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Original Article

Genetic characterization of extended-spectrum beta-lactamase-producing *Escherichia coli* and *Klebsiella pneumoniae* collected from healthy turkeys

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Abstract

Introduction: The spread of extended-spectrum beta-lactamase-producing *Enterobacteriaceae* (ESBL-En) from turkeys *via* food chain and environmental contamination is a human health concern.

Methodology: Seventy fecal samples were collected from healthy turkeys and streaked on Tryptone Bile X-Glucoronide (TBX) supplemented with 2 mg/L of cefotaxime and on TBX supplemented with 1 mg/L of imipenem. ESBL production and susceptibility to antibiotics were studied according to CLSI guidelines. Genes encoding for ESBLs (SHV, CTX-M, TEM), carbapenemases (IMI, KPC, OXA48, NDM), tetracyclines (*tetA*, *tetB*, *tetC*), colistin (*mcr*-1 to *mcr*-5), sulphonamides (*sul1*, *sul2*), quinolones (*qnr* A/B/S, *aac(6')-Ib*-cr, *qepA*) resistance, and class 1 and 2 integrons were determined by PCR.

Results: ESBL-En [n = 45 (64.3%): 41 *E. coli* and 4 *K. pneumoniae*] isolates were collected. In *E. coli*, $bla_{\text{CTX-M-1}}$, $bla_{\text{CTX-M-14}}$, $bla_{\text{CTX-M-15}}$, $bla_{\text{CTX-M-27}}$, and $bla_{\text{CTX-M-55}}$ genes were identified in 23, 2, 5, 16, and one isolate, respectively. The $bla_{\text{CTX-M-15}}$ gene was detected in two *K. pneumoniae* isolates, while each of $bla_{\text{CTX-M-1}}$ and $bla_{\text{CTX-M-27}}$ were detected in one isolate. Resistances to tetracyclines, sulfonamides, fluoroquinolones, and colistin were encoded by *tetA* (n = 21)/*tetB* (n = 1), *sul1* (n = 8)/*sul2* (n = 13), *aac(6')*-Ib-*cr* (n = 6), and *mcr*-1 (n = 2)/*mcr*-2 (n = 1) genes, respectively. Integrons of class 1 and class 2 were detected in 15 and six isolates, respectively. Five *E. coli* isolates belonged to the pandemic ST131 clone.

Conclusions: Our findings highlight the high occurrence of MDR/ESBL-En and demonstrate the possible transfer of these strains to humans *via* the food chain or direct contact.

Key words: Turkeys; ESBL; *E. coli*; *K. pneumoniae*; CTX-M; ST131.

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Introduction

Antimicrobial resistance (AR) is a persistent global public health issue that has grown in recent decades, with resistance spreading at an increasing rate in clinical/community human settings as well as animals (livestock and wild animals) and environments [1,2]. The misuse of antibiotics in humans and livestock, as well as the contamination of natural ecosystems by resistant bacteria, are the main causes of this phenomenon [3].

Several human bacterial pathogens with specific AR phenotypes have been reported as critically important to human health such as methicillin-resistant *S. aureus*, glycopeptides-resistant enterococci, and beta-lactams (including third generation cephalosporins and carbapenem) resistant *Enterobacteriaceae* and *Pseudomonas aeruginosa*. Beta-lactams/carbapenem-resistant *Enterobacteriaceae* have been reported globally from various origins and are mainly multidrug-resistant to several antimicrobials including aminoglycosides, quinolones/fluoroquinolones, tetracyclines, trimethoprim/sulfamethoxazole, and colistin [4,5]. The global spread of beta-lactams-resistant *Enterobacteriaceae* is linked to the wide dissemination of successful high clonal lineages and highly transferable plasmids bearing transposons, integrons and complex genetic structures encompassing genes encoding various antimicrobial agents [6]. In

enterobacteria, resistance to beta-lactams is primarily encoded by beta-lactamase enzymes, particularly extended-spectrum β-lactamases (ESBL), plasmid-borne AmpC (p-AmpC) and carbapenemases (CAR) [5]. A part of the high incidence of ESBL/p-AmpC/CAP-producing *Enterobacteriaceae* isolates in community and clinical settings, such isolates have been increasingly reported from livestock, especially in poultry, pork, and their food products [6,7]. Taken into account that some of the genera belonging to the enterobacteria family are known as zoonoses, ESBL/p-AmpC/CAR-producing isolates can infect humans through various ways mainly via the food chain, direct physical contact, aerosol, waste water, and environmental wastes [1,8,9]. Various molecular mechanisms encoding ESBL, p-AmpC, and CAR phenotypes have been characterized in *Enterobacteriaceae*. The genes encoding ESBL production were primarily from the CTX-M group, followed by TEM- and SHV-ESBL enzymes and they all encoded resistance to all beta-lactams except carbapenem [10]. However, carbapenemase enzymes have a versatile hydrolytic capacity and confer resistance to the vast majority of beta-lactam antibiotics [11]. *Klebsiella pneumoniae* carbapenemase (KPC), New Delhi metallo-lactamase (NDM), imipenem-resistant *Pseudomonas* (IMP), Verona integron-encoded metallo-lactamase (VIM), and oxacillinase OXA-48 are among the dominant carbapenemase enzymes worldwide [12]. The p-AmpC beta-lactamases are derived from chromosomal beta-lactamases of bacteria from the genera *Enterobacter*, *Serratia*, *Citrobacter*, *Pseudomonas*, and *Acinetobacter via* chromosomal gene escape to a plasmid [13]. They hydrolyze third-generation cephalosporins, monobactams, and cephamycins but not carbapenems and fourth generation cephalosporins. Unlike ESBLs, they are not inhibited by clavulanic acid, sulbactam, or tazobactam [13]. Based on the sequence, the commonly reported p-AmpC genotypes can be divided into six groups: CIT, EBC, DHA, ACC, FOX, and MOX [14]. Globally, these mechanisms, especially ESBL production, have been widely reported in poultry. In Tunisia, numerous studies reporting ESBL-producing enterobacteria from poultry have been published; however, little is known about their occurrence and their genetic traits in turkey [15]. Therefore, the objectives of this study were to investigate the occurrence of ESBL/CAR-producing *Enterobacteriaceae* and to determine their antimicrobial resistance phenotypes and genotypes. In addition, the occurrence of the pandemic sequence type ST131 *E. coli*, which is an extra-intestinal pathogenic *E. coli* clone causing millions of antimicrobial-resistant infections annually, was investigated by PCR-based method.

Methodology

Samples collection and selection of cefotaxime-resistant enterobacteria isolates

Seventy fecal samples from randomly selected healthy turkeys aged 71 days were collected from two flocks in an industrial poultry farm in northern Tunisia, in February 2022. In this farm, turkeys are usually slaughtered at 100–110 days; therefore, 71 days is an appropriate age to assess the occurrence of antimicrobial-resistant bacteria in their microbiota. Samples were aseptically collected in sterile plastic bags, immediately transported at a temperature of 4 °C to the laboratory and subjected to microbiological analysis. Five grams of each sample were placed in 5 mL of Brain Heart Infusion (BHI) Broth (Bio-Rad, Hercules, CA, USA) and incubated overnight at 37 °C. Then, 1 mL of the suspension was streaked onto i) Tryptone Bile X-Glucuronide (TBX) agar (Bio-Rad) containing 2 mg/L of cefotaxime (TBX-CTX) for the selection of cefotaxime-resistant *Enterobacteriaceae* (potential producers of ESBL and acquired pAmpC), and ii) TBX supplemented with 1 mg/L of imipenem (TBX-imipenem) to detect carbapenem-resistant isolates. Plates were incubated at 37 °C for 18-24 hours. Then, plates of each sample were examined, and one colony was picked up for further analysis. Collected isolates were identified by classical phenotypic and biochemical methods (Gram staining, oxidase, catalase, urease test, indole production, growth on Simmons citrate, lactose and glucose fermentation in Kligler-Hajna agar) and by API 20E system (bioMerieux, Marcy l'Etoile, France).

Antimicrobial susceptibility testing

Antimicrobial susceptibility was tested by disk-diffusion method on Mueller–Hinton agar (Bio-Rad) according to the recommendations of the Clinical Laboratory Standard Institute (CLSI) [16] and the European Committee on Antimicrobial Susceptibility Testing guidelines (EUCAST, http://mic.eucast.org/Eucast2/). The determination of the Minimum Inhibitory Concentration (MIC) for colistin was performed using the broth micro-dilution method (BMD) [16]. Isolates exhibiting a MIC ≥ 4 µg/mL were considered colistin-resistant. Screening of ESBL production was performed by the Double Disk Synergy Test (DDST) [16]. *K. pneumoniae* ATCC

700603 and *E. coli* ATCC 25922 were used as ESBL-positive and negative reference strains, respectively. An isolate was considered multidrug-resistant (MDR) if it showed resistance to at least one agent from three or more antimicrobial classes [17].

Detection of resistance genotype and occurrence of integrons by PCR experiments

Genomic DNA was extracted from each isolate using the boiling method as described previously [18] and used as the DNA template for all PCR experiments. Genes encoding the most clinically relevant ESBL (TEM, SHV, CTX-M-gr1, CTX-M-gr2, CTX-M-gr8, and CTX-M-gr9) and carbapenemases (KPC, OXA-48-like, VIM, IMP, IMI, and NDM) enzymes were investigated by PCR reactions (Supplementary Table 1) [18-20]. Amplicons were sequenced and analyzed using BLAST software (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to determine the ESBL variants. Genes encoding resistance to sulfonamides (*sul*1, *sul*2, and *sul*3), (tetracyclines (*tet*A, *tet*B, and *tet*C), fluoroquinolones (*qnrA*, *qnrB*, *qnrD*, *qnrS*, *aac(6')*-Ib, and *qepA*), and colistin (*mcr*-1 to *mcr*-5) (Supplementary Table 1) were investigated in all resistant isolates by PCR. The presence of class 1, class 2, and class 3 integrons as well as the 3′- conserved region (*qac*ΔE1-*sul*1) of class 1 integrons was examined by PCR (Supplementary Table 1). For the isolates harboring class 1 and class 2 integrons, the variable regions (VRs) were amplified by PCR to estimate the number of integrons per genomic DNA and to determine their molecular sizes (Supplementary Table 1) [18-20]. Positive control strains from our collection were included in all PCR reactions [15,18-20].

Phylogenetic group determination of E. coli isolates and the ST131 clone

All *E. coli* isolates were assigned to one of the four main phylogenetic groups and their sub-groups (A_0, A_1, B1, $B2_2$, $B2_3$, D_1 and D_2) by PCR [21]. Isolates belonging to the phylogroup B2 were screened for their affiliation to the pandemic ST131 clone using PCR detection of pabB3 and O25 alleles [22].

Results

Phenotypic antimicrobial susceptibility of collected isolates

On the TBX-cefotaxime medium, among the 70 samples analyzed, 45 (64.3%) showed the growth of cefotaxime-resistant enterobacteria. Generally, each positive sample contained colonies that are identical in morphology, size, and colour. One colony per sample was randomly selected and further studied. However, no growth was observed on TBX-imipenem plates. The DDST showed that all the 45 isolates were ESBL producers. They were identified as *E. coli* (41 isolates) and *K. pneumoniae* (four isolates). All isolates were resistant to colistin and the colistin MICs varied from 4 µg/mL to 16 µg/mL. Resistance to carbapenems (imipenem, meropenem, and ertapenem) was not detected in any isolate. All isolates showed phenotypes of multi-drug resistance; indeed, most of the isolates were resistant to tetracyclines, trimethoprim/sulfamethoxazole, nalidixic acid, ciprofloxacin, and colistin (Table 1).

Genes encoding ESBL enzymes and non-beta-lactam antibiotics

All the ESBL-producing *E. coli* harbored ESBL enzymes of CTX-M type. Indeed, the $bla_{\text{CTX-M-1}}$, $bla_{\text{CTX-M-14}}$, $bla_{\text{CTX-M-15}}$, $bla_{\text{CTX-M-27}}$, and $bla_{\text{CTX-M-55}}$ genes were identified in 23, 2, 5, 16, and one isolates, respectively. The $bla_{\text{TEM-1}}$ gene was associated with $bla_{\text{CTX-M}}$-type genes in 26 isolates. Interestingly, nine isolates harbored more than one ESBL-encoding gene and the following combinations were detected: $bla_{\text{CTX-M-1}}$ + $bla_{\text{CTX-M-27}}$ (n = 6), $bla_{\text{CTX-M-15}}$ + $bla_{\text{CTX-M-27}}$ (n = 2), and $bla_{\text{CTX-M-15}}$ + $bla_{\text{CTX-M-14}}$ (n = 1). For the four ESBL-producing *K. pneumoniae* isolates, $bla_{\text{CTX-M-15}}$ gene was detected in two isolates and each of $bla_{\text{CTX-M-1}}$ and $bla_{\text{CTX-M-27}}$ genes was detected in one isolate (Table 1).

Resistances to tetracyclines, sulfonamides, fluoroquinolones, and colistin were encoded by *tetA* (n = 21)/*tetB* (n = 1), *sul1* (n = 8)/*sul2* (n = 13), *aac(6')*-Ib-*cr* (n = 6), *mcr*-1 (n = 2), and *mcr*-2 (n = 1) genes, respectively. Integrons of class 1 and class 2 were detected in 15 and six isolates, respectively. All *int1*-positive isolates harbored the 3′- conserved region (*qac*ΔE1-*sul*1) of the classical class 1 integron. The variable regions of class 1 integron were amplified in 12 out of 15 *int1*-positive isolates, and the size of amplified DNA fragments varied from 200 bp to 2500 bp. Interestingly, eight isolates showed the amplification of two to four DNA fragments (Table 1). For class 2 integron, the size of the variable regions varied from 1200 bp to 2500. In addition, 5 isolates harbored two DNA fragments of 1200 bp and 2500 bp.

Phylogroups of E. coli isolates and occurrence of ST131 clone

The 41 ESBL-producing *E. coli* isolates belonged to the following subphylogroups: A_1 (n = 14), A0 (n = 9), B1 (n = 9), D_1 (n = 3), D_2 (n = 1), and $B2_2$ (n = 5).

Overall, the phylogroups A and B1 presented 78% of the ESBL-producing *E. coli* isolates, whilst, 22% presented the phylogroups B2 and D. The five $B2_2$ *E. coli* isolates were positive for PabB3 and O25 alleles and were classified as a member of the pandemic ST131 clone.

Discussion

During the last two decades, ESBL-producing *Enterobacteriaceae* have emerged as an important threat to human and animal health. Indeed, high rates of ESBL-producing *Enterobacteriaceae*, mainly *E. coli*, *Salmonella* spp., *K. pneumoniae*, and *K. oxytoca* have been reported in clinical settings and the community [23]. In addition, several studies have reported evident linkage between human ESBL-producing strains and animal-associated ones as well as the spread of mobile genetic elements encoding ESBL enzymes (plasmids and transposons) [24,25]. This was the result of the increasing spread of these strains in diseased and healthy livestock [15,26], as well as companion and wild animals [1]. The various environmental niches are widely interconnected; that is why several international organizations have adopted the 'One health' concept to fight the problem of antimicrobial resistance aiming to reduce or at least stop the increase of antimicrobial resistance rates. Food animal industries, especially those where antimicrobial agents are heavily used such

Table 1. Phenotypic and genotypic characteristics of the 45 ESBL-producing enterobacteria isolates collected from healthy turkeys.

Isolates	ESBL / Associated antibiotics resistance	Resistance genes	Integrons (size of variable region, bp)	Phylogroups
E. coli S1	ESBL/TET, TOB, NA, CIP, CS	$bla_{CTX-M-15}$, $bla_{CTX-M-27}$, bla_{TEM-1}, *tetA*	-	$B2_2$*
E. coli S2	ESBL/TET, SXT, NA, CIP, CS	$bla_{CTX-M-1}$, $bla_{CTX-M-27}$, bla_{TEM-1}, *tetA*, *tetB*	-	A_1
E. coli S3	ESBL/TET, SXT, NA, CIP, CS	$bla_{CTX-M-27}$, *tetA*	-	A_1
E. coli S4	ESBL/TET, NA, CIP, CS	$bla_{CTX-M-1}$, bla_{TEM-1}	-	A_1
E. coli S5	ESBL/TET, NA, CIP, CS	$bla_{CTX-M-1}$, bla_{TEM-1}	-	A_1
E. coli S6	ESBL/TET, NA, CIP, CS	$bla_{CTX-M-1}$, bla_{TEM-1}	-	A_1
E. coli S7	ESBL/TET, CS	$bla_{CTX-M-1}$, bla_{TEM-1}, *tetA*	-	B1
E. coli S8	ESBL/TET, NA, CIP, CS	$bla_{CTX-M-27}$, bla_{TEM-1}, *tetA*	-	B1
E. coli S9	ESBL/TET, NA, CIP, CS	$bla_{CTX-M-1}$, bla_{TEM-1}, *tetA*	Int1 (-)	B1
E. coli S10	ESBL/TET, NA, CIP, CS	$bla_{CTX-M-15}$, $bla_{CTX-M-27}$, *tetA*	-	A_1
E. coli S11	ESBL/TET, NA, CIP, CS	$bla_{CTX-M-15}$, bla_{TEM-1}	Int1 (300)	$B2_2$*
E. coli S12	ESBL/TET, TOB, NA, CIP, CS	$bla_{CTX-M-1}$, $bla_{CTX-M-27}$, bla_{TEM-1}, *tetA*	-	B1
E. coli S13	ESBL/TET, NA, CIP, CS	$bla_{CTX-M-27}$	-	A_0
E. coli S14	ESBL/TET, NA, CIP, CS	$bla_{CTX-M-1}$, bla_{TEM-1}	-	B1
E. coli S15	ESBL/TET, NA, CIP, CS	$bla_{CTX-M-55}$, bla_{TEM-1}, *tetA*	-	A_0
E. coli S16	ESBL/TET, SXT, CS	$bla_{CTX-M-1}$, bla_{TEM-1}, *sul2*, *aac(6')-Ib*-cr	Int1 (500/400/250)	B1
E. coli S17	ESBL/TET, SXT, NA, CIP, CS	$bla_{CTX-M-1}$, bla_{TEM-1}, *sul2*, *tetA*	Int1 (500/400/250)	A_1
E. coli S18	ESBL/TET, SXT, NA, CIP, CS	$bla_{CTX-M-1}$, $bla_{CTX-M-27}$, *sul2*, *aac(6')-Ib*-cr	Int2 (2500 /1200)	D_2
E. coli S19	ESBL/TET, SXT, NA, CIP, CS	$bla_{CTX-M-1}$, bla_{TEM-1}, *sul2*, *aac(6')-Ib*-cr	Int1 (500/400/250)	B1
E. coli S20	ESBL/TET, SXT, NA, CIP, CS	$bla_{CTX-M-1}$, *aac(6')-Ib*-cr	-	D_1
E. coli S21	ESBL/TET, SXT, NA, CIP, CS	$bla_{CTX-M-1}$, *sul2*	Int1 (500/400/250)	A_0
E. coli S22	ESBL/TET, NA, CIP, CS	$bla_{CTX-M-1}$, *sul2*	Int1 (1200)	A_0
E. coli S23	ESBL/TET, NA, CIP, CS	$bla_{CTX-M-1}$, $bla_{CTX-M-27}$, *sul1*, *sul2*, *tetA*, *aac(6')-Ib*-cr	Int1 (-)	A_0
E. coli S24	ESBL/TET, SXT, NA, CIP, CS	$bla_{CTX-M-14}$, *sul1*, *sul2*	Int1 (500/400/250)	A_0
E. coli S25	ESBL/TET, SXT, NA, CS	$bla_{CTX-M-27}$, *sul2*	Int2 (2500/1200)	D_1
E. coli S26	ESBL/TET, SXT, NA, CS	$bla_{CTX-M-1}$, *sul1*, *sul2*	Int2 (2000)	D_1
E. coli S27	ESBL/TET, SXT, NA, CS	$bla_{CTX-M-27}$, *sul1*, *sul2*	Int2 (2500/1200)	$B2_2$*
E. coli S28	ESBL/TET, SXT, CS	$bla_{CTX-M-27}$, *sul1*, *sul2*	Int2 (2500 /1200)	$B2_2$*
E. coli S29	ESBL/TET, SXT, CS	$bla_{CTX-M-27}$, *sul2*	Int2 (2500/1200)	$B2_2$*
E. coli S30	ESBL/TET, SXT, NA, CIP, CS	$bla_{CTX-M-1}$, $bla_{CTX-M-27}$, bla_{TEM-1}, *mcr*-1	-	A_1
E. coli S31	ESBL/TET, SXT, NA, CIP, CS	$bla_{CTX-M-1}$, bla_{TEM-1}, *tetA*, *mcr*-1	-	B1
E. coli S32	ESBL/TET, SXT, CS	$bla_{CTX-M-1}$, $bla_{CTX-M-27}$, bla_{TEM-1}, *mcr*-2	-	A_1
E. coli S33	ESBL/TET, SXT, NA, CIP, CS	$bla_{CTX-M-1}$, bla_{TEM-1}	-	B1
E. coli S34	ESBL/NA, CIP, CS	$bla_{CTX-M-1}$, bla_{TEM-1}	Int1 (1500)	A_0
E. coli S35	ESBL/TET, SXT, NA, CIP, CS	$bla_{CTX-M-15}$, $bla_{CTX-M-14}$, bla_{TEM-1}, *tetA*	Int1 (500/400/250)	A_0
E. coli S36	ESBL/TET, NA, CIP, CS	$bla_{CTX-M-27}$, bla_{TEM-1}, *tetA*	-	A_0
E. coli S37	ESBL/TET, SXT, NA, CIP, CS	$bla_{CTX-M-27}$, bla_{TEM-1}, *tetA*	-	A_1
E. coli S38	ESBL/TET, CS	$bla_{CTX-M-27}$, *tetA*	-	A_1
E. coli S39	ESBL/TET, SXT, NA, CIP, CS	$bla_{CTX-M-15}$, bla_{TEM-1}, *tetA*	Int1 (1200/500/400/250)	A_1
E. coli S40	ESBL/TET, SXT, NA, CS	$bla_{CTX-M-1}$, bla_{TEM-1}, *sul1*	Int1 (1500)	A_1
E. coli S41	ESBL/TET, SXT, NA, CIP, CS	$bla_{CTX-M-1}$, bla_{TEM-1}, *sul1, tetA*	Int1 (400)	A_1
K. pneumoniae S1	ESBL/TET, SXT, NA, CIP, CS	$bla_{CTX-M-1}$, bla_{TEM-1}, *tetA*	-	na
K. pneumoniae S2	ESBL/TET, NA, CS	$bla_{CTX-M-27}$, bla_{TEM-1}, *tetA*	Int1 (-)	na
K. pneumoniae S3	ESBL/TET, NA, CIP, CS	$bla_{CTX-M-15}$, bla_{TEM-1}	-	na
K. pneumoniae S4	ESBL/TET, SXT, NA, CIP, CS	$bla_{CTX-M-15}$, bla_{TEM-1}, *sul1, tetA*, *aac(6')-Ib*-cr	-	na

NAL: nalidixic acid; CIP: ciprofloxacin; SXT: trimethoprim/sulfamethoxazole; TET: tetracycline; CS: colistin; na: not applicable; ST: sequence type. * Strains belonging to the ST131 clone.

as intensive and extensive avian and pig production systems, have been recognized as important 'hot spot' reservoirs and vectors of antimicrobial-resistant bacteria and genes encoding antimicrobial resistance [27]. In Tunisia, several studies have reported high rates of ESBL-producing *E. coli* from healthy and diseased chickens; however, scarce or even an absence of studies dealing with the occurrence of ESBL-producers in turkeys have been noticed. This was unexplained particularly since consumption of turkey's meat is quite common in the country and turkey husbandry is a well-developed industry in Tunisia.

In our study, 45 (64.3%) out of 70 fecal samples were colonized with ESBL-producing *E. coli* (n = 41) and *K. pneumoniae* (n = 4) isolates. High rates of ESBL-producing *E. coli* from avian origins have been reported, worldwide and Tunisia [2,28]. In one Iranian study, 70 (35%) ESBL-producing *E. coli* were collected from 200 fecal samples of turkeys [29]; however, lower rates were reported in Egyptian (12.5%) [30] and Portuguese (2.2%) studies [31]. *K. pneumoniae* is an opportunistic pathogen colonizing the intestinal or respiratory tracts of animals and is a common cause of acquired infections and a major public health concern. ESBL-producing *K. pneumoniae* are rarely reported from avian origins [32]. This might be related to the categorization of *E. coli* as a sentinel of antimicrobial resistance in the human-animal-environment interface; thus, globally, most studies have been focused on *E. coli* rather than *K. pneumoniae*. Carbapenem-resistance was not detected in our study; indeed, carbapenem-resistant enterobacteria remain rare worldwide and in our country, it was only reported in *E. coli* isolates from rabbits [19] and ESBL- producing *E. coli* isolated from diarrheic calves [33].

The majority of isolates exhibited multidrug resistance phenotypes especially resistant against tetracyclines, trimethoprim/sulfamethoxazole, nalidixic acid, ciprofloxacin, and colistin. Multidrug resistance is a common trait of ESBL-producers strains; indeed, genes encoding ESBL enzymes are mainly plasmid-borne and embedded within complex molecular structures containing several genes encoding resistance to non-beta-lactam antimicrobials [34].

ESBLs of SHV and TEM types have been for a long time the predominant ESBL enzymes in *Enterobacteriaceae*; however, by the beginning of 2000s, the CTX-M enzymes have become pandemic [35]. That epidemiological scenario included the identification of multiple clones and genetic elements carrying $bla_{\text{CTX-M}}$ genes, as well as an increase in the number of different CTX-M enzymes [36]. In accordance, with the global epidemiological status of ESBL enzymes, in our isolates, ESBL were encoded by the $bla_{\text{CTX-M}}$ genes, with the predominance of $bla_{\text{CTX-M-1}}$ and $bla_{\text{CTX-M-27}}$. Moreover, $bla_{\text{CTX-M-14}}$, $bla_{\text{CTX-M-15}}$, and $bla_{\text{CTX-M-55}}$ genes were also identified in a few isolates. Worldwide, CTX-M-1 enzyme is one of the most predominant ESBLs in *E. coli* of animal origins [37]; however, CTX-M-27 enzyme has been recently reported as an important emerging ESBL enzyme in *E. coli* of various origins [38]. In Tunisia, $bla_{\text{CTX-M-27}}$ gene was first detected in 2002 in clinical ESBL-producing *K. pneumoniae* [39] and in ESBL-producing *Salmonella* Livingstone isolated from babies [40], then it was reported with low rates in *E. coli* from wastewater treatment plant [41, 42] and human clinical samples [43]. Interestingly, more than one ESBL-encoding gene was detected in nine *E. coli* isolates [$bla_{\text{CTX-M-1}}$ + $bla_{\text{CTX-M-27}}$ (n = 6), $bla_{\text{CTX-M-15}}$ + $bla_{\text{CTX-M-27}}$ (n = 2), and $bla_{\text{CTX-M-15}}$ + $bla_{\text{CTX-M-14}}$ (n = 1)]. Several studies from Asia, Europe, Latin America, and other countries have documented similar results [44,45].

As mentioned above, all isolates were multi-drug resistant and those phenotypes were associated with genes encoding several antimicrobials. Among the 44 tetracycline-resistant isolates, this resistance was associated with the *tetA* (n = 21) and the *tetB* (n = 1) genes. Both genes are mainly detected in tetracycline-resistant *E. coli* and *K. pneumoniae* isolates as reported by several studies [15,18,43,46]. Tetracyclines resistance is also encoded by other *tet* genes that, were not investigated in this study, which might be harbored by the resistant isolates lacking *tet*A/B/C genes. Similarly, sulfonamides- and fluoroquinolones resistance were encoded by the common *sul1* (n = 8)/*sul2* (n = 13) and *aac(6')-Ib*-cr (n = 6) genes, respectively. One of the alarming findings in this study, is the high rate of colistin resistance (100%), this resistance was mediated by *mcr*-1 (n = 2) and *mcr*-2 (n = 1) genes. After being neglected for a long time, colistin is currently one of the "last-resort" antimicrobial agents for the treatment of infections caused by ESBL/plasmidic AmpC/Carbapenemase-producing *Enterobacteriaceae*. The acquired *mcr*-genes encoding colistin resistance have become increasingly reported worldwide since a few years ago, especially from poultry [47]. Amongst at least ten *mcr*-variants characterized worldwide, the *mcr*-1 is the most prevalent especially in *E. coli* from animal origins. In Tunisia, *mcr*-1 has been increasingly reported among animal-derived ESBL-producing *E. coli* including poultry, dairy cattle, camels, and wastewater treatment plants [47] and recently from clinical CTX-M-1/TEM-

1- producing *E. coli* and one TEM-1 *E. coli* isolates [48]; however, our team has recently reported the *mcr*-2 gene in two ESBL-producing *E. coli* isolates isolated healthy free-range Chickens (*Gallus gallus domesticus*) in a rural region in Tunisia [49].

Integrons are genetic structures enabling the collection and expression of small gene cassettes containing promoter-less genes encoding antimicrobial resistance [50]. Consistent with other studies, the class 1 integron was more detected in our isolates than the class 2 integron (15 *versus* 6). In addition, all *int1*-positive isolated harbored the 3′- conserved region (*qac*ΔE1-*sul*1) of the classical class 1 integron. The variable regions (VRs) of class 1 integron were amplified in 12 isolates, and the size of amplified DNA fragments varied from 200 bp to 2500 bp. Interestingly, eight isolates showed the amplification of two to four DNA fragments implying the possible occurrence of more than one type of class 1 integron. The occurrence of more than one class 1 integron per strain is common in antimicrobial-resistant *E. coli* [46,51,52]. However, it should be noted that uncomplete amplification of large variable regions might lead to the observation of various DNA amplicons, thus, another molecular strategy has been used to characterize large VRs of class integrons such as the primer-walking strategy with further sequencing [51]. In addition, the VR was not detected in four *int1*-positive isolates. As previously documented, this finding could be due to an empty VR or variations in the conserved region targeted by the primers used to amplify the gene cassettes [52]. Class 2 integrons are not dynamic structures when compared to class 1 integrons since the *int2* gene encodes an un-functional integrase, which explains the reported homogeneity of the gene cassette arrangements in class 2 integrons [50]. Many studies reported sizes of the VRs in class 2 integrons of 2000 bp or 2500 bp, which is similar to our findings and in accordance with the harbored genes cassettes such as the gene cassette arrays *aadA1-dfrA1*, *sat2-aadA1*, and *dfrA1-sat2-aadA1* described by several studies [46,51,52]. Further investigations to characterize these VRs are needed to better characterize the role played by integrons in these isolates.

Several studies have shown that avian *E. coli* strains are primarily assigned to commensal phylogroups A and B1, with the majority of extraintestinal pathogenic *E. coli* isolates of human origin associated with phylogroup B2, and to a lesser extent, group D [53]. Similarly, in this study, phylogroups A and B1 presented 78% of the total ESBL-producing *E. coli* isolates. The five $B2_2$ *E. coli* isolates were classified as members of the pandemic ST131 clone, which is known as a multidrug-resistant extra-intestinal pathogenic *Escherichia coli* (ExPEC) clone responsible for millions of global urinary tract and bloodstream infections in both community and clinical settings [54]. This clone has also been detected in various food animals and wildlife [1,55] which poses a potential threat to human health after infection or consummation of food products contaminated by this *E. coli* clone. ST131 strains are known to harbor the CTX-M-15 enzyme, serotyped as O25b and belonged to phylogroup B2 [54] and thus designated O25b-B2-CTX-M-27-ST131; however, a divergent O25b-B2-CTX-M-27-ST131 *E. coli* clone has recently emerged globally [40]. *E. coli* ST131 containing $bla_{\text{CTX-M-27}}$ gene responsible for human infection has been reported from various continents and is common among ESBL-producing ExPEC in many countries [54,56]. This clone was recently reported in our country in ESBL-producing *E. c*oli from a wastewater treatment plant [41] indicating a possible human reservoir in the Tunisian population.

When combined, the high incidence of ESBL-producing *E. coli* and *K. pneumoniae* in turkeys' intestines and the occurrence of the pandemic extra-pathogenic E. *coli* ST131 clone are alarming for human and animal health. Humans can acquire such strains *via* food chain, close contact with turkeys (particularly by farmers), and environmental pollution caused by the discharge of manure and wastewater into the fields [1,25,57]. Restricted use of antimicrobial agents, particularly in agricultural sectors, is required both globally and in Tunisia where antibiotics are not applied properly. In addition, the immediate implementation of the One Health approach is crucial for the management of antimicrobial-resistance at the human-animal-environment interface for successful control and prevention. Furthermore, there is definitely a need for more research and application of antibiotic alternatives and feed additives to promote growth and enhance gut health, and reduce the use of antibiotics in animal production such as antimicrobial peptides, phytogenics (phytobiotics), prebiotics, synbiotics, probiotics, bacteriophages, clay, and metals.

Conclusions

Apart from the high prevalence of MDR *E. coli* and *K. pneumoniae* in fecal samples from turkeys in Tunisia, isolates harbored a combination of resistance, integrons, and β-lactamase-encoding genes. Moreover, this study describes for the first time the co-occurrence of the *mcr*-2 gene mediating colistin resistance and

ESBL-encoding genes in turkeys from Tunisia. Our findings showed an alarming prevalence of ESBL-producing *E. coli* and *K. pneumoniae* with an obvious similarity in ESBLs and non-beta-lactam resistance genes determinants from turkeys and chicken husbandries in Tunisia. Our investigation showed the presence of a high-risk clonal lineage ST131 *E. coli* which is a source of concern in human and animal health. Overall, our findings demonstrate the importance of giving more attention to the turkey breeding sector in Tunisia as this animal could be an important reservoir of clinically relevant *E. coli* and *K. pneumoniae*.

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Conflict of interests

No conflict of interests is declared.

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Annex – Supplementary Items

Supplementary Table 1. Primers and conditions of PCR amplification of investigated genes.

Gene	Primer sequences (5' to 3')	Conditions of PCR amplification	Product size (bp)
tetA	GTAATTCTGAGCACTGTCGC CTGCCTGGACAACATTGCTT	95°C 5 min, 25 cycles (94°C 30s, 62°C 30s, 72°C 45s), 72°C 7min	937
tetB	CTCAGTATTCCAGCCTTTG CTAAGCACTTGTCTCCTGTT	95°C 5 min, 25 cycles (95°C 30s, 57°C 30s, 72°C 20s), 72°C 7min	416
tetC	TCTAACAATGCGCTCATCGT GGTTGAAGGCTCTCAAGGGC	95°C 5 min, 25 cycles (94°C 30s, 62°C 30s, 72°C 45s), 72°C 7min	570
sul1	TGGTGACGGTGTTCGGCATTC GCGAGGGTTTCCGAGAAGGTG	94°C 5 min, 30 cycles (94°C 30s, 63°C 30s, 72°C 1 min), 72°C 8 min	789
sul2	CGGCATCGTCAACATAACC GTGTGCGGATGAAGTCAG	94°C 5 min, 30 cycles (94°C 30s, 50°C 30s, 72°C 90 s), 72°C 8 min	722
sul3	GAGCAAGATTTTTGGAATCG CATCTGCAGCTAACCTAGGGCTTTGGA	94°C 5 min, 30 cycles (94°C 1 min, 51°C 1 min, 72°C 1 min), 72°C 5 min	789
qnrA	AGAGGATTTCTCACGCCAGG TGCCAGGCACAGATCTTGAC	95°C 10 min, 35 cycles (95°C 1 min, 54°C 1 min, 72°C 1 min), 72°C 10 min	580
qnrB	GATCGTGAAAGCCAGAAAG ACGATGCCTGGTAGTTGTCC	95°C 10 min, 32 cycles (94°C 45s, 54°C 45s, 72°C 60s), 72°C 10 min	469
anrS	GCAAGTTCATTGAACAGGGT TCTAAACCGTCGAGTTCGGCG	95°C 10 min, 35 cycles (95°C 1 min, 54°C 1 min, 72°C 1 min), 72°C 10 min	428
aac(6')-Ib	TTGCGATGCTCTATGAGTGGCA CTCGAATGCCTGGCGTGTTT	95°C 5 min, 34 cycles (94°C 45s, 55°C 45s, 72°C 45s), 72°C 7 min	482
qepA	GCACGTCCAGCAGCGGGTAG CTTCCTGCCCGAGTATCGTG	96°C 5 min, 30 cycles (96°C 1 min, 60°C 1 min, 72°C 1 min), 72°C 5 min	199
int1	GGGTCAAGGATCTGGATTTCG ACATGGGTGTAAATCATCGTC	95°C 5 min, 30 cycles (94°C 30s, 62°C 30s, 72°C 1 min), 72°C 5 min	483
int2	CACGGATATGCGACAAAAAGGT GTAGCAAACGAGTGACGAAATG	95°C 5 min, 30 cycles (94°C 30s, 62°C 30s, 72°C 1 min), 72°C 5 min	788
*qac*ΔE1-*sul*1	GGCTGGCTTTTTCTTGTTATCG GCGAGGGTTTCCGAGAAGGTG	94°C 5 min, 30 cycles (94°C 30s, 63°C 30s, 72°C 1 min), 72°C 8 min	1125
Class 1 integron variable region	GGCATCCAAGCAGCAAG AAGCAGACTTGACCTGA	94°C 5 min, 30 cycles (94°C 1min, 55°C 1min, 65°C 8 min), 72°C 8 min	Variable
Class 2 integron variable region	CGGGATCCCGGACGGCATGCACGATTTGTA GATGCCATCGCAAGTACGAG	94°C 5 min, 30 cycles (94°C 1min, 60°C 1min, 72°C 6 min), 72°C 8 min	Variable
$bla_{\text{CTX-M-gr 1}}$	GTTACAATGTGTGAGAAGCAG CCGTTTCCGCTATTACAAAC	94°C 7 min, 30 cycles (94°C 50s, 50°C 50s, 72°C 1 min), 72°C 5 min	1041
$bla_{\text{CTX-M-gr 2}}$	CGACGCTACCCCTGCTATT CCAGCGTCAGATTTTTCAGG	94°C 5 min, 30 cycles (94°C 25s, 52°C 40s, 72°C 50s), 72°C 6 min	552
$bla_{\text{CTX-M-gr 8}}$	TCGCGTTAAGCGGATGATGC AACCCACGATGTGGGTAGC		666
$bla_{\text{CTX-M-gr 9}}$	CAAAGAGAGTGCAACGGATG ATTGGAAAGCGTTCATCA		205
bla_{SHV}	CACTCAAGGATGTATTGTG TTAGCGTTGCCAGTGCTCG	96°C 5 min, 24 cycles (96°C 15s , 60°C15s, 72°C 2 min), 72°C 5 min	885
bla_{TEM}	ATTCTTGAAGACGAAAGGGC ACGCTCAGTGGAACGAAAAC	94°C 5 min, 30 cycles (94°C 1 min, 60°C 1 min, 72°C 1 min), 72°C 7 min	1150
bla_{VIM}	GATGGTGTTTGGTCGCATA CGAATGCGCAGCACCAG	94°C 10 min, 30 cycles (94°C 40s, 55°C 40s, 72°C 1 min), 72°C 7 min	390
$bla_{\text{NDM-1}}$	GCTTTGGCGATCTGGTTTTC CGGAATGGCTCATCACGATC	94°C 10 min, 36 cycles (94°C 30s, 52°C 40s, 72°C 50 s), 72°C 5 min	621
$bla_{\text{OXA-48}}$	TTGGTGGCATCGATTATCGG GAGCACTTCTTTTGTGATGGC	96°C 5 min, 35 cycles (96°C 1 min, 61°C 1 min, 72°C 2 min), 72°C 10 min	743
bla_{IMP}	GGAATAGAGTGGCTTAAYTCTC CCAAACYACTASGTTATCT[a]	94°C 5 min, 36 cycles (94°C 30s, 52°C 40s, 72°C 50 s), 72°C 5 min	188
bla_{KPC}	GTATCGCCGTCTAGTTCTGC GGTCGTGTTTCCCTTTAGCC	94°C 5 min, 25 cycles (94°C 30s, 50°C 30s, 72°C 1 min), 72°C 7min	638
bla_{IMI}	TGC GGTCGATTGGAGATAAA CGATTCTTGAAGCTTCTGCG	94°C 5 min, 25 cycles (94°C 30s, 50°C 30s, 72°C 1 min), 72°C 7min	399
mcr-1	AGTCCGTTTGTTCTTGTGGC AGATCCTTGGTCTCGGCTTG		320
mcr-2	CAAGTGTGTTGGTCGCAGTT TCTAGCCCGACAAGCATACC		715
mcr-3	AAATAAAAATTGTTCCGCTTATG AATGGAGATCCCCGTTTTT	94°C 15 min, 25 cycles (94°C 30 s, 58°C 1 min, 72°C 1 min), 72°C 10 min	929
mcr-4	TCACTTTCATCACTGCGTTG TTGGTCCATGACTACCAATG		1116
mcr-5	ATGCGGTTGTCTGCATTTATC TCATTGTGGTTGTCCTTTTCTG		1644

[a] Y = C or T; S = G or C.