



NOTE

Avian Pathology

Isolation of *Escherichia coli* carrying the *bla*_{CTX-M-1} and *qnrS1* genes from reproductive organs of broiler breeders and internal contents of hatching eggs

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ABSTRACT. This study aimed to characterize two third-generation cephalosporins- and quinolone-resistant *Escherichia coli* (TGCs- and Q-R-*Ec*) isolates recovered from the ovaries of a broiler breeder flock and the internal contents of hatching eggs produced by the broiler breeder flock. Clonal relatedness was determined by multilocus sequence typing (MLST). The isolates displayed the same multidrug resistance profile, with resistance to ampicillin, ticarcillin, piperacillin, ceftazidime, cephalothin, cefotaxime, nalidixic acid, tetracycline and sulfonamides. Double disk synergy test demonstrated that the two isolates presented an ESBL phenotype. PCR and sequencing results showed that both the isolates harbored the *bla*_{CTX-M-1} and *qnrS1* genes. MLST revealed a novel allele combination, designated as ST461, in these isolates. This study would contribute to the molecular epidemiological understanding of TGCs- and/or Q-R-*Ec*.

KEY WORDS: antimicrobial resistance, molecular epidemiology, ovary, PCR, poultry

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The increasing trend of third-generation cephalosporins- and quinolone-resistant *Escherichia coli* (TGCs- and Q-R-*Ec*) in food-producing animals worldwide is worrisome [19, 22], due to the significance of TGCs and quinolones in human and veterinary medicine. The production of extended-spectrum β -lactamases (ESBLs) is the most encountered mechanism responsible for resistance to TGCs. TEM, SHV, and CTX-M-types are the three major families of ESBL [6]. CTX-M is the most extended group among ESBL enzymes, and due to its increasing prevalence (higher than TEM and SHV), some authors have announced the “CTX-M ESBL pandemic” [7]. Plasmid-mediated quinolone resistance (PMQR) determinants *qnr* gene has been subsequently reported [24]. In most cases, strains also had ESBLs, which raises the risk for transfer of multidrug resistant *E. coli* to humans [26].

Recently, TGCs- and/or Q-R-*Ec* isolates have been isolated at all levels of the broiler production pyramid, including chicken meat, in several countries [4, 7, 20, 21]. It has also been detected at commercial hatcheries, in one day old broiler chicks that had not been treated with antimicrobial before [10, 27], suggesting that particular TGCs- and/or Q-R-*Ec* clones are introduced through import of breeder stock and hatching eggs and disseminated via vertical transmission through the broiler production pyramid. Several experimental investigations have been employed to demonstrate possible colonization of hatching eggs by antimicrobial resistant (AMR) *E. coli* [8, 18]. Until now, there is no indication that trans-ovarian transmission plays a role in the dissemination of TGCs- and/or Q-R-*Ec*. To the author's knowledge, studies on vertical transmission of AMR *E. coli* from parent birds to their offspring have not been carried out so far since these bacteria have not been isolated from the reproductive organs of parent birds and/or internal contents of hatching eggs. The aim of this study was to characterize two TGCs- and Q-R-*Ec* strains isolated from the ovaries of a broiler breeder flock and the internal contents of hatching eggs produced by the broiler breeder flock.

Two ESBL-producing *Escherichia coli* strains (*E. coli* S1 and *E. coli* S2) were included in this study. The first strain (*E. coli* S1) was isolated from a pooled sample of the ovaries of broiler breeders collected from a breeder flock situated in Chlef province,

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Table 1. Characteristics of *E. coli* isolates

Isolate	Specimen	Resistance profile	MICs ($\mu\text{g/ml}$)									<i>bla</i> gene	Qnr determinant	Sequence type
			CTX	CAZ	FEP	ATM	CRO	NA	CIP	LEV	CT			
<i>E. coli</i> S1	Broiler breeders	AM, TIC, PIP, CZ, CF, CTX, NA, TE, SSS	>32	3	12	6	64	>256	0.25	0.5	0.125	CTX-M-1	<i>qnrS1</i>	ST461
<i>E. coli</i> S2	Hatching eggs	AM, TIC, PIP, CZ, CF, CTX, NA, TE, SSS	>32	3	12	6	128	>256	0.38	0.75	0.125	CTX-M-1	<i>qnrS1</i>	ST461

MIC, minimum inhibitory concentration; AM, ampicillin; TIC, ticarcillin; PIP, piperacillin; CZ, cefazolin; CF, cephalothin; CTX, cefotaxime; CAZ, ceftazidime; FEP: cefepime; ATM, aztreonam; CRO, cefriaxone; NA, nalidixic acid; CIP, ciprofloxacin; LVX, levofloxacin; TE, tetracycline; SSS, sulfonamides; CT, colistin.

Western Algeria, and the second one (*E. coli* S2) was recovered from a sample of internal contents of broiler hatching eggs collected from a commercial broiler hatchery located in Mostaganem province, North-western Algeria. Eggs produced by the breeder flock in Chlef province were transported to the hatchery in Mostaganem province. Hatching eggs were collected one week after the sampling of the ovaries. The samples were collected following the instructions of veterinarians in those facilities and submitted to the Regional Veterinary Laboratory of Mostaganem, Algeria, for routine antimicrobial resistance monitoring. According to the veterinarian, a drop in egg production (12.53%) was observed in the breeder flock sampled.

The samples were processed immediately upon arrival using aseptic techniques. Ten birds, randomly sampled from the breeder flock, were necropsied in the laboratory and only the ovaries were collected. The ovaries were pooled in a sterile tube and were then flamed for 5 sec using a Bunsen burner, for disinfecting their surface, and cut into small dice. Visually clean hatching eggs (20 eggs) were also randomly collected in sterile plastic bags and transported to the laboratory on ice in an ice box. After disinfection of the surface of the eggshell, by dipping the eggs into 2% tincture iodine for 1 min, the egg internal contents were examined as described previously [8]. One ml or g of the samples (egg internal contents or ovaries) was mixed with 9 ml of buffered peptone water, vortexed and incubated at 37°C overnight. To isolate *E. coli*, a drop of broth was streaked on MacConkey's agar. Isolates were further identified using the API 20E System (BioMérieux, Marcy l'Etoile, France).

Antimicrobial susceptibility tests of the *E. coli* isolates were carried out using the disk diffusion method and minimal inhibitory concentrations (MICs) following Clinical and Laboratory Standards Institute guidelines [9]. The isolates were tested against a panel of 23 antimicrobials including many that are important to both human and veterinary medicine: nalidixic acid (30 μg), flumequin (30 μg), ciprofloxacin (5 μg), levofloxacin (5 μg), ampicillin (10 μg), amoxicillin-clavulanic acid (20/10 μg), ticarcillin (75 μg), piperacillin (100 μg), cefazolin (30 μg), cephalothin (30 μg), ceftazidime (30 μg), cefotaxime (30 μg), aztreonam (30 μg), cefepime (30 μg), cefriaxone (30 μg), tetracycline (30 μg), sulfonamides (300 μg), trimethoprim (5 μg), trimethoprim-sulfamethoxazole (1.25/23.75 μg), imipenem (10 μg), gentamicin (10 μg), chloramphenicol (30 μg), and nitrofurantoin (300 μg) (Bio-Rad, Marnes-la-Coquette, France). The MICs against cefotaxime, ceftazidime, aztreonam, cefepime, cefriaxone, nalidixic acid, ciprofloxacin and levofloxacin were determined using E-test (AES, AB Biodisk, Solna, Sweden). The MIC value for colistin was determined by broth microdilution method following EUCAST recommendations [25]. *E. coli* ATCC 25922 (American Type Culture Collection, Rockville, MD, U.S.A.) was used as a quality control strain.

Double disk synergy test (DDST) was performed to confirm ESBL production as described by Jarlier *et al.* [15] using a central amoxicillin/clavulanic acid disk, 30 mm away from cefotaxim, cefriaxone, ceftazidim, and aztreonam disks. The presence of ESBL was revealed by a champagne cork aspect.

The *E. coli* isolates were also subjected to PCR for the detection of *bla*_{ESBL} as described previously for *bla*_{CTX-M} genotype groups 1, 2, 8 and 9 [1], *bla*_{SHV} [1], and *bla*_{TEM} [17]. The isolates were also screened for *qnr*-type genes (*qnrA*, *qnrB* and *qnrS*) as previously described [11, 12, 14]. PCR amplicons were confirmed by sequencing and the DNA sequences obtained were compared with those in the GenBank using the BLAST program.

To analyze the possible clonal relatedness between the *E. coli* isolates, molecular typing by multilocus sequence typing (MLST) was used. Internal fragments of eight housekeeping genes (*dinB*, *icdA*, *pabB*, *polB*, *putP*, *trpA*, *trpB* and *uidA*) were studied by PCR and sequencing according to the method of Jaureguy *et al.* [16] Allele sequences and sequence typing were assigned according to Institute Pasteur's MLST scheme for *E. coli*.

Congestion and swelling of the ovaries were observed on post mortem examination in 3 out of the 10 ovaries examined. *E. coli* was isolated in pure culture on MacConkey's agar from the two specimens. The two isolates displayed the same antimicrobial resistance profile, with resistance to ampicillin, ticarcillin, piperacillin, cefazolin, cephalothin and cefotaxime. Both isolates were susceptible to ceftazidime and imipenem. The MIC values indicated that these isolates showed reduced susceptibility to cefepime and aztreonam (Table 1). On another hand, the isolates were resistant to nalidixic acid, tetracycline and sulfonamides, but susceptible to ciprofloxacin, trimethoprim-sulfamethoxazole, chloramphenicol, nitrofurantoin and colistin. DDST demonstrated that both isolates presented an ESBL phenotype. In addition, PCR screening for *bla*_{ESBL} and *qnr* genes showed that the two isolates coproduced *bla*_{CTX-M} and *qnrS*-encoding genes. According to sequencing results, the *bla*_{CTX-M} and *qnr* genes harbored by the two isolates were *bla*_{CTX-M-1} and *qnrS1*. By MLST, a novel allele combination (25-47-10-10-16-57-4-50) was found in both the isolates and is designated as ST461.

In recent years, there has been increasing concern in the scientific community about the emergence and dissemination of TGCs- and Q-R-*Ec* within the broiler production pyramid [4, 5, 20]. Import of breeding animals has been suggested as the most

likely source for introduction of antimicrobial resistance to the broiler production [10, 20]. In this study, *E. coli* isolates harboring the *bla*_{CTX-M-1} and *qnrS1* genes belonging to ST461 were detected in the ovaries of a broiler breeder flock and subsequently in the internal contents of hatching eggs originating from the broiler breeder flock. The presence of *E. coli* in egg internal contents may be due to penetration through the eggshell during or after oviposition as previously demonstrated [8]. A recent study has reported the presence of TEM- and SHV-producing Enterobacteriaceae isolates in the shell surface and shell crushed of broiler hatching eggs [18]. Another explanation is by direct contamination before oviposition, originating from the infection of reproductive organs with *E. coli*. Isolation of *E. coli* harboring the *bla*_{CTX-M-1} and *qnrS1* genes from the ovaries of the broiler breeders in this study strongly suggests the latter hypothesis which may be responsible for ovaritis and associated with reduced egg production. Indeed, TGCs-R-*Ec* has also been detected, in commercial hatcheries, in one-day-old chicks that had not been treated with antimicrobial before [10].

In Algeria, a few investigations have demonstrated the presence of *qnr* determinants in isolates from animals and food of animal origin. Among them, *qnrA* has been recently identified in SHV-12-producing *E. coli* isolates from poultry [2], and *qnrS1* and *qnrB5* in ESBL-producing *E. coli* isolates from companion animals [28]. In this study *qnrS1* was reported in CTX-M-1 producing *E. coli*. Recently, *qnrS1* has also been detected in non-ESBL-producing *E. coli* and *Enterobacter cloacae* of poultry origin [3].

The results of this study revealed that the isolates showed a high level of resistance to nalidixic acid (MIC>256), but they remained susceptible to ciprofloxacin and levofloxacin. Generally, the plasmid-mediated mechanisms provide only low-level resistance that by itself does not exceed the clinical breakpoint for susceptibility [13]. MICs of nalidixic acid in the isolates from ovary and egg content samples may be due to a mutation(s) in the quinolone resistance-determining regions including *gyrA* and *parC* genes. Resistance to other antibiotics was also observed in the two *E. coli* isolates. It has been reported that the prevalence of co-resistance to nalidixic acid and sulfonamides was high among ESBL-producing *E. coli* [2]. This can be explained by the ESBL-plasmids often carry other genes of resistance (particularly to aminosides, tetracyclins and sulfamides) suggesting co-resistance, co-expression, and co-selection as previously reported [23].

In conclusion, this is the first report demonstrating the presence of TGCs- and Q-R-*Ec* in the reproductive organs of broiler breeders and internal contents of hatching eggs. The present results would contribute to the molecular epidemiological understanding of TGCs- and Q-R-*Ec*. However, further molecular epidemiological studies are needed in order to characterize a large number of AMR isolates.

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