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Detection of multidrug resistant *Escherichia coli* in the ovaries of healthy broiler breeders with emphasis on extended-spectrum β-lactamases producers

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ABSTRACT

In the last few years, antimicrobial resistant (AMR) *Escherichia coli* have been detected in newborn chickens suggesting their vertical transmission from breeding birds to their offspring. However, little is known about the presence of AMR *E. coli* in the reproductive organs of broiler breeders. The aim of this study was to investigate the presence of *E. coli* in the ovaries of healthy broiler breeders and to study their antimicrobial resistance. Samples from broiler breeders (n = 80) collected from 80 different broiler breeder flocks were included in this study. Antibiotic susceptibility testing was performed using disk diffusion method according to Clinical and Laboratory Standards Institute guidelines. Minimal inhibitory concentrations (MICs) of five antimicrobial agents were determined by Etest. PCR and sequencing were used to detect the *bla*$_{ESBL}$ genes. *E. coli* were detected in the ovaries of thirty seven out of 80 (46.25%) sampled flocks. High levels of resistance to various first-line antimicrobial agents were recorded in *E. coli* isolates. This study showed that 89.18% of *E. coli* isolates were multidrug resistant (MDR). Furthermore, MDR extended-spectrum β-lactamases (ESBL)-producing *E. coli* were detected in the ovaries of four different broiler breeder flocks. Molecular characterization revealed that three isolates harboured *bla*$_{CTX-M-1}$ gene and one isolate expressed *bla*$_{SHV-12}$ gene. In addition, one *bla*$_{CTX-M-1}$ -producing *E. coli* co-harboured the *bla*$_{TEM-1}$ gene. These findings would contribute to a better epidemiological understanding of MDR *E. coli* for improve existing preventive strategies in order to reduce the dissemination of antimicrobial resistance in the broiler production system.

1. Introduction

Escherichia coli is as a major pathogen of widespread importance in commercially produced poultry [1], contributing to heavy economic losses in broiler chickens both in broiler breeders and their progeny. Simultaneously, *E. coli* is a highly versatile microorganism and is used as a model organism for detecting the presence of antimicrobial resistance (AMR) [2]. The surveillance data shows that the prevalence of multidrug resistant *E. coli* (resistant to three or more classes of therapeutic antibiotics such as β-lactams and quinolone) in food-producing animals increased in the last decade [3], constituting an important reservoir for transmissible resistance genes. The production of extended-spectrum β-lactamases (ESBLs) is the most encountered mechanism responsible for resistance to third-generation cephalosporins. In the last years, CTX-M-type ESBL have been frequently isolated, and due to its increasing prevalence (higher than TEM and SHV), some authors have announced the "CTX-M ESBL pandemic" [4]. In veterinary medicine, resistance to multiple antimicrobials was found more often in *E. coli* from broiler chickens compared to *E. coli* from other meat producing animals or from laying hens [5,6]. Various authors have reported a relatively high prevalence of AMR *E. coli* in birds sampled from all levels of broiler production pyramid, including broilers sampled few days before slaughtering and/or retail meat, and also in several countries with a low antimicrobial usage profile [7,8]. It has been hypothesized that particular AMR *E. coli* clones are introduced through imported breeding birds, and disseminated via vertical transmission through the broiler production pyramid. Indeed, cephalosporins resistant *E. coli* were recently detected in newborn broilers in several countries [9–11].

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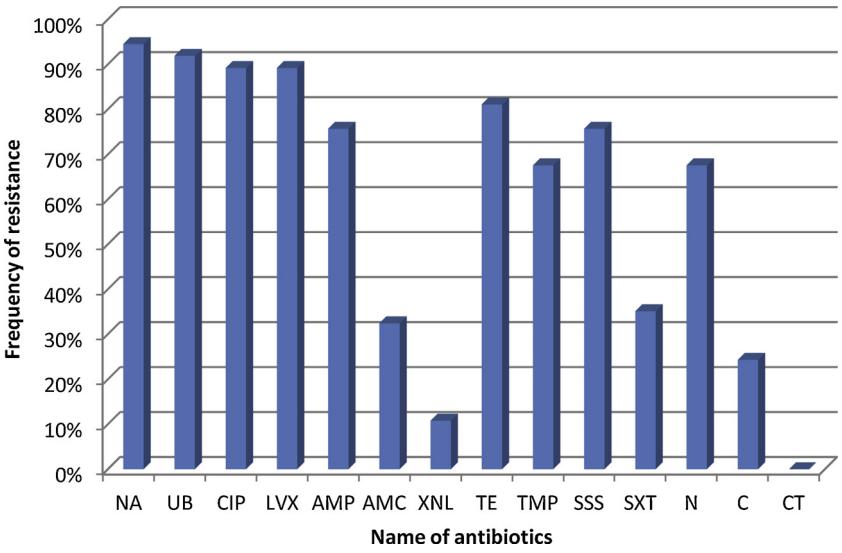

Fig. 1. Antimicrobial resistance rates of *E. coli* strains isolated from the ovaries of healthy broiler breeders.

All previous studies conducted to investigate the detection of AMR *E. coli* in broiler production used samples from the digestive tract of birds or their environment. However, very few studies have been conducted to investigate the ability of AMR *E. coli* to colonize the reproductive organs of broiler breeder chickens. The objective of this study was to demonstrate the colonization of the ovaries of healthy broiler breeders by *E. coli* and to evaluate their antimicrobial resistance.

2. Materials and methods

2.1. Study area and sampling

Eighty broiler breeder flocks situated in seven provinces of western Algeria (including Mostaganem, Oran, Mascara, Relizane, Chlef, Tiaret, and Tissemsilt) were chosen to carry out this study. A total of 400 healthy broiler breeders collected from 80 different broiler breeder flocks, between February 2017 and March 2018, were analyzed in this study. Healthy broiler breeder samples (five broiler breeders by sample) were randomly taken from different broiler breeder flocks. In general, one broiler breeder flock was sampled at one time point to represent the status of the flock. Samples were transported to the Laboratoire Vétérinaire Régional de Mostaganem, Algeria, and processed immediately for bacterial isolation.

2.2. Bacterial strains

The samples were processed immediately upon arrival using aseptic techniques. Broiler breeders were necropsied in the laboratory and only the ovaries were collected. On each occasion, 5 ovaries originating from the same sample were pooled together in a sterile tube. The organs were examined using a previously described method [12], with slight modifications. Briefly, the organs were flamed using a Bunsen burner and cut into a small dice. One gram of the pooled sample of the ovaries was inoculated 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 agar. *E. coli* isolates were identified using the API 20E System (BioMérieux, Marcy l'Etoile, France).

2.3. Antimicrobial susceptibility testing

Antimicrobial susceptibility of the isolates was tested using the disk diffusion method on Muller–Hinton agar following Clinical and Laboratory Standards Institute guidelines [13]. The isolates were tested against a panel of fourteen antimicrobials including many critically important antimicrobials for public and veterinary health: nalidixic acid (NA, 30 µg), flumequin (UB, 30 µg), ciprofloxacin (CIP, 5 µg), levofloxacin (LVX, 5 µg), ampicillin (AMP, 10 µg), amoxicillin-clavulanic acid (AMC, 20/10 µg), ceftiofur (XNL, 30 µg), tetracycline (TE, 30 µg), trimethoprim (TMP, 5 µg), sulfonamides (SSS, 300 µg), trimethoprim-sulfamethoxazole (SXT, 1,25/23,75 µg), neomycin (N, 30 µg), chloramphenicol (C, 30 µg), and colistin (CT, 50 µg) (Bio-Rad, Marnes-la-Coquette, France). Minimum inhibitory concentrations (MICs) against cefotaxime, ceftazidime, aztreonam, cefepime and cefriaxone were determined by the E-test method (AES, AB Biodisk, Solna, Sweden). *E. coli* ATCC® 25922 (American Type Culture Collection, Rockville, MD, USA) was used as a quality control strain.

2.4. Phenotypic detection of ESBL

Isolates showing a phenotype of resistance (or reduced susceptibility) to ceftiofur were identified as potential ESBL producers. Screening of the isolates to test for ESBL secretion in *E. coli* strains was performed by double disk synergy test (DDST) as previously described [14]. The presence of ESBL was revealed by a champagne cork aspect.

2.5. Characterization of resistance genes

The phenotypically confirmed ESBL-producing isolates were screened using PCR as described previously for $bla_{\text{CTX-M}}$ genes of groups 1, 2, 8 and 9 [15], bla_{SHV} [15], and bla_{TEM} [16], after extraction of total DNA by the boiling method. PCR amplicons were confirmed by sequencing and the DNA sequences obtained were compared with those in the GenBank using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST).

3. Results

3.1. Study area and sampling/bacterial strains

In total, the reproductive organs of 37 out of 80 sampled flocks were found colonized by *E. coli* isolates. However, no apparent lesion was observed in the ovaries on post-mortem examination.

3.2. Antimicrobial susceptibility testing

Resistance rates of *E. coli* isolates are displayed in Fig. 1.

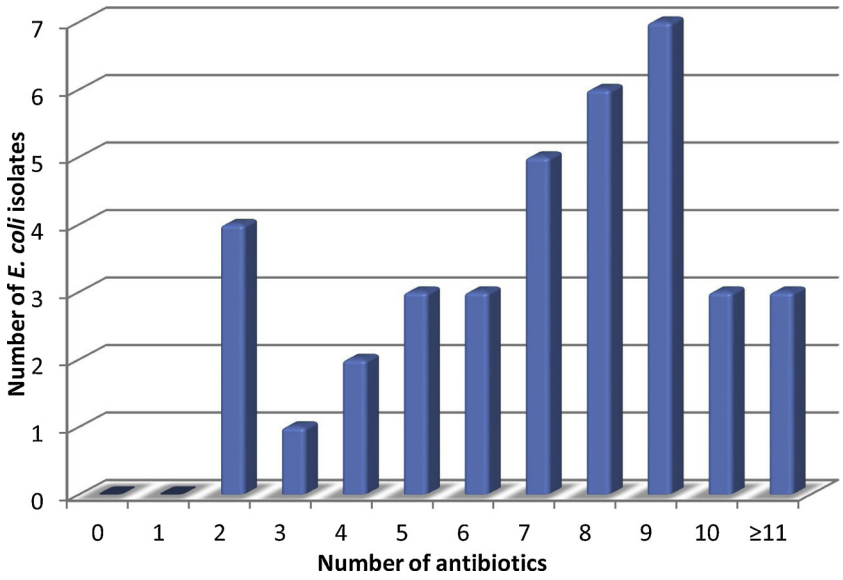

Fig. 2. Multidrug resistant *E. coli* strains isolated from the ovaries of healthy broiler breeders.

Interestingly, the most frequent resistance rates were observed against nalidixic acid 94.5% (n = 35), flumequin 91.8% (n = 34), ciprofloxacin 89.1% (n = 33), levofloxacin 89.1% (n = 33) and tetracycline 81% (n = 30) and the lowest resistance rate was observed against ceftiofur 10.8% (n = 4). All isolates remained susceptible to colistin. Nine *E. coli* isolates (24.3%) showed resistance to chloramphenicol. *E. coli* found resistant to three or more different antimicrobial agents belonging to different classes of antibiotics were considered as multidrug resistant (MDR) isolates. This study showed that 89.18% (n = 33) of *E. coli* isolates were MDR. Most of the *E. coli* isolates were resistant to more than 6 antibiotics as indicated in Fig. 2. Three isolates was found to be resistant to more than 10 antibiotics out of 14 antibiotics tested.

3.3. Phenotypic description of ESBL

Four phenotypicaly confirmed ESBL-producing *E. coli* were detected in different broiler breeder flocks. All of these isolates were MDR. MICs for cefotaxime, ceftazidime, aztreonam, cefepime and cefriaxone ranged from 1.5 to > 32 µg/mL, 2–16 µg/mL, 4–12 µg/mL, 1–16 µg/mL and 3 to > 256 µg/mL, respectively (Table 1).

3.4. Characterization of resistance genes

Molecular characterization revealed that three isolates harboured $bla_{\text{CTX-M-1}}$ gene and one isolate produced $bla_{\text{SHV-12}}$ gene. In addition, one $bla_{\text{CTX-M-1}}$-producing *E. coli* co-harboured the $bla_{\text{TEM-1}}$ gene.

4. Discussion

4.1. E. coli strains isolated from the ovaries show resistance against many first line antimicrobial agents

The epidemiology of AMR *E. coli* is both evolving and complex, and the potential role of breeding birds as a possible reservoir of AMR *E. coli* is currently being analyzed from different perspectives [7,8,17]. The results of the present study demonstrated the ability of AMR *E. coli* isolates to colonize the ovaries of healthy broiler breeders. In the recent last years, several findings highlighted the presence of AMR *E. coli* at all levels of broiler production pyramid also in several countries with a low antimicrobial usage profile, suggesting vertical transmission of AMR *E. coli* from broiler breeders to their offspring [7–9,18]. However, there is little published literature on the colonization of the reproductive organs by *E. coli*. The low antimicrobial use in several countries indicates that factors other than selective pressure must have impact on the success of the resistant bacteria.

Antibiotic susceptibility testing was performed for all *E. coli* isolates with 14 antibiotics including many that are frequently used in human and veterinary medicine. The high resistance rates to the first-line antimicrobial agents tested in *E. coli* from broiler breeders were troubling given their important use in poultry. Compared to our study, nalidixic acid-resistant *E. coli* have been detected in Swedish broiler population and also in imported breeding birds, despite the lack of a known selective pressure [7]. Cephalosporins-resistant *E. coli* have been detected in broiler production chain in the Netherland and Norway [8,9]. Most recently, the clonal spread of cephalosporins- and quinolones-resistant *E. coli* has been reported in the broiler production in the three Nordic

Table 1
Characteristics of ESBL-producing *E. coli* strains isolated from the ovaries of healthy broiler breeders.

Isolate	Region	Resistance profile	MICs (µg/ml)					Bla genes
			CTX	CAZ	FEP	ATM	CRO	
E. coli [S1]	Tiaret	AMP, XNL, NA, UB, CIP, LVX, TE, SSS.	> 32	2	8	4	64	CTX-M-1, TEM-1
E. coli [S2]	Oran	AMP, XNL, NA, UB, TE, SSS, TMP, SXT.	> 32	3	16	12	> 256	CTX-M-1
E. coli [S3]	Chlef	AMP, XNL, NA, UB, TE, SSS.	> 32	3	12	6	64	CTX-M-1
E. coli [S4]	Tiaret	AMP, XNL, NA, UB, CIP, LVX, TE, SSS, TMP, SXT.	1.5	16	1	8	3	SHV-12

MIC, minimum inhibitory concentration ; AMP, ampicillin ; XNL, ceftiofur; CTX, cefotaxime ; CAZ, ceftazidime ; FEP : cefepime ; ATM, aztreonam ; CRO, cefriaxone ; NA, nalidixic acid ; UB, flumequin; CIP, ciprofloxacin ; LVX, levofloxacin; TE, tetracycline; SSS, sulfonamides; TMP, trimethoprim; SXT, trimethoprim-sulfamethoxazole.

countries; Sweden, Norway and Iceland [17]. Resistance to chloramphenicol was also observed in our study. This drug is not approved for use in food animals in Algeria. It is approved only for human clinical use. Persistence of chloramphenicol resistance in *E. coli* has also been observed previously [19,20], and this could be explained by co-selection of mobile resistance elements for the chloramphenicol-resistance [21].

4.2. E. coli isolates exhibit resistance to multiple antibiotics

To our knowledge, very few studies have been conducted to investigate coresistance in AMR *E. coli* isolates from broiler breeders. Among them, one study on third generation cephalosporin-resistant *E. coli* reported coresistance with tetracyclines, sulfamethoxazole, trimethoprim, gentamycin and streptomycin with very low rates [8]. The presence of ampicillin- and nalidixic acid-resistant *E. coli* have also been detected in faecal samples in all the flocks sampled from the broiler production pyramid in South Africa, despite the absence of antibiotics usage [18]. However, in that study, resistance to ciprofloxacin was detected at a very low frequency and resistance towards ceftiofur was not recorded. This multidrug resistance could be explained by the presence of mobile genetic elements contributing to co-resistance, co-expression, and co-selection [22].

4.3. Extended spectrum beta-lactamase genes

In Algeria, the occurrence of bla_{ESBL}-producing *E. coli* has been increasingly reported for broilers over the past few years [23,24]. However, the genetic background for cephalosporins resistance in *E. coli* from poultry in Algeria has not been extensively investigated. CTX-M-1, SHV-12 and TEM-1 have been detected in *E. coli* strains isolated from slaughtered broiler intestines [23], and CTX-M-1- and CTX-M-15-producing *E. coli* have been reported in samples of poultry origin in Algerian central regions [24]. The results of this study are in a good agreement with the study conducted in the Netherlands which showed the presence of $bla_{CTX\text{-}M\text{-}1}$-producing *E. coli* in commercial hatchery, in one day old parent broilers that had not been treated with antimicrobial before [9]. Furthermore, treatment with third-generation cephalosporin has also been reported as an important risk factor for occurrence and persistence of cephalosporin-resistant *E. coli* in one-day-old chicks [25]. All the previously cited investigations reported the presence of AMR *E. coli* in the gastrointestinal tract of birds or their environment. There is very little published data regarding the presence of AMR *E. coli* in the reproductive organs of broiler breeders. Recently, ESBLs-producing *E. coli* have been detected in the reproductive tract of broiler breeding roosters [26]. More recently, Benameur et al. (2018) described the first isolation of *E. coli* carrying $bla_{CTX\text{-}M\text{-}1}$ gene in a pooled ovaries sample of a diseased broiler breeder flock [27].

Our finding showed that broiler breeders may be a reservoir for these isolates and they could play an important role in the transmission and dissemination of AMR *E. coli* within broiler production pyramid. Vertical transmission can be the result of infection of the ovaries of a laying hen via systemic infection, or also from an ascending infection from the contaminated cloaca to the vagina and lower regions of the oviduct [28,29]. These could lead to contamination of the yolk, albumen, eggshell membranes or eggshells before oviposition. Thus, 3GC-resistant *E. coli* may be introduced in the hatchery facilities, either from true vertical transmission when parental poultry stocks are contaminated or from very early contamination in the hatchery itself. Dissemination and persistence of AMR *E. coli* might also be contributed by other possible contamination events. i.e; horizontal transmission in breeder and broiler farms and hatcheries [9], national transport and transfer of similar chickens and also through international trade to many different countries around the world [7]. Genes encoding cephalosporins resistance from broilers are commonly located on self-transmissible plasmids [30,31]. These plasmids may be promiscuous and are able to disseminate into a broad range of hosts. The global spread of MDR *E. coli* in broiler production pyramid is a real concern in human and veterinary medicine and the transferability of AMR *E. coli* or mobile resistance determinants from chickens to humans has been indicated in several studies [32,33].

In conclusion, this study demonstrated the contamination of the ovaries of healthy broiler breeders by MDR *E. coli*. It is therefore worrying that MDR *E. coli* isolates are already present in the reproductive organs of healthy broiler breeder chickens. Contamination of the ovaries with MDR *E. coli* could lead to contamination of hatching eggs and could be an important factor in the dissemination of MDR *E. coli* in the poultry industry. Therefore, implementation of new and improve existing prevention strategies in broiler production system are becoming mandatory.

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Ethical approval

Not required.

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