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Molecular characterisation of extended-spectrum β-lactamase- and plasmid AmpC-producing *Escherichia coli* strains isolated from broilers in Béjaïa, Algeria

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ABSTRACT

This study aimed to characterise the molecular support of antibiotic resistance in expanded-spectrum cephalosporin (ESC)-resistant *Escherichia coli* isolates recovered from healthy broilers in Béjaïa, northeast Algeria. A total of 61 intestinal swabs from slaughtered broilers from four regions in Béjaïa locality, Algeria, were collected between February and April 2014, from which 20 ESC-resistant *E. coli* strains were isolated. *Escherichia coli* isolates were identified by classical biochemical and MALDI-TOF methods. Antibiotic susceptibility testing was performed using disk diffusion and Etest methods. Screening for β-lactamases, aminoglycoside-modifying enzyme (AME)-encoding genes and *qnr* determinants was performed by PCR and sequencing. Clonal relatedness was determined using molecular typing by multilocus sequence typing (MLST). Antibiotic susceptibility testing revealed that the isolates showed high rates of resistance (>90%) to amoxicillin, amoxicillin/clavulanic acid, piperacillin/tazobactam, aztreonam, ceftazidime, streptomycin, tobramycin, nalidixic acid and ciprofloxacin. Low rates of resistance were observed for kanamycin (35%), amikacin (30%), cefoxitin (20%) and cefotaxime (15%). Molecular characterisation revealed that all of the isolates expressed the bla_{TEM-1} gene. Fourteen of them harboured the bla_{SHV-12} gene, two harboured the $bla_{CTX-M-1}$ gene and four isolates harboured bla_{CMY-2}. Screening for AME-encoding genes demonstrated that all isolates contained the *aadA* gene. In addition, *qnrA* was detected as the quinolone resistance determinant in 13 isolates. MLST revealed four known sequence types (STs), including ST744, ST38, ST1011 and ST2179, as well as one new sequence type (ST5086). Here we report the first study describing the clonal diversity of extended-spectrum β-lactamase (ESBL)- and plasmid AmpC-producing *E. coli* isolated from healthy broilers in Algeria.

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1. Introduction

Escherichia coli is a common commensal of the intestinal tract of animals and humans but is also an important human pathogen [1]. Currently, several studies have reported many cases of infection caused by multidrug-resistant *E. coli* in humans and animals [1]. Some studies have raised an alarm about the wide presence of extended-spectrum β-lactamases (ESBLs) in bacteria recovered from a wide diversity of animals and food products in different countries [2]. This resistance has been observed in strains originating from different animal species, but is significantly higher in strains isolated from intensive broiler production around the world [3]. Thus, it is well established that antibiotic-resistant bacteria that are selected in chickens, pigs and cattle may be transmitted to the human intestine via the food chain as well as in environmental settings [1]. The role of pets and wild animals as reservoirs of antibiotic resistance genes has been also documented [4]. Resistant bacteria and plasmids bearing resistance genes could

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be transferred from chicken to chicken and from chicken to humans [5]. This raises public health concerns as the intestinal microbiome of these animals might serve as a reservoir for ESBL/AmpC-encoding resistance genes capable of being transmitted to humans [5]. Transmission via the food chain has been suggested, but transmission resulting from close contact between humans and animals on livestock farms is also plausible [5]. This suggests that contact with broilers and/or the farm environment could be a risk factor for ESBL/AmpC carriage among humans [5]. *Escherichia coli* isolates resistant to oxyimino-cephalosporins owing to the production of ESBLs have emerged worldwide and a number of different ESBL genes, such as the bla_{SHV}, bla_{TEM} and bla_{CTX-M}, have been identified [1]. In Algeria, the first report of the presence of CTX-M-like enzymes in clinical Enterobacteriaceae isolates was made by Touati et al., from Béjaïa, northeast Algeria [6]. Detection of the SHV-12 enzyme was reported for the first time in clinical isolates in Algeria in 2008 by Iabadene et al. [7]. In addition, the CMY-2 enzyme was reported for the first time in a *Salmonella enterica* serotype Senftenberg clinical isolate recovered from stools of an Algerian child [8]. In poultry, one study on ESBL-producing *E. coli* from avian isolates was conducted by Meguenni et al. [9]. The authors cited the presence of the ESBL enzymes CTX-M-1 and CTX-M-15 in *E. coli* isolates from birds in the Algerian central regions [9].

The objective of this study was to characterise the antibiotic resistance determinants in expanded-spectrum cephalosporin (ESC)-resistant *E. coli* isolates recovered from healthy broilers in Béjaïa, Algeria.

2. Materials and methods

2.1. Sampling and strain isolation

A total of 61 intestinal swabs (inside of the caecum) of slaughtered broilers were collected from four poultry farms in four regions in Béjaïa, Algeria (30 from the Béjaïa region, 15 from the Akbou region, 13 from the Feraoun region and 3 from the Souk El-Ténine region) and were screened for the presence of ESBL-producing *E. coli* isolates between February and April 2014. Each swab was incubated for 24 h at 37 °C in nutrient broth (Fluka, St Louis, MO) for enrichment. They were subsequently seeded on eosin–methylene blue agar (Fluka) plates supplemented with 2 μg/mL ceftazidime and were incubated for 24 h at 37 °C. Isolates with typical *E. coli* morphology were selected (one per sample) and were identified by classical biochemical methods using the API 20E identification system (bioMérieux, Marcy-l'Étoile, France) and were confirmed using the matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF/MS) method (Microflex; Bruker Daltonics GmbH, Bremen, Germany) [10].

2.2. Antibiotic susceptibility testing

Antibiotic susceptibility testing was performed on Mueller–Hinton (Fluka) agar by the standard disk diffusion procedure as described by the Antibiogram Committee of the French Society for Microbiology (CA-SFM) (http://www.sfm-microbiologie.org/). Eighteen antibiotics were tested, including amoxicillin, amoxicillin/clavulanic acid, piperacillin/tazobactam, cefoxitin, cefotaxime, ceftazidime, cefepime, aztreonam, meropenem, imipenem, amikacin, tobramycin, streptomycin, kanamycin, gentamicin, nalidixic acid, ciprofloxacin and colistin (all from Bio-Rad, Marnes-la-Coquette, France). Minimum inhibitory concentrations (MICs) of cefotaxime, ceftazidime, cefepime and ciprofloxacin were determined using the Etest method (AB BIODISK, Solna, Sweden). Results were interpreted according to CA-SFM breakpoints.

2.3. Phenotypic extended-spectrum β-lactamase detection

A screening test for ESBL production was carried out on Mueller–Hinton agar by the double-disk synergy test by placing disks of aztreonam, cefepime, ceftazidime and cefotaxime (30 μg each) at a distance of 20 mm centre-to-centre from a disk with amoxicillin/clavulanic acid (20/10 μg). Enhancement of the inhibition zone between the disks containing amoxicillin/clavulanic acid and cefotaxime or ceftazidime indicated the presence of ESBL production [11].

2.4. Molecular detection of antibiotic resistance-encoding genes

PCR amplification of bla_{CTX-M}, bla_{TEM}, bla_{SHV}, bla_{PER}, bla_{VEB}, bla_{GES}, bla_{CMY} and bla_{DHA} genes was carried out by PCR as previously described [12]. PCR screening was also performed for aminoglycoside-modifying enzyme (AME) genes [*aac(3)-Ia*, *aac(6′)-Ib*, *aadA*, *ant(2″)-I* and *aph(3′)-VI*] and 16S rRNA methylase genes (*armA* and *rmtA–F*) as previously described [13]. In addition, the plasmid-mediated quinolone resistance-encoding genes *qnr* and *aac(6′)-Ib*-cr were screened as previously described [14,15].

All positive PCR products obtained were sequenced using BigDye® terminator chemistry on an automated ABI 3730 automated sequencer (Applied Biosystems, Foster City, CA).

2.5. Multilocus sequence typing (MLST)

MLST was performed on the 20 ESC-resistant *E. coli* isolates using seven housekeeping genes (*adk*, *fumC*, *gyrB*, *icd*, *mdh*, *purA* and *recA*) according to the *E. coli* MLST database (http://mlst.warwick.ac.uk/mlst/dbs/Ecoli).

3. Results

3.1. Bacterial strains and antibiotic susceptibility

Among the 61 broilers sampled, 20 ESC-resistant *E. coli* isolates were selected and identified by API 20E and MALDI-TOF/MS. Using the double-disk synergy test, ESBL production was confirmed in 16 of the 20 ESC-resistant *E. coli* isolates.

Results of antibiotic susceptibility testing revealed that the isolates showed high rates of resistance (>90%) to amoxicillin, amoxicillin/clavulanic acid, piperacillin/tazobactam, aztreonam, ceftazidime, streptomycin, tobramycin, nalidixic acid and ciprofloxacin. Low rates of resistance were observed for kanamycin (35%), amikacin (30%), cefoxitin (20%) and cefotaxime (15%). All isolates remained susceptible to gentamicin, meropenem, imipenem and colistin. MICs for cefotaxime, ceftazidime, cefepime and ciprofloxacin ranged from 1 to >256 μg/mL, 2–48 μg/mL, 0.25–64 μg/mL and 0.125–16 μg/mL, respectively (Table 1).

3.2. Resistance gene determination

All *E. coli* isolates tested contained the bla_{TEM-1} gene. Among them, two co-expressed the $bla_{CTX-M-1}$ gene, 14 co-expressed the bla_{SHV-12} gene and 4 co-expressed bla_{CMY-2} (Table 1). Screening for genes encoding AMEs demonstrated that all of the isolates contained the adenylyltransferase gene *aadA* (Table 1). None of the isolates contained either the *aac(3)-Ia*, *aac(6′)-Ib*, *aph(3′)-VI*, *ant(2″)-I* or 16S rRNA methylase genes. In addition, 13 of the isolates tested produced the *qnrA*-like gene (Table 1). None of the isolates produced the variant *aac(6′)-Ib*-cr.

Table 1
Characteristics of 20 extended-spectrum β-lactamase (ESBL)-producing *Escherichia coli* strains isolated in broilers from Béjaïa regions, Algeria.

Strain	Date of isolation	Locality	Resistance profile	MIC (μg/mL)				*bla* genes	AME and *qnr* genes	ST
				CTX	CAZ	FEP	CIP			
AC14	25/03/2014	Akbou	AMX/AMC/TZP/CTX/FEP/ATM/TOB/STR/KAN/NAL/CIP	>256	4	64	6	CTX-M-1 + TEM-1	*aadA*	38
BC23	13/03/2014	Béjaïa	AMX/AMC/TZP/FOX/CAZ/ATM/TOB/STR/KAN/NAL/CIP	4	16	0.25	8	CMY-2 + TEM-1	*aadA*	744
BC28	13/03/2014	Béjaïa	AMX/AMC/TZP/FOX/CAZ/TOB/STR/KAN/NAL/CIP	4	16	0.25	16	CMY-2 + TEM-1	*aadA*	744
BC3	13/03/2014	Béjaïa	AMX/AMC/TZP/FOX/CAZ/ATM/AMK/TOB/STR/KAN/NAL/CIP	4	16	0.25	12	CMY-2 + TEM-1	*aadA*	744
BC8	13/03/2014	Béjaïa	AMX/AMC/TZP/FOX/CAZ/ATM/TOB/STR/KAN/NAL/CIP	4	16	0.25	12	CMY-2 + TEM-1	*aadA*	744
FC1	02/04/2014	Feraoun	AMX/AMC/TZP/CAZ/ATM/AMK/TOB/STR/NAL/CIP	2	16	0.5	12	SHV-12 + TEM-1	*aadA* + *qnrA*	1011
FC10	02/04/2014	Feraoun	AMX/AMC/TZP/CAZ/ATM/TOB/STR/NAL/CIP	2	24	0.75	16	SHV-12 + TEM-1	*aadA* + *qnrA*	1011
FC11	02/04/2014	Feraoun	AMX/AMC/TZP/CTX/CAZ/ATM/AMK/TOB/STR/NAL/CIP	3	24	0.75	12	SHV-12 + TEM-1	*aadA* + *qnrA*	1011
FC12	02/04/2014	Feraoun	AMX/AMC/TZP/CAZ/ATM/TOB/STR/NAL/CIP	3	24	0.75	8	SHV-12 + TEM-1	*aadA* + *qnrA*	1011
FC13	02/04/2014	Feraoun	AMX/AMC/TZP/CAZ/ATM/TOB/STR/AMK/NAL/CIP	2	16	0.5	16	SHV-12 + TEM-1	*aadA* + *qnrA*	1011
FC2	02/04/2014	Feraoun	AMX/AMC/TZP/CTX/FEP/ATM/STR/AMK/NAL/CIP	32	2	6	8	CTX-M-1 + TEM-1	*aadA* + *qnrA*	2179
FC3	02/04/2014	Feraoun	AMX/AMC/TZP/CAZ/ATM/TOB/STR/NAL/CIP	2	16	0.5	16	SHV-12 + TEM-1	*aadA* + *qnrA*	1011
FC4	02/04/2014	Feraoun	AMX/AMC/TZP/CAZ/ATM/TOB/STR/NAL/CIP	2	24	0.75	8	SHV-12 + TEM-1	*aadA* + *qnrA*	1011
FC5	02/04/2014	Feraoun	AMX/AMC/TZP/CAZ/ATM/TOB/STR/NAL/CIP	3	48	0.75	8	SHV-12 + TEM-1	*aadA* + *qnrA*	1011
FC6	02/04/2014	Feraoun	AMX/AMC/TZP/CAZ/ATM/TOB/STR/NAL/CIP	2	16	0.5	16	SHV-12 + TEM-1	*aadA* + *qnrA*	1011
FC7	02/04/2014	Feraoun	AMX/AMC/TZP/CAZ/ATM/TOB/STR/NAL/CIP	2	16	0.5	16	SHV-12 + TEM-1	*aadA* + *qnrA*	1011
FC8	02/04/2014	Feraoun	AMX/AMC/TZP/CAZ/ATM/AMK/TOB/STR/NAL/CIP	3	32	0.75	8	SHV-12 + TEM-1	*aadA* + *qnrA*	1011
FC9	02/04/2014	Feraoun	AMX/AMC/TZP/CAZ/ATM/TOB/STR/NAL/CIP	3	24	0.75	16	SHV-12 + TEM-1	*aadA* + *qnrA*	1011
SEC1B	30/03/2014	Souk El-Ténine	AMX/AMC/TZP/CAZ/ATM/TOB/STR/KAN/NAL	1	12	0.38	0.13	SHV-12 + TEM-1	*aadA*	5086
SEC1J	30/03/2014	Souk El-Ténine	AMX/AMC/TZP/CAZ/ATM/TOB/STR/KAN	1.5	16	0.25	0.13	SHV-12 + TEM-1	*aadA*	5086

MIC, minimum inhibitory concentration; AME, aminoglycoside-modifying enzyme; ST, sequence type; CTX, cefotaxime; CAZ,; ceftazidime; FEP, cefepime; CIP, ciprofloxacin; AMX, amoxicillin; AMC, amoxicillin/clavulanic acid; TZP, piperacillin/tazobactam; ATM, aztreonam; TOB, tobramycin; STR, streptomycin; KAN, kanamycin; NAL, nalidixic acid; FOX, cefoxitin; AMK, amikacin.

3.3. Multilocus sequence typing results

Five different sequence types (STs) were assigned to the 20 ESC-resistant *E. coli* strains isolated from four Béjaïa localities, including four known STs [ST744 (4 strains), ST38 (1 strain), ST1011 (12 strains) and ST2179 (1 strain)] and one new ST (ST5086) in two *E. coli* isolates (Table 1; Fig. 1).

4. Discussion

Escherichia coli is a common inhabitant of the intestinal tract in humans and animals and can be easily disseminated in different ecosystems through the food chain and via water [16]. Overuse of antibiotics in food-source animal production has been shown to increase the risk of spread of antibiotic resistance [4]. ESBL-producing Enterobacteriaceae have been detected in patients, individuals from the community, meat, livestock, companion animals and the environment. Transmission between humans and animals might occur through the food chain [17]. In this context, our study was conducted to investigate the occurrence of the ESBL-producing *E. coli* in slaughtered broiler intestines. The results of this study show that 20 ESBL-positive *E. coli* strains were isolated from intestinal swabs of 61 slaughtered broilers in four regions in Béjaïa, Algeria (Fig. 1). The SHV-1, TEM-1 and OXA-type β-lactamases have been frequently described in *E. coli* and *Salmonella* spp. from animals and food of animal origin in Spain, Germany, the USA and the UK [18]. In Tunisia, Ben Slama et al. have reported the presence of CTX-M-1, CTX-M-8, CTX-M-14 and TEM-1 in food samples (poultry and other animals) [19].

In Algeria, very few studies have been conducted to characterise antibiotic-resistant strains from animals and food of animal origin. Among them, one study on ESBL-producing avian *E. coli* was conducted by Meguenni et al. [9]. In that study, the authors reported the presence of CTX-M-1- and CTX-M-15-producing *E. coli* in Algerian central regions [9]. Hence, the originality of the present study is the detection, in addition to CTX-M-1, of the presence of SHV-12 associated with the TEM-1 β-lactamase from *E. coli* isolates from healthy broilers for consumption after slaughtering. The TEM-1 detected in all of the strains was associated with SHV-12 from the Feraoun and Souk El-Ténine cities, or with CTX-M-1 from the Akbou and Feraoun cities, or with CMY-2 from Béjaïa City (Table 1). Dissemination and persistence of CMY-2-encoding plasmids are evident, and this might have also been contributed by the geographical transfer of the CMY-2 isolates through international trade in food animals [20]. In 2006, Blanc et al. reported the presence of CMY-2-encoding plasmids in *E. coli* from poultry, pig and rabbit farms in Spain [21]. In Algeria, this β-lactamase has only been detected from isolates recovered in the clinical setting [22]. CMY-2 was detected in four isolates of *E. coli* recovered from Béjaïa City.

Co-resistance to non-β-lactam antibiotics such as aminoglycosides in ESBL-producing Enterobacteriaceae is commonly described [20]. In addition, the presence of ESBL and plasmid AmpC (pAmpC) genes associated with AME genes has been reported in other studies [16,19].

Resistance to aminoglycosides is generally mediated by AMEs, including aminoglycoside phosphotransferases, acetyltransferases and nucleotidyltransferases, and 16S rRNA methylases have been reported recently among Enterobacteriaceae, *Pseudomonas* spp. and *Acinetobacter* spp. [23]. In the current study, the acetyltransferase *aadA* gene was identified in all strains. None of the isolates contained either the *aac(3)-Ia*, *aac(6′)-Ib*, *aph(3′)-VI*, *ant(2″)-I* or 16S rRNA methylase genes. The *aadA* gene was detected, associated with CTX-M-14a, CTX-M-32 or TEM 52, from *E. coli* in poultry by Costa et al. in their prevalence study of ESBL-producing *E. coli* isolates in faecal samples from broilers [24]. Plasmid-mediated quinolone resistance was first identified in a clinical isolate of *Klebsiella pneumoniae*. Recently, a new mechanism of quinolone resistance has been identified: transfer from species to species of a plasmid encoding *aac(6′)-Ib*-cr, a variant of aminoglycoside acetyltransferase that confers reduced susceptibility to ciprofloxacin and norfloxacin by *N*-acetylation of the amino nitrogen on its piperazinyl substituent. Genes responsible for plasmid-mediated quinolone resistance are thought to be linked to ESBL genes [25]. The *aac(6′)-Ib*-cr gene was detected from *E. coli* in poultry by Agabou et al. in their study [1]. In the current study, in addition to ESBLs, 13 isolates producing CTX-M-1 or SHV-12 were found to carry a *qnrA*-like gene, from Feraoun locality. To our knowledge, the *qnrA* gene has not yet been reported in Algeria. In China, Xie et al. reported the presence of the *qnrA* gene in *E. coli* recovered

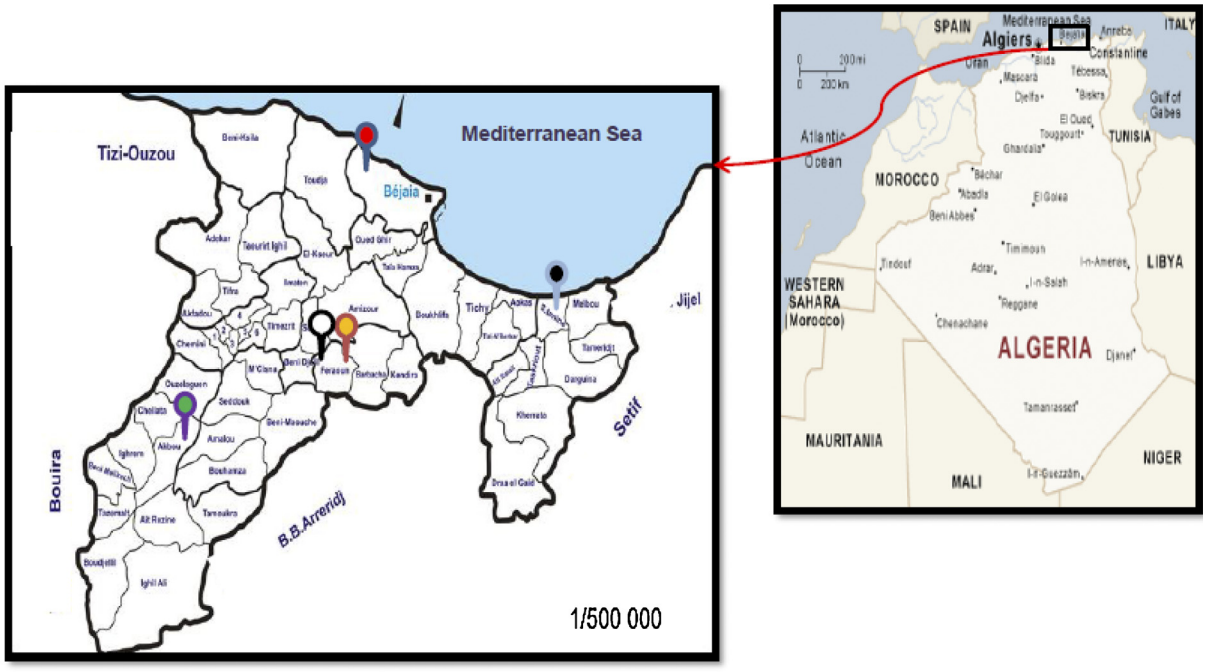

Fig. 1. Different localities in Béjaïa region where the five groups of extended-spectrum β-lactamase (ESBL)-producing *Escherichia coli* were isolated.

from septicaemic broilers. Thus, broilers might have a role in the transmission of resistance genes to humans [26].

The study of antibiotic resistance in strains of animal origin in Algeria has been focused on cases of illness caused by *E. coli* [27,28]. The STs identified in the current study were ST744 (from Béjaïa locality), ST38 (from Akbou locality), ST1011 and ST2179 (from Feraoun locality) and a new ST5086 (from Souk El-Ténine locality) (Fig. 1). Hasan et al. isolated CTX-M-1- and CTX-M-15-producing *E. coli* ST744 from domestic chicken in Bangladesh [29]. Then, ST744 with CTX-M-1 was reported by Guenther et al. in a bird of prey *E. coli* in Germany [30]. ST38 was also reported by Vogt et al. in meat-packing plants in Switzerland [31], and recently by Al Bayssari et al. in an *E. coli* OXA-48-producer in fowl from Lebanon [32]. ST2179 was detected in CTX-M-8-producing *E. coli* in buffalo [33] and in CTX-M-15-producing *E. coli* in horses from Brazil [34]. ST744, ST38 and ST2179 have been identified in clinical isolates in three hospitals in Hangzhou, China [35]. *Escherichia coli* ST1011 should be evaluated further for its zoonotic potential. It was detected in human extraintestinal pathogens from Brazil by Maluta et al. [36]. In the present study, ST1011 was the predominant ST because it was found in 12 strains (60%) isolated from the Feraoun locality. Interestingly, in addition to the four known STs obtained in this study, we have detected a new ST (ST5086). It was detected in two TEM-1- and SHV-12-producing *E. coli* strains isolated from Souk El-Ténine locality.

Antibiotic usage is considered the most important factor promoting the emergence, selection and dissemination of antibiotic-resistant micro-organisms both in veterinary and human medicine. Antibiotic usage selects for resistance not only in pathogenic bacteria but also in the endogenous flora of exposed individuals (animals and humans) or populations [37].

Several studies have shown that antimicrobial use in food animals contributes to the selection of antimicrobial resistance and poses risks to humans because of transmission of resistant zoonotic bacteria via the food chain and by indirect transfer of resistance genes from animals to man [38]. At slaughter, resistant strains from the gut readily soil poultry carcasses and as a result poultry meats are often contaminated with multiresistant *E. coli*; likewise, eggs become contaminated during laying. Hence, resistant faecal *E. coli* from poultry can infect humans both directly and via food. These resistant bacteria may colonise the human intestinal tract and may also contribute resistance genes to human endogenous flora [37].

Currently, when a severe clinical infection due to a multidrug-resistant pathogen appears, it is treated with colistin [39]. In animals, colistin is used to prevent or treat infections caused by *E. coli* isolates. In addition, this antibiotic is administered with food during or post weaning of animals [40]. Colistin-resistant bacteria in animals have been identified in many countries. Recently, the emergence of plasmid-mediated colistin resistance involving the *mcr-1* gene from bacteria was reported in many countries [41]. In Algeria, colistin-resistant *E. coli* isolates with the associated *mcr-1* gene were isolated from poultry. This is worrying because colistin is used as a last resort to treat multidrug-resistant pathogenic infections [41].

Finally, we conclude that broilers can be a reservoir of ESBL- and pAmpC-producing bacteria. These can be transmitted by direct contact or by the food chain. Surveillance of antibiotic resistance in

commensal bacteria from food-producing animals is considered to be one of the main priorities.

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Conflict of interest

None declared.

Ethical approval

Not required.

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