM}, and bla_{SHV} β-lactamase-encoding genes. *E. coli* isolates were assigned to different phylogroups but most of them (74%) belonged to the pathogenic phylogroup B2. Molecular typing by PFGE showed that some *E. coli* isolates harbouring ESBL-*mcr-1* genes were clonally related. MLST revealed the presence of four different ST lineages among ESBL- and *mcr-1*-carrying *E. coli*: ST4187, ST3882; ST5693, and ST8932 with clonal dissemination of *E. coli* ST4187 between two of the farms.
Conclusion: This is the first report of ESBL-*mcr-1*-carrying *E. coli* isolates of a clinically relevant phylogenetic group (B2) from chickens that died due to colibacillosis in Tunisian poultry farms.

1. Introduction

Although most of the *Escherichia coli* isolates colonising chickens' intestines are commensal and normal inhabitants, some are responsible for poultry diseases and designated as Avian Pathogenic *E. coli* (APEC) belonging to the category of extra-intestinal pathogenic *E. coli* (ExPEC) [1–3]. Colibacillosis caused by APEC is considered a major hindrance in poultry farming worldwide because of increased mortality, high medication costs, and condemnation of carcasses in slaughterhouses [4].

Antibiotherapy remains important in reducing both incidence and mortality associated with this disease. However, the overuse and misuse of antibiotics, including β-lactams and polypeptides in

* Corresponding author at: Manouba University, Biotechpole Sidi Thabet, ISBST, BVBGR-LR11ES31, 2020 Ariana, Tunisia.
E-mail address: ramzi.b.landolsi@gmail.com (R.B. Landolsi).

http://dx.doi.org/10.1016/j.jgar.2020.03.017

poultry farming industries, has increased the selection and emergence of multidrug-resistant (MDR) *E. coli* exhibiting resistance to at least one agent belonging to three or more antimicrobial families [5], resulting in limited therapeutic options being available to clinicians [4].

Extended-spectrum β-lactamases (ESBLs) are enzymes that confer resistance to most β-lactam antibiotics, especially to third-generation cephalosporins. Currently, more than 350 ESBL genes have been reported, and these genes are commonly developed through point mutations of the classical SHV-1 and TEM-1 β-lactamases and the more increasingly prominent CTX-M types [6].

Colistin is considered a last-resort antibiotic used to treat bacterial infections caused by carbapenemases and ESBL-producing Enterobacteriaceae including *E. coli*. However, the use of colistin in animal husbandry has led to an increased incidence of colistin-resistant *E. coli* (CREC) [7]. Until recently, colistin resistance was mainly encoded by mutations or interruptions in certain chromosomal genes. Nonetheless, plasmid-mediated colistin resistance has recently been described [7]. In recent studies, the plasmid-borne colistin resistance gene *mcr-1* and other resistance determinants such as ESBLs were simultaneously detected in *E. coli* from poultry [8,9].

In Tunisia, *E. coli* isolated from healthy poultry with various antibiotic resistance and virulence patterns have been reported [10–12] and *mcr-1* gene has been detected in *E. coli* from apparently healthy chickens [13–15] and from camel faeces in southern Tunisia [16]. Conversely, there are no data targeting *mcr-1* and ESBL-producing *E. coli* (ESBL-EC) from diseased chickens. Thus, the aims of this study were to assess the extent of *E. coli* isolates from chickens that died due to colibacillosis as reservoirs of antibiotic-resistance determinants, and further to determine the genetic relatedness among these isolates.

2. Methods

2.1. Sample collection

From January to June 2017, a total of 100 broiler chicken fresh carcasses were collected from 3 industrial poultry farms in 3 governorates in northern Tunisia: farm I (FI) (Nabeul; n = 40), FII (Ben Arous; n = 36), and FIII (Zaghouan; n = 34). Farms included in the survey were characterized by the number of chickens of 12 500 on average, moderate morbidity and mortality rates (15% and 7%, respectively), and an antibiotherapy based on florfenicol, doxycycline, and enrofloxacin for the treatment of some infections, including colibacillosis. Only chickens that died after exhibiting an episode of various signs compatible with colibacillosis (growth delay, decreased hatching rates, weight and egg production, and respiratory syndrome) were included in this study. After post-mortem examination by veterinarians in the 3 farms, samples of the liver (n = 40), pericardium (n = 30), and bone marrow (n = 30) showing fibrinous hepatitis, pericarditis, and osteomyelitis, characteristic of colibacillosis, were aseptically collected separately in sterile plastic bags and immediately transported to the laboratory. Only one organ (showing the most infection) was sampled from each animal.

2.2. Bacteria isolation and identification

A section from each infected organ (10 g) was placed in brain–heart infusion broth (Oxoid Ltd., Basingstoke, UK) and incubated aerobically at 37 °C for 24 h. Then, 100 μL were streaked on MacConkey agar (Merck, Darmstadt, Germany) plates and incubated at 37 °C overnight. Isolates with typical *E. coli* morphology were selected and seeded onto Endo agar (Merck) and incubated overnight at 37 °C. Colonies characteristic of *E. coli* were revealed by their red colour with a metallic sheen. Presumptive *E. coli* colonies were further identified using Gram staining and biochemical tests such as oxidase, catalase, urea-Indole, lactose, and glucose fermentation gas production ability in Kigler-Hajna agar and by API 20E system (BioMerieux, Marcy l'Etoile, France). Bacterial DNA for polymerase chain reaction (PCR)analysis was prepared by boiling a loopful of bacteria in 400 μL of Tris–EDTA for 10 min, followed by centrifugation for 15 min at 10 000 × *g*. Subsequently, isolates were confirmed as *E. coli* using species-specific PCR targeting the *uidA* gene encoding for β-glucuronidase structural protein [17].

2.3. Antimicrobial susceptibility test and screening for ESBL production

Antimicrobial susceptibility of all *E. coli* isolates was determined using the disc-diffusion method and interpreted according to the Clinical and Laboratory Standards Institute and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines [18,19]. The following antibiotics (Oxoid) commonly used in poultry farming were applied (amount in μg perdisk): nalidixic acid (NAL, 30 μg), flumequine (FLU, 30 μg), enrofloxacin (ENF, 5 μg), tetracycline (TET, 30 μg), doxycycline (DOX, 30 μg), trimethoprim–sulfamethoxazole (SXT, 1.25/23.75 μg), florfenicol (FFC, 30 μg), streptomycin (STR, 10 μg), amoxicillin (AMX, 25 μg), ceftiofur (CTF, 30 μg), and colistin (COL, 50 μg). The isolates were defined as multidrug resistant (MDR) if they exhibited resistance to at least one agent belonging to three or more antimicrobial families, according to Magiorakos et al. [5]. The double-disk synergy test (DDST) with cefotaxime (CTX, 30 μg), ceftazidime (CAZ, 30 μg), aztreonam (ATM, 30 μg), and cefepime (FEP, 30 μg) in the proximity of amoxicillin–clavulanic acid (AMC, 20/10 μg) was used for the screening of ESBL production [18]. *E. coli* ATCC25922 and *Klebsiella pneumoniae* ATCC700603 were used as ESBL-negative and -positive control strains, respectively.

Colistin susceptibility testing and screening of *mcr-1* and *mcr-2* genes

Colistin minimum inhibitory concentration (MIC) was determined using the broth microdilution method (BMD) according to the CLSI guidelines [18]. Dilution methods were performed using colistin sulfate (Sigma–Aldrich, Merck KGaA, Darmstadt, Germany) tested over a range from 0.25 to 128 μg/mL. All experiments were repeated in triplicate. *E. coli* ATCC 25922 was used as a quality control strain. The *mcr-1* and *mcr-2* genes encoding for colistin resistance were investigated by PCR in all isolates with MIC ≥ 2 μg/mL as described elsewhere [7,20].

2.4. Detection of β-lactamase-encoding genes in ESBL-producing E. coli

All ESBL-EC isolates were screened for the presence of five β-lactamase-encoding genes (bla_{TEM}, bla_{SHV}, $bla_{CTX\text{-}M\text{-}g\text{-}1}$, $bla_{CTX\text{-}M\text{-}g\text{-}8}$, and $bla_{CTX\text{-}M\text{-}g\text{-}9}$) using PCR conditions as previously described [17,21] (Table 1). Four isolates were selected and the blaCTX-M-1 group amplicons were sequenced.

2.5. Detection of resistance-encoding genes and integrons

The presence of 14 antimicrobial resistance genes conferring resistance to streptomycin (*aadA*, *strA*, *strB*), phenicols (*cmlA*, *floR*), tetracycline (*tetA*, *tetB*), trimethoprim (*dfrAI*, *dfrVII*), and sulfonamide (*sul1*, *sul2*) were investigated by PCR as previously described [21,22]. The detection of *intI1*, *intI2*, and *intI3* genes was performed by PCR [23] (Table 1).

Table 1
Primer and PCR conditions used for the detection of resistance genes and phylogenetic groups.

Primer name	Oligonucleotide sequence (5′–3′)	Amplicon size (bp)	Annealing temp. °C	Resistance specificity	Reference
UidA	ATCACCGTGGTGACGCATGTCGC CACCACGATGCCATGTTCATCTGC	486	51	β-Glucuronidase enzyme	[17]
bla_{TEM}	ATTCTTGAAGACGAAAGGGC ACGCTCAGTGGAACGAAAAC	1150	60	β-Lactamases	[21]
tet(A)	AATTCTGAGCACTGTCGC CTGCCTGGACAACATTGCTT	937	62	Tetracycline	[21]
tet(B)	CTCAGTATTCCAAGCCTTTG CTAAGCACTTGTCTCCTGTT	416	57	Tetracycline	
aadA	GCAGCGCAATGACATTCTTG ATCCTTCGGCGCGATTTTG	282	60	Streptomycin	[21]
strA	ATTCTGACTGGTTGCCTGTC CGCAGATAGAAGGCAAGG	1562	55	Streptomycin	[22]
strB	TTCTCATTGCGGACAACCT TAGATCGCGTTGCTCCTCTT			Streptomycin	
DfrAI	GTGAAACTATCACTAATGG TTAACCCTTTTGCCAGATTT	474	55	Trimethoprim	[21]
DfrVII	TTGAAAATTTCATTGATT TTAGCCTTTTTTCCAAATCT	474	55	Trimethoprim	
sul1	TGGTGACGGTGTTCGGCATTC GCGAGGGTTTCCGAGAAGGTG	789	63	Sulfamides	[21]
sul2	CGGCATCGTCAACATAACC GTGTGCGGATGAAGTCAG	722	50	Sulfamides	
floR	CACGTTGAGCCTCTATAT ATGCAGAAGTAGAACGCG	868	55	Florfenicol	–
cmlA	TGTCATTTACGGCATACTCG ATCAGGCATCCCATTCCCAT	455	55	Chloramphenicol	[21]
mcr-1	CGGTCAGTCCGTTTGTTC CTTGGTCGGTCTGTAGGG	309	58	Colistin	[7]
mcr-2	TGTTGCTTGTGCCGATTGGA AGATGGTATTGTTGGTTGCTG	567	58	Colistin	[20]
*Int*1	GGGTCAAGGATCTGGATTTCG ACATGGGTGTAAATCATCGTC	483	62	Class 1 integron	[21,23]
*Int*2	CACGGATATGCGACAAAAAGGT GTAGCAAACGAGTGACGAAATG	788	62	Class 2 integron	
*Int*3	GCCTCCGGCAGCGACTTTCAG ACGGATCTGCCAAACCTGACT	979	62	Class 3 integron	
bla_{SHV}	CACTCAAGGATGTATTGTG TTAGCGTTGCCAGTGCTCG	885	52	β-Lactamases	[17]
$bla_{CTX\text{-}M\text{-}g\text{-}1}$	GTTACAATGTGTGAGAAGCAG CCGTTTCCGCTATTACAAAC	1041	50	β-Lactamases	[17]
$bla_{CTX\text{-}M\text{-}g\text{-}8}$	TGATGAGACATCGCGTTAAG TAACCGTCGGTGACGATTTT	666	52	β-Lactamases	[17]
$bla_{CTX\text{-}M\text{-}g\text{-}9}$	GTGACAAAGAGAGTGCAACGG ATGATTCTCGCCGCTGAAGCC	856	62	β-Lactamases	[17]
chuA	GACGAACCAACGGTCAGGAT TGCCGCCAGTACCAAAGACA	279	55	Phylogenetic groups	[26]
yjaA	TGAAGTGTCAGGAGACGCTG ATGGAGAATGCGTTCCTCAAC	211	55		
*tsp*E4.C2	GAGTAATGTCGGGGCATTCA CGCGCCAACAAAGTATTACG	152	55		

bp, base pairs.

2.6. Phylogenetic groups and genetic relatedness by PFGE

Phylogenetic groups (A, B_1, B_2, or D) and sub-groups (A_0, A_1, B_1, $B2_2$, $B2_3$, D_1, and D_2) in all isolates were determined using a triplex PCR targeting the *chuA* and *yjaA* genes and the DNA fragment *tsp*E4.C2 as described by Clermont et al. [24] and Escobar-Paramo et al. [25]. The genotyping of 50 *E. coli* isolates was performed by pulsed field gel electrophoresis (PFGE) using the restriction enzyme *Xba*I (Promega Madison, Fitchburg, WI, USA) as reported by Kaufmann [26]. DNA fingerprints generated visually by PFGE were analysed visually and digitally following Tenover criteria [27]. Then the phylogenetic tree was established using MVSP 3.2 software (Kovach Computing Services, Anglesey, Wales, UK).

2.7. Multilocus sequence typing of mcr-1-positive isolates

The molecular typing of the *mcr-1*-carrying isolates was performed by multilocus sequence typing (MLST) which was based on seven standard housekeeping genes (*adk, fumC, gyrB, icd, mdh, purA,* and *recA*) as previously described [28].

3. Results

3.1. Bacteria isolation and identification

Of the 100 samples of infected chickens' organs—liver (n = 40), pericardium (n = 30), and bone marrow (n = 30)—100 isolates belonging to different genera were collected (one per sample). *E. coli* was the most prevalent species recovered from chickens' organs (50/100; 50%). The remaining 50 isolates (50/100; 50%) belonged to other species and were excluded from this study. Of the 50 *E. coli* isolates, 20 were recovered from the liver (40%), 18 from the bone marrow (36%), and 12 from the pericardium (24%). The highest rate of *E. coli* was observed in FI (32/50; 64%) followed by both FII and FIII (9/50; 18%).

3.2. Antimicrobial susceptibility test and screening of ESBL production

Antibiotic-resistance rates were as follows: nalidixic acid (46/50; 92%), flumequine (43/50; 86%), enrofloxacin (34/50; 68%), doxycycline (41/50; 82%), tetracycline (38/50; 76%), florfenicol (28/50; 56%), trimethoprim–sulfamethoxazole (26/50; 52%), streptomycin (22/50; 44%), amoxicillin (39/50; 78%), ceftiofur (10/50; 20%), amoxicillin–clavulanic acid (37/50; 74%), cefotaxime (34/50; 68%), ceftazidime (33/50; 66%), aztreonam (31/50; 62%), cefepime (29/50; 58%), and colistin (12/50; 24%). All 50 isolates were resistant to at least 2 antibiotics (100%) and 48/50 (96%) were MDR. Fifteen of 50 (30%) showed a positive ESBL screening test (Fig. 1; Table 2). These isolates were recovered from FI (n = 11), FII (n = 1), and FIII (n = 3).

3.3. Colistin susceptibility testing and detection of mcr-1 and mcr-2 genes

Of the 50 *E. coli* isolates, 12 were classified as CREC as they showed an MIC value of 4 μg/mL (6 isolates) and 8 μg/mL (6 isolates). Among these, nine, one, and two isolates were recovered from chickens belonging to FI, FII, and FIII, respectively. Seven isolates among the 12 CREC isolates carried the *mcr-1* gene and none of them were positive for the *mcr-2* gene (Table 2). The distribution of the *mcr-1* gene depending on farms was as follows: five from FI, and one in each of FII and FIII.

3.4. Detection of β-lactamase-encoding genes

All 15 ESBL-EC isolates carried β-lactamase-encoding genes. The $bla_{\text{CTXM-1}}$, bla_{TEM}, and bla_{SHV} genes were found in 14, 6, and 2 isolates, respectively. None of the 15 ESBL strains carried the $bla_{\text{CTX-M-8}}$ and $bla_{\text{CTX-M-9}}$ genes. Four combinations of ESBL-encoding genes were observed among the isolates: $bla_{\text{CTX-M-1}}$ (n = 8), bla_{SHV} (n = 1), $bla_{\text{CTX-M-1}}$ + bla_{TEM} (n = 5), and $bla_{\text{CTX-M-1}}$ + bla_{TEM} + bla_{SHV} (n = 1) (Fig. 1, Table 2).

3.5. Detection of resistance genes and integrons

Of the 38 tetracycline-resistant isolates, 23 (60.5%) harboured the *tetA* gene. The *dfrAI* gene was detected in 21 of 26 (80.7%) trimethoprim-resistant isolates, whereas *floR* and *cmlA* genes were identified in 78.5% (22/28) and 7.1% (2/28) of florfenicol-resistant isolates, respectively. Among the 22 streptomycin-resistant isolates, *aadA*, *strA*, and *strB* genes were found in 19 (86.4%), five (2.3%), and three (1.3%) isolates, respectively. The *sul1* gene encoding sulfonamide resistance was detected in nine isolates (3.2%) (Table 2). However, all isolates were negative for *tetB*, *sul2*, and *dfrIIV* genes. A class 1 integron was detected in 9 of 50 *E. coli* isolates (18%), whereas only 1 isolate (2%) harboured a class 2 integron. All isolates were negative for class 3 integrons.

3.6. E. coli phylogenetic group typing

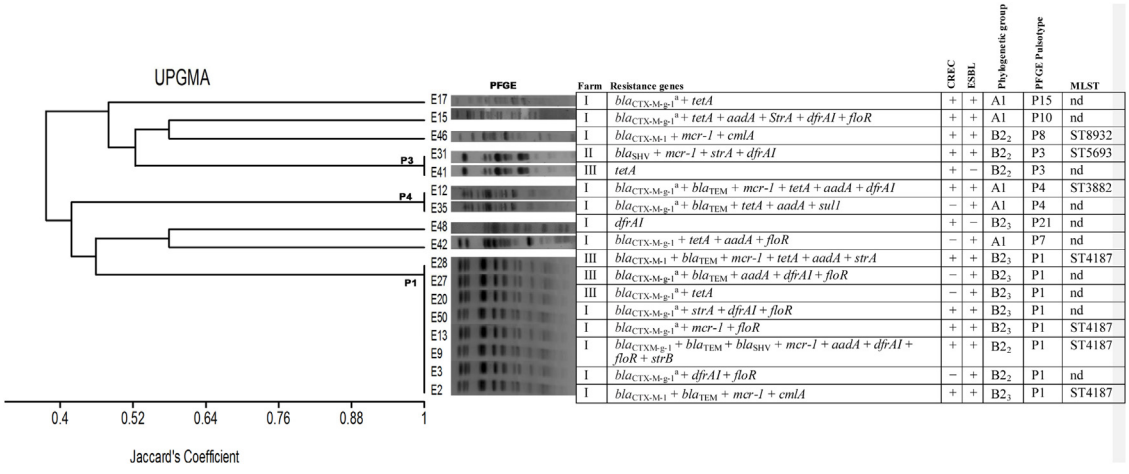
Phylogenetic analysis of the 50 *E. coli* isolates showed that they belonged to three groups: group A (10; 20%), B2 (37; 74%), and D (3; 6%). Regarding subgroups, isolates were allotted as follows: $B2_3$ (19; 38%); $B2_2$ (18; 36%); A_1 (8; 16%); D_2 (3; 6%); and A_0 (4; 8%). The 12 CREC isolates were placed in three subgroups: A_1 (n = 3), sub-group $B2_2$ (n = 2), and sub-group $B2_3$ (n = 7) (Table 2). Various phylogenetic sub-groups ($B2_3$, $B2_2$ and A_1) were observed among the ESBL-EC isolates (Fig. 1).

3.7. Genetic relatedness between E. coli isolates using PFGE

PFGE analysis of the 50 *E. coli* isolates demonstrated 26 pulsotypes (assigned as P1–P26). Thirty-three *E. coli* isolates obtained from multiple farms belonged to identical pulsotypes, including P1 (11), P3 (4), P4 (2), P5 (2), P6 (4), P7 (3), P10 (2), P11 (2), and P14, whereas 17 isolates showed unrelated pulsotypes (Table 2). The pulsotype P1 (n = 11) was not only the most prevalent pulsotype among ESBL-EC (8/15), but also among CREC and ESBL-*mcr-1*-carrying isolates (5/12 and 4/10, respectively) (Fig. 1). The 12 CREC isolates displayed related and unrelated pulsotypes within the 3 farms: FI (P1; P4; P8; P10; P15; P21); FII (P3); and FIII (P1 and P3) (Fig. 1).

3.8. Molecular typing of mcr-1-positive E. coli by MLST

MLST demonstrated that the seven *mcr-1*-carrying *E. coli*-positive isolates were assigned to four sequence types (STs): ST4187 (n = 4 isolates from FI and FIII); ST3882 (n = 1 isolate from FI); ST5693 (n = 1 isolate from FII); and ST8932 (n = 1 isolate from FI) (Fig. 1).



Farm	Resistance genes	CREC	ESBL	Phylogenetic group	PFGE Pulsotype	MLST
I	$bla_{\text{CTX-M-g-1}}$[a] + *tetA*	+	+	A1	P15	nd
I	$bla_{\text{CTX-M-g-1}}$[a] + *tetA* + *aadA* + *StrA* + *dfrAI* + *floR*	+	+	A1	P10	nd
I	$bla_{\text{CTX-M-1}}$ + *mcr-1* + *cmlA*	+	+	$B2_2$	P8	ST8932
II	bla_{SHV} + *mcr-1* + *strA* + *dfrAI*	+	+	$B2_2$	P3	ST5693
III	*tetA*	+	−	$B2_2$	P3	nd
I	$bla_{\text{CTX-M-g-1}}$[a] + bla_{TEM} + *mcr-1* + *tetA* + *aadA* + *dfrA1*	+	+	A1	P4	ST3882
I	$bla_{\text{CTX-M-g-1}}$[a] + bla_{TEM} + *tetA* + *aadA* + *sul1*	−	+	A1	P4	nd
I	*dfrA1*	+	−	$B2_3$	P21	nd
I	$bla_{\text{CTX-M-g-1}}$ + *tetA* + *aadA* + *floR*	−	+	A1	P7	nd
III	$bla_{\text{CTX-M-1}}$ + bla_{TEM} + *mcr-1* + *tetA* + *aadA* + *strA*	+	+	$B2_3$	P1	ST4187
III	$bla_{\text{CTX-M-g-1}}$[a] + bla_{TEM} + *aadA* + *dfrA1* + *floR*	−	+	$B2_3$	P1	nd
III	$bla_{\text{CTX-M-g-1}}$[a] + *tetA*	−	+	$B2_3$	P1	nd
I	$bla_{\text{CTX-M-g-1}}$[a] + *strA* + *dfrA1* + *floR*	+	+	$B2_3$	P1	nd
I	$bla_{\text{CTX-M-g-1}}$[a] + *mcr-1* + *floR*	+	+	$B2_3$	P1	ST4187
I	$bla_{\text{CTXM-g-1}}$ + bla_{TEM} + bla_{SHV} + *mcr-1* + *aadA* + *dfrA1* + *floR* + *strB*	+	+	$B2_2$	P1	ST4187
I	$bla_{\text{CTX-M-g-1}}$[a] + *dfrA1* + *floR*	−	+	$B2_2$	P1	nd
I	$bla_{\text{CTX-M-1}}$ + bla_{TEM} + *mcr-1* + *cmlA*	+	+	$B2_3$	P1	ST4187

Fig. 1. PFGE dendrogram representing the genetic relatedness and characteristics of CREC, ESBLs, and CREC-ESBL isolates in chickens with colibacillosis from three farms in Tunisia, using UPGMA (unweighted pair group method using Jaccard's coefficient).
*Sequencing not done.
CREC, colistin-resistant *Escherichia coli;* ESBL, extended-spectrum β-lactamase; MLST, multilocus sequence typing; nd, not done; P1, P3 and, P4, PFGE groups.

Table 2
Phenotypic and genotypic characteristics of the 50 *E. coli* strains isolated from broiler chickens with colibacillosis within 3 farms in Tunisia.

Farm	ID	Resistance profile	Resistance genes	Virulence genes	Integron class	Phylogenetic group	PFGE group	MLST
FI	E1	AMX/NAL/FLU/ENF/SXT/FFC/AMC/CAZ/CTX/FEP/ATM	*bla*$_{TEM}$ + *aadA* + *dfrAI* + *floR* + *sul1*	*fimA* + *aer*	*int*1	$B2_2$	P16	ND
	E2[a]	AMX/AMC/CAZ/CTX/FEP/ATM/NAL/FLU/ENF/SXT/DOX/TET/FFC/STR/COL[b]	*bla*$_{CTX-M-1}$ + *bla*$_{TEM}$ + *mcr-1* + *cmlA*	*fimA* + *stx1* + *stx2* + *papC* + *aer*	*int*1	$B2_3$	P1	ST4187
	E3[a]	AMC/CAZ/CTX/FEP/ATM/CTF/NAL/ENF/SXT/DOX/FFC	*bla*$_{CTX-M-g-1}$[c] + *dfrAI* + *floR*	—	—	$B2_2$	P1	ND
	E4	AMX/CTF/NAL/FLU/ENF/SXT/DOX/TET/FFC/AMC/CAZ/CTX/FEP/ATM	*dfrAI*	*fimA*	—	$B2_2$	P11	ND
	E5	AMX/AMC/NAL/FLU/ENF/SXT/FFC	*dfrAI* + *floR*	*aer*	—	$B2_2$	P5	ND
	E7	AMX/AMC/CAZ/CTX/FEP/ATM/NAL/FLU/ENF/FFC	*bla*$_{TEM}$	*fimA*	—	$B2_2$	P6	ND
	E8	AMX/NAL/FLU/ENF/SXT/DOX/TET/FFC/STR/AMC/CAZ/CTX/FEP/ATM	*bla*$_{TEM}$ + *tetA* + *aadA* + *dfrAI* + *floR* + *strB*	*fimA* + *stx2*	—	A1	P17	ND
	E9[a]	AMX/CTF/NAL/FLU/ENF/SXT/DOX/TET/FFC/STR/COL[b]/AMC/CAZ/CTX/FEP/ATM	*bla*$_{CTX-M-g-1}$[c] + *bla*$_{TEM}$ + *bla*$_{SHV}$ + *mcr-1* + *aadA* + *dfrAI* + *floR* + *strB*	*fimA*	—	$B2_2$	P1	ST4187
	E12[a]	AMX/NAL/FLU/SXT/DOX/TET/COL[b]	*bla*$_{CTX-M-g-1}$[c] + *bla*$_{TEM}$ + *mcr-1* + *tetA* + *aadA* + *dfrAI*	*fimA* + *stx1* + *aer*	—	A1	P4	ST3882
	E13[a]	AMC/CAZ/CTX/FEP/ATM/NAL/FLU/ENF/DOX/TET/FFC/COL[b]	*bla*$_{CTX-M-g-1}$[c] + *mcr-1* + *floR*	*fimA* + *stx2*	—	$B2_3$	P1	ST4187
	E14	AMX/NAL/FLU/ENF/DOX/AMC/CAZ/CTX/FEP/ATM	*bla*$_{TEM}$ + *aadA*	*fimA* + *stx2* + *aer*	—	$B2_2$	P6	nd
	E15[a]	AMX/NAL/FLU/ENF/SXT/DOX/TET/FFC/AMC/CAZ/CTX/ATM/COL[b]	*bla*$_{CTX-M-g-1}$[c] + *tetA* + *aadA* + *StrA* + *dfrAI* + *floR*	*fimA*	*int*1	A1	P10	nd
	E16	AMX/AMC/CAZ/CTX/CTF/NAL/ENF/STR	*bla*$_{TEM}$ + *aadA*	*fimA*	—	$B2_2$	P3	nd
	E17[a]	AMX/CTF/NAL/FLU/ENF/DOX/TET/FFC/AMC/CAZ/CTX/FEP/ATM/COL[b]	*bla*$_{CTX-M-g-1}$[c] + *tetA*	—	—	A1	P15	nd
	E19	AMX/NAL/FLU/ENF/AMC/CAZ/CTX/FEP/ATM	—	—	—	D2	P13	nd
	E21	AMX/NAL/FLU/ENF/SXT/DOX/TET/AMC/CTX/CAZ	*bla*$_{TEM}$ + *tetA* + *aadA* + *dfrAI*	*fimA*	—	D2	P14	nd
	E22	NAL/FLU/ENF/SXT/DOX/FFC	*strA* + *sul1*	*fimA* + *aer*	—	A0	P18	nd
	E23	AMX/DOX/TET/AMC/CAZ/CTX/FEP/ATM	*bla*$_{TEM}$ + *tetA* + *sul1*	—	*int*1	$B2_3$	P1	nd
	E24	AMX/AMC/CAZ/CTX/FEP/ATM/NAL/FLU/ENF/SXT/DOX/TET/FFC/STR	*bla*$_{TEM}$ + *tetA* + *aadA* + *dfrAI* + *floR*	*fimA*	—	$B2_2$	P6	nd
	E26	NAL/FLU/DOX/TET	*dfrAI* + *floR* + *sul1*	—	*int*1	$B2_3$	P1	nd
	E29	AMX/NAL/FLU/DOX/TET/STR/AMC/CAZ/CTX/FEP/ATM	*bla*$_{TEM}$ + *tetA*	*fimA*	—	$B2_3$	P14	nd
	E30	AMX/NAL/FLU/DOX/TET/STR/AMC/CAZ/CTX/FEP/ATM	*bla*$_{TEM}$ + *tetA* + *sul1*	*fimA*	*int*1	$B2_3$	P7	nd
	E35[a]	AMX/AMC/CAZ/CTX/FEP/ATM/CTF/NAL/FLU/ENF/SXT/DOX/TET/STR	*bla*$_{CTX-M-g-1}$[c] + *bla*$_{TEM}$ + *tetA* + *aadA* + *sul1*	*fimA*	—	$B2_3$	P4	nd
	E37	AMX/NAL/FLU/SXT/DOX/TET/FFC/STR	*cmlA* + *sul1*	—	—	$B2_3$	P1	nd
	E40	AMX/SXT/DOX/TET/FFC	*tetA* + *dfrAI* + *floR* + *sul1*	*fimA* + *aer*	—	A1	P4	nd
	E42[a]	AMX/NAL/FLU/ENF/SXT/DOX/TET/FFC	*bla*$_{CTX-M-g-1}$ + *tetA* + *aadA* + *floR*	*fimA*	—	A1	P7	nd
	E45	AMX/NAL/FLU/ENF/SXT/DOX/TET/FFC/STR	*dfrAI* + *floR*	*fimA* + *aer*	—	A1	P19	nd
	E46[a]	AMX/AMC/CAZ/CTX/FEP/ATM/NAL/FLU/ENF/SXT/DOX/TET/FFC/COL[b]	*bla*$_{CTX-M-1}$ + *mcr-1* + *cmlA*	*fimA* + *aer*	—	$B2_3$	P8	ST8932
	E47	NAL/FLU/DOX/TET/STR	*aadA*	—	—	$B2_2$	P20	nd
	E48	AMX/NAL/FLU/DOX/TET/STR/AMC/CTX/FEP/ATM/COL[b]	*dfrAI*	*aer*	*int*1	$B2_3$	P21	nd
	E50[a]	AMX/NAL/FLU/ENF/SXT/DOX/TET/FFC/STR/AMC/CAZ/CTX/FEP/ATM/COL[b]	*bla*$_{CTX-M-g-1}$[c] + *strA* + *dfrAI* + *floR*	*fimA* + *aer*	—	$B2_3$	P1	nd
FII	E6	AMX/AMC/NAL/FLU/ENF/DOX/TET/FFC	*floR*	—	—	$B2_3$	P7	nd
	E10	AMX/AMC/NAL/FLU/ENF/SXT/DOX/TET/FFC/STR	*tetA* + *aadA* + *dfrAI* + *floR* + *strB*	*aer*	—	$B2_3$	P9	nd
	E18	AMX/NAL/FLU/ENF/SXT/DOX/TET/FFC/STR	*tetA* + *aadA* + *dfrAI*	—	*int*2	$B2_2$	P5	nd
	E25	NAL/FLU/ENF	*strB*	—	—	A0	P22	nd
	E32	NAL/FLU/SXT/DOX/TET/FFC/STR/AMC/CAZ/CTX/FEP/ATM	*floR*	*fimA*	—	$B2_3$	P23	nd
	E31[a]	NAL/FLU/SXT/DOX/TET/STR/COL[b]/AMC/CAZ/CTX/FEP/ATM	*bla*$_{SHV}$ + *mcr-1* + *strA* + *dfrAI*	*fimA* + *aer*	—	$B2_2$	P3	ST5693
	E33	AMX/NAL/FLU/ENF	*aadA*	—	—	$B2_2$	P24	Nd
Farm	ID	Resistance profile	Resistance genes	Virulence genes	Integron class	Phylogenetic group	PFGE group	MLST
	E36	AMX/NAL/FLU/DOX/TET/FFC	*dfrAI* + *floR*	*aer*	—	$B2_3$	P25	nd
	E38	SXT/SPC/FFC/FLU/DOX/NAL/ERY/STR/TET	*tetB* + *dfrIA* + *floR*	—	—	A1	P10	nd
	E39	AMX/AMC/CAZ/CTX/FEP/ATM/NAL/FLU/ENF/DOX/TET	*bla*$_{TEM}$ + *tetA*	*aer*	—	$B2_2$	P11	nd

FIII	E11	AMX/AMC/CAZ/CTX/FEP/ATM/NAL/FLU/ENF	bla_{TEM}	*fimA*	–	$B2_3$	P3	nd	
	E20[a]	AMX/AMC/CAZ/CTX/FEP/ATM/NAL/FLU/ENF/DOX/TET	$bla_{CTX\text{-}M\text{-}g\text{-}1}$[c] + *tetA*	*fimA*	–	$B2_3$	P1	nd	
	E27[a]	AMX/AMC/CAZ/CTX/FEP/ATM/CTF/NAL/FLU/ENF/SXT/DOX/TET/FFC/STR	$bla_{CTX\text{-}M\text{-}g\text{-}1}$[c] + bla_{TEM} + *aadA* + *dfrAI* + *floR*	*fimA* + *aer*	–	$B2_3$	P1	nd	
	E28[a]	AMX/CTF/AMC/CAZ/CTX/FEP/ATM/DOX/TET/STR/COL[b]	$bla_{CTX\text{-}M\text{-}1}$ + bla_{TEM} + *mcr-1* + *tetA* + *aadA* + *strA*	*fimA* + *aer*	–	$B2_3$	P1	ST4187	
	E34	DOX/TET/STR	*tetA*	–	–	$B2_2$	P26	nd	
	E41	AMC/CAZ/CTX/FEP/ATM/NAL/FLU/ENF/SXT/DOX/TET/FFC/COL[b]	*tetA*	–	–	$B2_2$	P3	nd	
	E43	AMX/AMC/CAZ/CTX CTF/NAL/ENF/DOX/FFC	*floR*	*fimA* + *aer*	–	D2	P12	nd	
	E44	NAL/FLU/TET/AMC/CAZ/CTX/ATM	*tetA*	–	–	$B2_2$	P2	nd	
	E49	AMX/CTF/NAL/FLU/DOX/TET/STR/AMC/CAZ/CTX/FEP/ATM	*tetA*	–	–	$B2_2$	P6	nd	

AMC, amoxicillin–clavulanic acid; AMX, amoxicillin; ATM, aztreonam; CAZ, ceftazidime; COL, colistin; CTF, ceftiofur; CTX, cefotaxime; DOX, doxycycline; ENF, enrofloxacin; FEP, cefepime; FFC, florfenicol; FLU, flumequine; MLST, multilocus sequence typing; NAL, nalidixic acid; nd, sequencing not done; STR, streptomycin; SXT, trimethoprim-sulfamethoxazole; TET, tetracycline.

[a] Extended-spectrum β-lactamase-producing strains.

[b] Colistin-resistant strains (MIC of 4 and 8 ug/mL).

[c] Sequencing not done.

4. Discussion

Avian colibacillosis caused by APEC is a major problem in poultry industries worldwide [4]. To the best of our knowledge, this study is the first to reveal a high percentage of MDR *E. coli* harbouring *mcr-1* and ESBL-encoding genes recovered from broiler chickens that died due to colibacillosis in Tunisia and the genetic relatedness of isolates.

In this study, 50 *E. coli* isolates were collected from infected organs from chickens (50/100; 50%). Similar to previous studies, our *E. coli* isolates were highly resistant to β-lactams, tetracyclines, quinolones, phenicols, and trimethoprim–sulfamethoxazole [29,30]. The majority of isolates showed an MDR phenotype (48/50; 96%), which corroborates previous findings [29,31].

A high number of isolates (94%) harboured one or more resistance genes (RGs) (*tetA*, bla_{TEM}, *aadA*, *dfrIA*, *sul1*, *floR*, *strB*, *strA*, and *cmlA*) which demonstrates their role as reservoirs of β-lactam, sulfonamide, and tetracycline-encoding genes, as previously reported [12,29,30]. Thus, the MDR phenotypes exhibited by our isolates might be due to the acquisition of various genetic elements such as plasmids, transposons, and integrons which are important vehicles of resistance genes [29].

In this study, class 1 and class 2 integrons were detected in nine isolates (18%) and one isolate (2%), respectively. These findings are different from that of Soufi et al. [10] who found a higher prevalence of integrons (60%) and from Kilani et al. [11] who reported the predominance of *int2* in avian *E. coli* isolates. The predominance of the class 1 integron was not surprising as it has been known as the most ubiquitous and commonly reported integron among clinical *E. coli* isolates [32].

All previous data regarding *E. coli* from chickens in Tunisia were investigated in healthy animals. To the best of our knowledge, this study is the first to identify the *mcr-1* gene in diseased chickens from Tunisia. The *mcr-1*-positive isolates were recovered from dead chickens already subjected to antibiotherapy including colistin which may explain in part the relatively high percentage of CREC (24%). Isolates harbouring the *mcr-1* gene co-carried a combination of genes (*tetA*, *aadA*, *dfrIA*, *floR*, *strA*, *strB*, *sul1*, and *cmlA*), demonstrating the high genetic pool they constitute for other *E. coli* isolates [9,30].

The percentage of ESBL-EC in this study (28%) was higher than that found by Halfaoui et al. in APEC from Algeria (1.9%) [33] but lower than that recorded by Maamar et al. in 2016 (35%) [12]. Nonetheless, it is important to note that ESBL isolates found in this study were detected without using a selective protocol for the screening of ESBL-EC, in contrast with the study by Maamar et al. in 2018 [14], which may reflect the high frequency of ESBL-EC from chickens with colibacillosis. β-Lactamase-encoding genes that predominated in our isolates belonged to the $bla_{CTX\text{-}M\text{-}1}$ group. This finding is in agreement with those reported in Tunisia by Kilani et al. [11] and Maamar et al. [12] where CTX-M-1 is so far the predominant ESBL enzyme in avian *E. coli*.

It is a matter of concern that the majority of ESBL-EC were CREC and vice versa. This finding indicates the potential co-location and co-transfer of *mcr-1* and ESBL-encoding genes as previously reported [8,9] and highlights the impact of the overuse of both β-lactams and colistin in chicken production systems in Tunisia. This also suggests that ESBL-EC are more likely to recruit the mcr-1 gene than other non-ESBL-EC as previously reported [34]. In other studies, Grami et al. [13] and Saidani et al. [15] have reported the co-carriage of *mcr-1* and $bla_{CTX\text{-}M\text{-}1}$ genes on the same IncHI2-type and IncI1 and IncI2 plasmids in *E. coli* from healthy chickens in Tunisia, whereas Maamar et al. [12] detected *mcr-1*-positive *E. coli* in CMY-2-producing isolates. In other studies, the *mcr-1* gene has been observed on a variety of plasmid types containing other antimicrobial resistance genes such as those encoding

carbapenemases [35] and ESBLs [36]. In this study, we report the simultaneous occurrence of ESBL and CREC isolates from broiler chickens with colibacillosis from Tunisia. This is probably due to the fact that the aforementioned genes are commonly found in mobile elements such as conjugative plasmids that also harbour resistance determinants to different groups of antimicrobials and confer the MDR phenotype [9]. Previous studies have reported that the co-carriage of ESBL and *mcr-1* genes on the same plasmid facilitates the dissemination of CREC isolates by the co-selective pressure applied via the use of colistin and other non β-lactam antibiotics. It is highly likely that food animals have become one of the most important sources for the spread of these resistance-gene-carrying bacteria to humans through horizontal gene transfer [34].

Therefore, further studies are necessary to search for a probable colocation of the *mcr-1* gene along with ESBL genes on a single plasmid among our isolates. Consistent with global and national trends, the *mcr-1* gene was the most common colistin-resistant gene found in poultry [13,31]. The widespread dissemination of this plasmid carrying the *mcr-1* gene poses a significant public health risk, as these can spread rapidly by horizontal gene transfer [37,38].

Phylogroup distribution in *E. coli* isolates showed that most of the isolates (74%) were allotted to the phylogroup B2 which is in accordance with that of Cordoni et al. [39] but in disagreement with the findings of Kilani et al. and Maamar et al. [11,12]. This finding can be explained by the difference in the health status of chickens included in studies. All CREC isolates examined in this study were of either phylogenetic type B2 or A1. Phylogenetic types B2 and D were considered virulent by Clermont et al. [24]. The B2 group was frequently detected in ExPEC incriminated in severe human infections [1] which demonstrates the high zoonotic potential of these isolates and the possibility of their transmission to humans [2]. Further studies are necessary to assess the content of virulence determinants in *E. coli* from chickens with colibacillosis.

PFGE analysis showed a high level of genetic diversity among our isolates (26 pulsotypes). On the one hand (i) isolates displayed identical pulsotypes including P1 (n = 11), P3 (n = 4), and P6 (n = 4), demonstrating a clonal dissemination among chickens with colibacillosis within different farms, and (ii) P1 pulsotype was not only found in *mcr-1*-carrying isolates, but also in ESBL-EC which strongly suggests a clonal spread among chickens as reported by Maamar et al. [14]. On the other hand, PFGE showed unrelated PFGE patterns among CREC in the three farms which may reflect the diversity of *E. coli* clones harbouring *mcr-1* gene as shown by Grami et al. [13]. PFGE analysis showed a close relationship between ESBL- and mcr-1-carrying E. coli among chickens from the same and different farms (FI and FIII, respectively). In addition, MLST revealed that isolates of the P1 PFGE group in isolates from FI and FIII belonged to ST4187 lineage, highlighting the importance of this ST in the spread of *mcr-1*-mediated colistin resistance and ESBL-encoding genes among *E. coli* isolates in broiler chickens with colibacillosis from Tunisia. Seven different PFGE pulsotypes were observed among ESBL-EC isolates (Fig. 1) with P1 being the most predominant pattern (8/15) from 2 different farms (FI and FIII), indicating the importance of this clone in the dissemination of ESBL-encoding genes among clinical *E. coli* isolates. In this study, the seven mcr-*1*-producing isolates were assigned by MLST to four different STs: ST4187 (n = 4), ST3882 (n = 1), ST5693 (n = 1), and ST8932 (n = 1) (Fig. 1). Finding identical lineages in different farms (FI and FIII) from different regions may indicate that genetically related isolates of *E. coli* had been spread in chickens with colibacillosis. Worldwide, many studies have reported mcr-1-carrying *E. coli* isolates with multiple ST types (ST46, ST1286, ST10, ST29, ST101, and ST354) [34]. To the best of our knowledge, the two ST lineages (ST8932 and ST3882) have never been reported in Tunisia or worldwide; however, ST5693 and ST4187 were reported in *E. coli* isolated from hospitalization units in Angola and birds from Chile, respectively [40,41]. Finding no previously detected ST lineages is relevant because it shows the emergence of other lineages of colistin-resistant and ESBL-EC. However, whether these ST lineages are associated with colibacillosis outcomes other than ST lineage isolates is still unclear. These findings highlight the importance of prudent use of antibiotics in chicken production systems and underscore the urgent need to identify all probable ST lineages in ESBL-*mcr-1*-carrying *E. coli* in poultry to better understand the epidemiology of these clinically relevant isolates.

5. Conclusion

This study describes for the first time the co-occurrence of the *mcr-1* gene mediating colistin resistance and ESBL-encoding genes (bla_{TEM}, bla_{SHV}, and $bla_{CTX\text{-}M\text{-}1}$) in *E. coli* from broiler chickens with colibacillosis in Tunisia. Apart from the high prevalence of MDR *E. coli* (96%), ESBL- and *mcr-1*-carrying *E. coli* isolates harboured a combination of resistance, virulence, and β-lactamase-encoding genes and were assigned to four ST lineages: ST4187 (n = 4 isolates from FI and FIII); ST3882 (n = 1 isolate from FI); ST5693 (n = 1 isolate from FII); and ST8932 (n = 1 isolate from FI), suggesting clonal dissemination of *E. coli* ST4187 in FI and FIII. (ST4187, ST3882, ST5693, and ST8932). The presence of the *mcr-1* and ESBL genes in genetically related isolates raises the possibility of clonal dissemination between chickens. Thus, continuously monitoring ESBL-EC and CREC in chickens is essential to ensure food safety and to assess risk factors for their entrance into the food chain.

Funding

None.

Competing interests

None declared.

Ethical approval

This study was subjected to ethical review and given approval by the ethics committee in animal experimentation (CEEA, ENMV) at the National School of Veterinary Medicine of Sidi Thabet, Ariana, Tunisia, ref: 01. 2019/ISBST.

Acknowledgements

The authors thank the veterinarians who participated in sampling. This study was supported by the Tunisian Ministry of Higher Education and Scientific Research,Ceva Santé Animale (Tunisia), and theUniversidad de La Rioja (Logroño, Spain).

References

[1] Ewers C, Antao EM, Diehl I, Philipp HC, Wieler LH. Intestine and environment of the chicken as reservoirs for extraintestinal pathogenic *Escherichia coli* strains with zoonotic potential. Appl Environ Microbiol 2009;75:184–92, doi: http://dx.doi.org/10.1128/AEM.01324-08.
[2] Ewers C, Janssen T, Wieler LH. Avian pathogenic *Escherichia coli* (APEC). Berl Munch Tierarztl 2003;116:381–95.
[3] Ewers C, Li G, Wilking H, Kiessling S, Alt K, Antáo EM, et al. Avian pathogenic, uropathogenic, and newborn meningitis-causing *Escherichia coli*: how closely related are they? Int J Med Microbiol 2007;297:163–76.
[4] Guabiraba R, Schouler C. Avian colibacillosis: still many black holes. FEMS Microbiol Lett 2015;362:fnv118.
[5] Magiorakos AP, Srinivasan A, Carey RB, Carmeli Y, Falagas ME, Giske CG, et al. Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacte-

ria: an international expert proposal for interim standard definitions for acquired resistance. Clin Microbiol Infect 2012;18:268–81, doi:http://dx.doi.org/10.1111/j.1469-0691.2011.03570.x.
[6] Shafiq M, Huang J, Ur Rahman S, Shah JM, Chen L, Gao Y, et al. High incidence of multidrug-resistant *Escherichia coli* coharboring mcr-1 and bla$_{CTX-M-15}$ recovered from pigs. Infect Drug Resist 2019;12:2135–49, doi:http://dx.doi.org/10.2147/IDR.S209473.
[7] Liu YY, Wang Y, Walsh TR, Yi LX, Zhang R, Spencer J, et al. Emergence of plasmid-mediated colistin resistance mechanism MCR-1 in animals and human beings in China: a microbiological and molecular biological study. Lancet Infect Dis 2016;16:161–8, doi:http://dx.doi.org/10.1016/S1473-3099(15)00424-7.
[8] Haenni M, Poirel L, Kieffer N, Chatre P, Saras E, Metayer V, et al. Co-occurrence of extended spectrum beta lactamase and MCR-1 encoding genes on plasmids. Lancet Infect Dis 2016;16:281–2, doi:http://dx.doi.org/10.1016/S1473-3099(16)00007-4.
[9] Dominguez JE, Redondo LM, Figueroa Espinosa RA, Cejas D, Gutkind GO, Chacana PA, et al. Simultaneous carriage of mcr-1 and other antimicrobial resistance determinants in *Escherichia coli* from poultry. Front Microbiol 2018;9:1679, doi:http://dx.doi.org/10.3389/fmicb.2018.01679.
[10] Soufi L, Saenz Y, Vinue L, Abbassi MS, Ruiz E, Zarazaga M, et al. *Escherichia coli* of poultry food origin as reservoir of sulphonamide resistance genes and integrons. Int J Food Microbiol 2011;144:497–502, doi:http://dx.doi.org/10.1016/j.ijfoodmicro.2010.11.008.
[11] Kilani H, Abbassi MS, Ferjani S, Mansouri R, Sghaier S, Ben Salem R, et al. Occurrence of bla$_{CTX-M-1,}$ qnrB1 and virulence genes in avian ESBL-producing *Escherichia coli* isolates from Tunisia. Front Cell Infect Microbiol 2015;5:38, doi:http://dx.doi.org/10.3389/fcimb.2015.00038.
[12] Maamar E, Hammami S, Alonso CA, Dakhli N, Abbassi MS, Ferjani S, et al. High prevalence of extended-spectrum and plasmidic AmpC beta-lactamase-producing *Escherichia coli* from poultry in Tunisia. Int J Food Microbiol 2016;231:69–75, doi:http://dx.doi.org/10.1016/j.ijfoodmicro.2016.05.001.
[13] Grami R, Mansour W, Mehri W, Bouallegue O, Boujaafar N, Madec JY, et al. Impact of food animal trade on the spread of mcr-1-mediated colistin resistance, Tunisia, July 2015. Euro Surveill 2016;21:, doi:http://dx.doi.org/10.2807/1560-7917.ES.2016.21.8.30144.
[14] Maamar E, Alonso CA, Hamzaoui Z, Dakhli N, Abbassi MS, Ferjani S, et al. Emergence of plasmid-mediated colistin-resistance in CMY-2-producing *Escherichia coli* of lineage ST2197 in a Tunisian poultry farm. Int J Food Microbiol 2018;269:60–3, doi:http://dx.doi.org/10.1016/j.ijfoodmicro.2018.01.017.
[15] Saidani M, Messadi L, Chaouechi A, Tabib I, Saras E, Soudani A, et al. High genetic diversity of Enterobacteriaceae clones and plasmids disseminating resistance to extended-spectrum cephalosporins and colistin in healthy chicken in Tunisia. Microbial Drug Resist 2019;25:1507–13, doi:http://dx.doi.org/10.1089/mdr.2019.0138.
[16] Saidani M, Messadi L, Sahmin E, Zouaoui S, Soudani A, Daaloul-Jedidi M, et al. ESBL- and mcr-1-producing *Escherichia coli* in veal calves in Tunisia. J Glob Antimicrob Resist 2019;19:104–5, doi:http://dx.doi.org/10.1016/j.jgar.2019.08.009.
[17] Jouini A, Vinue L, Slama KB, Saenz Y, Klibi N, Hammami S, et al. Characterization of CTX-M and SHV extended-spectrum beta-lactamases and associated resistance genes in *Escherichia coli* strains of food samples in Tunisia. J Antimicrob Chemother 2007;60:1137–41, doi:http://dx.doi.org/10.1093/jac/dkm316.
[18] Clinical and Laboratory Standards Institute (CLSI). Performance standards for antimicrobial susceptibility testing; twenty-third information supplement CLSI document M100-S23. Philadelphia: CLSI; 2013.
[19] The European Committee on Antimicrobial Susceptibility Testing (EUCAST). Breakpoint tables for interpretation of MICs and zone diameters, v7.1. 2017. http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Breakpoint_tables/v_7.1_Breakpoint_Tables.pdf.
[20] Xavier BB, Lammens C, Ruhal R, Kumar-Singh S, Butaye P, Goossens H, et al. Identification of a novel plasmid-mediated colistin-resistance gene, mcr-2, in *Escherichia coli*, Belgium, June 2016. Euro Surveill 2016;21:, doi:http://dx.doi.org/10.2807/1560-7917.ES.2016.21.27.30280.
[21] Saenz Y, Brinas L, Dominguez E, Ruiz J, Zarazaga M, Vila J, et al. Mechanisms of resistance in multiple-antibiotic-resistant *Escherichia coli* strains of human, animal, and food origins. Antimicrob Agents Chemother 2004;48:3996–4001, doi:http://dx.doi.org/10.1128/AAC.48.10.3996-4001.2004.
[22] Bean DC, Livermore DM, Hall LMC. Plasmids imparting sulfonamide resistance in *Escherichia coli*: implications for persistence. Antimicrob Agents Chemother 2009;53:1088–93, doi:http://dx.doi.org/10.1128/AAC.00800-08.
[23] Mazel D, Dychinco B, Webb VA, Davies J. Antibiotic resistance in the ECOR collection: integrons and identification of a novel aad gene. Antimicrob Agents Chemother 2000;44:1568–74, doi:http://dx.doi.org/10.1128/aac.44.6.1568-1574.2000.
[24] Clermont O, Bonacorsi S, Bingen E. Rapid and simple determination of the *Escherichia coli* phylogenetic group. Appl Environ Microbiol 2000;66:4555–8, doi:http://dx.doi.org/10.1128/aem.66.10.4555-4558.2000.
[25] Escobar-Paramo P, Le Menac'h A, Le Gall T, Amorin C, Gouriou S, Picard B, et al. Identification of forces shaping the commensal *Escherichia coli* genetic structure by comparing animal and human isolates. Environ Microbiol 2006;8:1975–84, doi:http://dx.doi.org/10.1111/j.1462-2920.2006.01077.x.
[26] Kaufmann ME. Pulsed-field gel electrophoresis. Methods Mol Med 1998;15:17–31.
[27] Tenover FC, Arbeit RD, Goering RV, Mickelsen PA, Murray BE, Persing DH, et al. Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. J Clin Microbiol 1995;33:2233–9.
[28] Liu X, Thungrat K, Boothe DM. Multilocus sequence typing and virulence profiles in uropathogenic *Escherichia coli* isolated from cats in the United States. PLoS One 2015;10:e0143335, doi:http://dx.doi.org/10.1371/journal.pone.0143335.
[29] Soufi L, Abbassi MS, Saenz Y, Vinue L, Somalo S, Zarazaga M, et al. Prevalence and diversity of integrons and associated resistance genes in *Escherichia coli* isolates from poultry meat in Tunisia. Foodborne Pathog Dis 2009;6:1067–73, doi:http://dx.doi.org/10.1089/fpd.2009.0284.
[30] Awad A, Arafat N, Elhadidy M. Genetic elements associated with antimicrobial resistance among avian pathogenic *Escherichia coli*. Ann Clin Microb Anti 2016;15:59.
[31] Lima Barbieri N, Nielsen DW, Wannemuehler Y, Cavender T, Hussein A, Yan SG, et al. mcr-1 identified in Avian Pathogenic *Escherichia coli* (APEC). PLoS One 2017;12:e0172997, doi:http://dx.doi.org/10.1371/journal.pone.0172997.
[32] Machado E, Coque TM, Cantón R, Sousa JC, Peixe L. Antibiotic resistance integrons and extended-spectrum beta-lactamases among Enterobacteriaceae isolates recovered from chickens and swine in Portugal. J Antimicrob Chemother 2008;62:296–302.
[33] Halfaoui Z, Menoueri NM, Bendali LM. Serogrouping and antibiotic resistance of *Escherichia coli* isolated from broiler chicken with colibacillosis in center of Algeria. Vet World 2017;10:830–5.
[34] Wu C, Wang Y, Shi X, Wang S, Ren H, Shen Z, et al. Rapid rise of the ESBL and mcr-1 genes in *Escherichia coli* of chicken origin in China, 2008-2014. Emerg Microbes Infect 2018;7:30, doi:http://dx.doi.org/10.1038/s41426-018-0033-1.
[35] Poirel L, Kieffer N, Liassine N, Thanh D, Nordmann P. Plasmid-mediated carbapenem and colistin resistance in a clinical isolate of *Escherichia coli*. Lancet Infect Dis 2016;16:281, doi:http://dx.doi.org/10.1016/S1473-3099(16)00006-2.
[36] Zhang XF, Doi Y, Huang X, Li HY, Zhong LL, Zeng KJ, et al. Possible transmission of mcr-1-harboring *Escherichia coli* between companion animals and human. Emerg Infect Dis 2016;22:1679–81, doi:http://dx.doi.org/10.3201/eid2209.160464.
[37] Carattoli A. Plasmids and the spread of resistance. Int J Medical Microbiol Suppl 2013;303:298–304, doi:http://dx.doi.org/10.1016/j.ijmm.2013.02.001.
[38] Kluytmans JA, Overdevest IT, Willemsen I, Kluytmans-van den Bergh MF, van der Zwaluw K, Heck M, et al. Extended-spectrum beta-lactamase-producing *Escherichia coli* from retail chicken meat and humans: comparison of strains, plasmids, resistance genes, and virulence factors. Clin Infect Dis 2013;56:478–87, doi:http://dx.doi.org/10.1093/cid/cis929.
[39] Cordoni G, Woodward MJ, Wu H, Alanazi M, Wallis T, La Ragione RM. Comparative genomics of European avian pathogenic *E. coli* (APEC). BMC Genomics 2016;17:960, doi:http://dx.doi.org/10.1186/s12864-016-3289-7.
[40] Báez J, Hernández-García M, Guamparito C, Díaz S, Olave A, Guerrero K, et al. Molecular characterization and genetic diversity of ESBL-producing *Escherichia coli* colonizing the migratory Franklin's gulls *(Leucophaeus pipixcan)* in Antofagasta, North of Chile. Microb Drug Resist 2015;21:111–6.
[41] Kieffer N, Nordmann P, Aires-de-Sousa M, Poirel L. High prevalence of carbapenemase-producing Enterobacteriaceae among hospitalized children in Luanda, Angola. Antimicrob Agents Chemother 2016;60:6189–92.