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Extended-spectrum β-lactamase-producing Enterobacteriaceae from animal origin and wastewater in Tunisia: first detection of O25b-B2$_3$-CTX-M-27-ST131 *Escherichia coli* and CTX-M-15/OXA-204-producing *Citrobacter freundii* from wastewater

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ABSTRACT

Objectives: This study aimed to isolate and characterise extended-spectrum β-lactamase-producing Enterobacteriaceae (ESBL-E) isolates from animals and wastewater in Tunisia.
Methods: ESBL-E from wastewater (n = 123 samples), faeces of healthy animals (poultry, sheep, goats and calves) (n = 140) and raw milk from healthy cows (n = 42) and goats (n = 20) were investigated. Antimicrobial susceptibility was determined according to CLSI recommendations. The *bla*$_{TEM}$, *bla*$_{SHV}$, *bla*$_{CTX-M}$ and *bla*$_{OXA-48}$ genes were analysed by PCR and sequencing. Phylogenetic groups were determined by PCR for *Escherichia coli* isolates. The clonality of *E. coli* and *Klebsiella pneumoniae* isolates was determined by *Xba*I-PFGE and MLST.
Results: A total of 81 *E. coli*, 20 *K. pneumoniae*, 4 *Enterobacter cloacae*, 1 *Citrobacter freundii* and 1 *Citrobacter braakii* were isolated. The *bla*$_{CTX-M-1}$ and *bla*$_{CTX-M-15}$ genes were predominant in *E. coli and K. pneumoniae* isolates. *E. cloacae* and *C. braakii* isolates harboured the *bla*$_{SHV-12}$ gene. The *C. freundii* isolated from wastewater carried *bla*$_{CTX-M-15}$, *bla*$_{TEM-1}$ and *bla*$_{OXA-204}$. *E. coli* isolates belonged to phylogroups A (37), B1 (25), B2 (7) and D (12). Seventy-eight *E. coli* isolates were typeable by PFGE and were classified into 34 pulsotypes. The *K. pneumoniae* isolates belonged to 11 pulsotypes. The *E. coli* isolates belonged to sequence types ST131, ST224, ST162, ST845, ST5204, ST69, ST141 and ST10. The *K. pneumoniae* isolates belonged to ST405, ST147, ST564, ST307, ST152, ST45, ST661 and ST1564.
Conclusion: This is the first report of O25b-B2$_3$-CTX-M-27-ST131 *E. coli* isolates and of *C. freundii* carrying *bla*$_{CTX-M-15}$, *bla*$_{TEM-1}$ and *bla*$_{OXA-204}$ in Tunisia.

1. Introduction

The emergence and dissemination of extended-spectrum β-lactamase-producing Enterobacteriaceae (ESBL-E) in clinical settings as well as in animals has become a serious problem worldwide. Use of extended-spectrum cephalosporins has been recognised as a major driving force for the selection and spread of enzymes conferring resistance to these antibiotics [1,2]. Aquatic environments receiving large quantities of urban wastewater, animal waste and hospital effluent represent a reservoir of diverse Enterobacteriaceae. In aquatic environments, pathogenic bacteria from different origins (humans, animals, industry, soil) are able to mix, enhancing the exchange of antimicrobial resistance genes and mobile genetic elements such as plasmids, transposons and integrons. In addition, aquatic environments serve as a route by

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which antimicrobial resistance genes are introduced into natural bacterial ecosystems, transforming non-pathogenic bacteria into reservoirs of resistance genes and mobile genetic elements [3]. In the last years, numerous reports have documented the presence of ESBLs in municipal urban water, sludge, rivers and lakes, representing a potential infection risk and a public-health problem. Before the year 2000, bla_{SHV} and bla_{TEM} genes were the predominant ESBL variants; however, during the last decade CTX-M-type enzymes have spread worldwide and are now the most prevalent ESBLs [4]. Similar to other ESBL-encoding genes, bla_{CTX-M} genes are mainly located on large plasmids co-harbouring genes encoding resistance to other antimicrobial families [4]. Therefore, CTX-M-producing enterobacteria strains often exhibit a multidrug-resistant (MDR) phenotype including resistance to aminoglycosides, quinolones, tetracycline and trimethoprim/sulfamethoxazole (SXT) [5–7]. In Tunisia, like in other parts of the world, ESBL-E have been increasingly reported in humans, animals and vegetables [5,6,8]. However, little is known about the occurrence of ESBL-E in aquatic environments and especially in effluent water from municipal wastewater treatment plants (WWTPs).

The aim of this study was to investigate the occurrence of ESBL-E in samples from WWTPs as well as various animals. Genetic characterisation of the isolates was performed by determining the genes encoding ESBLs and clonality was determined by pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST).

2. Materials and methods

2.1. Bacterial isolates

ESBL-E isolates from samples of wastewater ($n = 123$), faeces of healthy animals ($n = 140$) (60 poultry, 40 sheep, 20 goat and 20 calves) as well as raw milk from healthy cows ($n = 42$) and goats ($n = 20$) were investigated. Faeces and milk samples were collected from apparently healthy animals from different farms in Tunisia. Wastewater samples were collected from two municipal WWTPs located in the greater area of Tunis City. The study was conducted during 2013–2015. Approximately 5 g of faeces was vigorously homogenised with 50 mL of brain–heart infusion (BHI) broth (Oxoid Ltd., Basingstoke, UK) and was incubated for 24 h at 37 °C. Then, 1 mL of the suspension was streaked onto MacConkey agar plates (Oxoid Ltd.) supplemented with 2 mg/L cefotaxime (MC-CTX) and was incubated overnight at 37 °C for recovery of cefotaxime-resistant Enterobacteriaceae (potential producers of ESBLs and acquired plasmid-mediated AmpC β-lactamases). For raw milk and wastewater samples, approximately 5 mL was streaked onto MC-CTX plates and was incubated overnight at 37 °C. One colony per sample was selected and was identified using an API 20E system (bioMérieux, La Balme-les-Grottes, France) and was confirmed by matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF/MS) (Bruker Daltonik, Bremen, Germany) [9].

2.2. Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed by the agar disk diffusion method according to Clinical and Laboratory Standards Institute (CLSI) guidelines [10]. The tested antibiotics (Bio-Rad, Marnes-la-Coquette, France) were amoxicillin, amoxicillin/clavulanic acid (AMC), cefotaxime, ceftazidime, piperacillin/tazobactam (TZP), aztreonam, cefepime, cefoxitin, tetracycline, streptomycin, nalidixic acid, ciprofloxacin, imipenem, gentamicin, tobramycin, amikacin and SXT. All isolates showing resistance to TZP were tested for susceptibility to temocillin. The double-disk synergy test using cefotaxime and ceftazidime in proximity to AMC was used to screen for ESBL production [10]. *E. coli* ATCC 25922 was used as an ESBL-negative reference strain and *K. pneumoniae* ATCC 700603 as an ESBL-positive reference strain. An isolate was considered MDR when it was resistant to three or more antibiotics from different families [5,6].

2.3. Detection of extended-spectrum β-lactamase genes

The bla_{TEM}, bla_{SHV}, $bla_{CTX-Mgroup1}$ and $bla_{CTX-Mgroup9}$ genes [11] were analysed by PCR and sequencing in all ESBL-positive isolates. The bla_{OXA-48} gene was also tested by PCR for strains showing resistance to temocillin [12]. Amplified DNA fragments were sequenced on both strands and the nucleotide and deduced amino acid sequences were compared with those in the GenBank database to confirm the specific type of β-lactamase gene. Positive and negative control strains were provided from the collection of the Microbiology Department of School of Medicine, University of Seville (Seville, Spain).

2.4. Determination of phylogenetic groups

All *E. coli* isolates were assigned to one of the four main phylogenetic groups and their subgroups (A_0, A_1, B1, $B2_2$, $B2_3$, D_1 and D_2) by PCR [13]. Isolates belonging to phylogroup $B2_3$ were screened for their affiliation to ST131 by PCR detection of *pabB3* and O25 alleles [14].

2.5. Pulsed-field gel electrophoresis

The clonal relationship among *E. coli* and *K. pneumoniae* isolates was determined by *Xba*I-PFGE following the method described by Sáenz et al. [15]. *Xba*I-digested DNA of *Salmonella* Braenderup H9812 was included as a size marker. PFGE patterns were analysed using BioNumerics fingerprinting software (Applied Maths, St-Martens-Latem, Belgium). Cluster analysis of the Dice similarity indices based on the unweighted pair-group method with average linkages (UPGMA) was performed to generate a dendrogram describing the relationship among the PFGE profiles.

2.6. Multilocus sequence typing

E. coli isolates presenting relevant PFGE profiles containing two or more isolates were studied by MLST. Seven housekeeping genes (*adk*, *fumC*, *gyrB*, *icd*, *mdh*, *purA* and *recA*) were amplified by PCR [16]. All of the amplicons were sequenced and were compared with the MLST database (http://enterobase.warwick.ac.uk/species/ecoli/allele_st_search). MLST was also performed for representative *K. pneumoniae* isolates as reported previously by Diancourt et al. [17] by PCR amplification of the standard seven housekeeping loci (*rpoB*, *gapA*, *mdh*, *pgi*, *phoE*, *infB* and *tonB*). All of the amplicons were sequenced and were compared with the sequences deposited in the MLST database (http://bigsdb.pasteur.fr/klebsiella/klebsiella.html) to determine the specific allele combination and sequence type (ST).

3. Results

3.1. Extended-spectrum β-lactamase-producing isolates and antimicrobial susceptibility

In this study, only cefotaxime-resistant isolates showing a positive synergy test were investigated. Among the 325 samples analysed, 107 (32.9%) contained an ESBL-producing isolate, comprising 34 poultry faeces (56.7% of the poultry faeces samples), 58 wastewater samples (47.2%), 9 sheep faeces (22.5%), 1 calf faeces

(5.0%), 1 goat faeces (5.0%), 3 raw bovine milk (7.1%) and 1 raw goats milk (5.0%). The 107 isolates were identified as follows: 81 *E. coli* (31 poultry faeces, 8 sheep faeces, 1 calf faeces, 1 goat faeces, 1 raw bovine milk and 39 wastewater samples); 20 *K. pneumoniae* (17 wastewater samples, 2 raw bovine milk and 1 raw goats milk); 4 *E. cloacae* (3 poultry faeces and 1 sheep faeces); and 1 *C. freundii* and 1 *C. braakii* (both from wastewater samples) (Table 1).

All ESBL-producing isolates showed resistance to aztreonam and cefepime but remained susceptible to cefoxitin and imipenem. Only the *C. braakii* isolate showed resistance to temocillin. Independent of their origin, the majority of isolates were MDR, mainly to nalidixic acid, SXT, tetracycline and streptomycin (Table 1).

3.2. Identification of extended-spectrum β-lactamase-encoding genes

Amongst the 81 *E. coli* isolates, $bla_{\text{CTX-M-1}}$ and $bla_{\text{CTX-M-15}}$ were found in 39 and 37 isolates, respectively. $bla_{\text{TEM-1}}$ co-occurred in three $bla_{\text{CTX-M-15}}$-producing *E. coli* isolates. In addition, $bla_{\text{CTX-M-3}}$, $bla_{\text{CTX-M-14}}$ and $bla_{\text{CTX-M-27}}$ were found in one, two and two isolates, respectively. The $bla_{\text{CTX-M-15}}$ gene was found in the 20 *K. pneumoniae* isolates and the following *bla* gene combinations were found (no. of isolates): $bla_{\text{CTX-M-15}} + bla_{\text{SHV-76}}$ (1); $bla_{\text{CTX-M-15}} + bla_{\text{TEM-1}}$ (7); $bla_{\text{CTX-M-15}} + bla_{\text{SHV-1}}$ (2); $bla_{\text{CTX-M-15}} + bla_{\text{SHV-1}} + bla_{\text{TEM-1}}$ (3); and $bla_{\text{CTX-M-15}} + bla_{\text{SHV-27}}$ (3). The $bla_{\text{SHV-12}}$ gene was found alone in the four *E. cloacae* isolates as well as in the *C. braakii* isolate. The *C. freundii* isolate carried $bla_{\text{CTX-M-15}}$, $bla_{\text{TEM-1}}$ and $bla_{\text{OXA-204}}$ genes (Table 1).

3.3. Phylogenetic grouping of the E. coli isolates

The 81 *E. coli* isolates were classified in the following phylogroups: A_0 (25 isolates); A_1 (12 isolates); B1 (25 isolates); $B2_3$ (7 isolates); D_1 (11 isolates); and D_2 (1 isolate) (Table 1).

3.4. Genetic relationship of E. coli and K. pneumoniae isolates

Of the 81 *E. coli* isolates, 78 were typeable by PFGE and were classified into 34 pulsotypes (assigned as E1–E34). Seven pulsotypes each contained two isolates and five pulsotypes each contained one, three, five, six and seven isolates, whereas 28 isolates appeared as unrelated. Three isolates were not typeable by PFGE despite many attempts using thiourea as an inhibitor of DNA degradation (Table 1). The 20 *K. pneumoniae* isolates were classified into 11 PFGE pulsotypes (assigned K1–K11). Seven isolates were unrelated (pulsotypes K3–K9), whereas pulsotypes K1, K2, K10 and K11 included five, two, three and three isolates, respectively (Table 1).

3.5. Genetic relationship of E. coli and K. pneumoniae isolates

Six of the seven $B2_3$ *E. coli* isolates were positive for PabB3 and O25 alleles and were classified as a member of ST131 clone. The following *E. coli* STs were identified: ST224 (strains belonging to pulsotypes E1, E2, E3, E5, E11, E16, E17 and E33); ST162 (pulsotype E14); ST845 (pulsotype E6); ST5204 (pulsotype E8); ST69 (pulsotype E14); ST141 (pulsotype E9); and ST10 (pulsotype E15) (Table 1). The *K. pneumoniae* isolates of pulsotypes K1 (five isolates) and K7 (one isolate) belonged to ST405. The remaining STs were as follows: ST147 (pulsotype K5); ST564 (pulsotype K8); ST307 (pulsotype K2); ST152 (pulsotype K4); ST45 (pulsotype K10); ST661 (pulsotype K11); and ST1564 (pulsotype K3).

4. Discussion

In this study, ESBL-E from faeces of healthy animals (poultry, sheep, goats and calves), raw milk samples from cows and goats, and wastewater samples were investigated. The findings showed that ESBL-E isolates where more frequent in poultry faeces (56.7%) and wastewater (47.2%), followed by sheep faeces (22.5%), calf and goat faeces (each 5.0%), raw bovine milk (7.1%) and raw goats milk (5.0%). These findings are similar to other studies in Tunisia and worldwide reporting high rates of ESBL-E from poultry and wastewater and a low prevalence of ESBL-producing isolates from bovine, caprine and ovine species as well as food products from these livestock [18–20]. Worldwide, high rates of ESBL-producing isolates, mainly *E. coli*, have been reported in poultry and porcine in contrast to other livestock species [1].

In this study, amongst the 107 ESBL-E isolates, *E. coli* was predominant (81 isolates), followed by *K. pneumoniae* (20 isolates), *E. cloacae* (4 isolates), *C. freundii* and *C. braakii* (1 isolate each). These finding are in accordance with worldwide results where *E. coli*, *Salmonella* spp. and *Klebsiella* spp. are the most important ESBL-producers in animals as well as in urban wastewater [18,20,21]. ESBL-producing *E. cloacae* and *Citrobacter* spp. (mainly *C. freundii*) are rarely reported either from humans or animals or from animal food products [22,23]. To the best of our knowledge, we report here the first case in the world of ESBL-producing *K. pneumoniae* from goats milk and from non-mastitis bovine milk in Tunisia [8,24]. Indeed, worldwide rare ESBL-producing *K. pneumoniae* isolates have been reported from milk samples of either healthy cows or cows suffering from mastitis [25]. So far, ESBL-producing *K. pneumoniae* isolates have not been reported in studies on livestock animals in Europe. Bovine mastitis is the most common disease affecting dairy cattle. Both *E. coli* and *K. pneumoniae* often cause life-threatening clinical mastitis, therefore their occurrence in milk samples might suggest undiagnosed subclinical mastitis. The high occurrence of ESBL-E (47.2%) in wastewater samples from the WWTPs is expected, indeed many studies have reported high frequencies of ESBL-producing isolates in samples from WWTPs [19,25,26]. Wastewater from municipal WWTPs as well as hospital effluent may contain several and diverse pathogenic and MDR bacteria.

Regarding the epidemiology of ESBL-encoding genes, $bla_{\text{CTX-M}}$ genes were the most prevalent, and the major $bla_{\text{CTX-M}}$ variants were $bla_{\text{CTX-M-15}}$ (58/107; 54.2%) followed by $bla_{\text{CTX-M-1}}$ (39/107; 36.4%). Analysis of the distribution of these *bla* genes highlights two important findings. First, the epidemiology of ESBL-encoding genes differed according to the Enterobacteriaceae species; and second, the ESBL gene distribution in *E. coli* isolates from animal origin differed to that from wastewater samples. Indeed, $bla_{\text{CTX-M}}$ genes dominated in *E. coli* and *K. pneumoniae* isolates, whereas *the* $bla_{\text{SHV-12}}$ gene was the unique ESBL-encoding gene in *E. cloacae* and *C. braakii* isolates. Actually, in Tunisia the major ESBL-encoding genes identified in human *E. coli* and *K. pneumoniae* isolates are $bla_{\text{CTX-M}}$ types with a predominance of $bla_{\text{CTX-M-15}}$. The $bla_{\text{CTX-M-1}}$, $bla_{\text{CTX-M-14}}$ and $bla_{\text{CTX-M-9}}$ genes remain scarce [5,22]. Amongst human and animal isolates in Western European countries and Japan, the most common CTX-M types were the CTX-M-1 group (CTX-M-1/-15/-55) and CTX-M-9 group (CTX-M-9/-14/-27) [27]. However, in Tunisia, in *E. coli* isolates from animals or food products of animal origin, $bla_{\text{CTX-M-1}}$ is so far the most prevalent ESBL-encoding gene, followed by scarce reports of $bla_{\text{CTX-M-15}}$, $bla_{\text{CTX-M-14}}$, $bla_{\text{CTX-M-9}}$ and $bla_{\text{SHV/TEM}}$ genes [28]. Interestingly, the $bla_{\text{CTX-M-3}}$ gene, found in one *E. coli* isolate from wastewater, has never been reported previously in our country either in human or animal enterobacterial isolates [29]. In addition, the $bla_{\text{CTX-M-27}}$ gene (two *E. coli* isolates) has been reported only twice, in 2005 and 2010, from *Salmonella* Livingstone isolates causing a nosocomial outbreak in a neonatal unit and in clinical *K. pneumoniae* isolates, respectively [29]. The second important finding was the difference in ESBL gene distribution between *E. coli* isolates of animal origin and from wastewater samples. Indeed, 36 (86%) of the 42

Table 1
Phenotypic and genotypic characteristics of extended-spectrum β-lactamase-producing Enterobacteriaceae isolates.

bla genes (no. of isolates)	Origin	No. of isolates	MLST	PFGE	Phylogroup	Resistance to non-β-lactams
Escherichia coli						
$bla_{CTX-M-1}$ (39)	FP	1	nd	E32	B1	TET
		1		E23	A_0	SXT/NAL
		1		E24		SXT/TET
		1		E4		SXT/TET/STR/NAL
		1		E30		TET
		1		E34		TET
		2	ST224	E1	B1	SXT/TET/STR
		1		E2		SXT/STR/NAL/CIP
		14				SXT/TET/STR/NAL
		3				SXT/STR/NAL
		1		E3		SXT/STR/NAL
		1				SXT/TET/STR/NAL
		1		E5	A_0	SXT/TET/STR/NAL
	FS	1		E2	B1	SXT/TET/STR/NAL/CIP
		1				SXT/TET/STR/NAL
		2		E5	A_0	SXT/TET/STR/NAL
		2				SXT/STR/NAL
	BM	1		E11	D_2	SXT/STR/NAL
	WW	1		E33	A_0	TET/NAL/CIP
		1		E16	A_1	SXT/NAL/TET
		1		E17		TET/STR/NAL/CIP
$bla_{CTX-M-15}$ + bla_{TEM-1} (3)	FS	1	ST10	E15	A_1	NAL
	WW	2				TET/NAL/GEN
$bla_{CTX-M-15}$ (34)	FS	1	ST69	E12	D_1	SXT/TET/STR/NAL
	FP	1	nd	E25	A_0	SXT/TET/STR/NAL
	FC	1	nd	E18	A_0	SXT/TET/STR/NAL/CIP/GEN/TOB
	FG	1	ST5204	E8	A_0	SXT/TET/STR
	FP	1				SXT/TET/STR
	WW	1				SXT/TET
		2				SXT/NAL
		1				SXT/TET/STR
		2	ST845	E6	A_1	SXT/TET/STR/NAL/CIP/GEN/TOB
		1	ST141	E9	$B2_3$	SXT/TET/STR
		1	ST69	E12	D_1	SXT/TET/GEN/TOB
		1				SXT/TET/GEN/TOB/(TZP)
		2				GEN/TOB
		2				SXT/TET/STR
		2	ST162	E14	A_0	SXT/NAL/TET
		1	ST131	E27	$B2_3$	SXT/TET/STR
		2		E28		SXT/STR
		1		nt		SXT/STR
		2	nd	E7	A_0	SXT/TET/STR
		1		E31		TET/NAL/CIP
		2		E10	A_1	SXT/TET/STR
		1		E29		SXT/STR/TET/NAL
		1		E20	D_1	SXT/TET/GEN/TOB
		1		E13		SXT/TET/STR
		1		nt		SXT/TET/STR
		1		nt		TET/NAL/GEN
$bla_{CTX-M-3}$ (1)	WW	1	nd	E19	A_1	GEN
$bla_{CTX-M-14}$ (2)	WW	1	nd	E21	A_0	TET/NAL/GEN/TOB
		1		E22	A_1	TET/NAL/GEN
$bla_{CTX-M-27}$ (2)	WW	2	ST131	E26	$B2_3$	SXT/TET/STR
Klebsiella pneumoniae						
$bla_{CTX-M-15}$ + bla_{SHV-76} (1)	GM	1	ST405	K7	na	SXT/TET/GEN/TOB
$bla_{CTX-M-15}$ + bla_{TEM-1} (7)	BM	2	ST307	K2		SXT/STR/NAL
	WW	5	ST405	K1		SXT/STR/NAL
$bla_{CTX-M-15}$ + bla_{SHV-1} (2)	WW	1	ST152	K4		SXT/TOB/NAL/GEN
		1	ST147	K5		SXT/TET/STR/NAL/CIP/GEN/TOB
$bla_{CTX-M-15}$ (4)	WW	1	ST1564	K3		SXT/STR/NAL
		1	ST564	K8		SXT/TET/STR
		1	nd	K9		SXT/TET/GEN
		1	nd	K6		SXT/TET
$bla_{CTX-M-15}$ + bla_{SHV-27} (3)	WW	3	ST661	K11		SXT/TET/STR
$bla_{CTX-M-15}$ + bla_{SHV-1} + bla_{TEM-1} (3)	WW	3	ST45	K10		SXT/TET/STR
Enterobacter cloacae						
bla_{SHV-12} (4)	FP	1	nd	nd	na	TET/STR
		1				TET/STR/SXT/GEN/TOB/AMK
		1				SXT/TET/STR/TOB
	FS	1				TET/STR

Citrobacter freundii $bla_{CTX-M-15} + bla_{TEM-1} + bla_{OXA-204}$ (1)	WW	1	nd	nd	na	TET/CIP/GEN/TOB/TZP
Citrobacter braakii bla_{SHV-12} (1)	WW	1	nd	nd	na	NAL/TZP

MLST, multilocus sequence typing; PFGE, pulsed-field gel electrophoresis; FP, poultry faeces; FS, sheep faeces; BM, raw bovine milk; WW, wastewater; FC, calf faeces; FG, goat faeces; GM, raw goat milk; nd, not determined; nt, not typeable; na, not applicable; TET, tetracycline; SXT, trimethoprim/sulfamethoxazole; STR, streptomycin; NAL, nalidixic acid; CIP, ciprofloxacin; GEN, gentamicin; TOB, tobramycin; TZP: piperacillin/tazobactam; AMK, amikacin.

ESBL-producing *E. coli* isolates of animal origin harboured the $bla_{CTX-M-1}$ gene and the remaining 6 isolates harboured the $bla_{CTX-M-15}$ gene. In contrast, amongst the 39 wastewater *E. coli* isolates; 31 (79.5%) harboured $bla_{CTX-M-15}$ and the others had $bla_{CTX-M-1}$ (3 isolates), $bla_{CTX-M-14}$ (2 isolates), $bla_{CTX-M-27}$ (2 isolates) and $bla_{CTX-M-3}$ (1 isolate). This finding might be linked to the origins of the treated wastewaters, mainly coming from urban municipal and hospital wastewaters. Therefore, it reflects the predominance of these genes, particularly $bla_{CTX-M-15}$, in human *E. coli* isolates in Tunisia [22,28].

The association of $bla_{CTX-M-15}$ with bla_{SHV-1} and/or bla_{TEM-1} was observed in some of the isolates in this study. This finding has been previously reported in $bla_{CTX-M-15}$-producing isolates [5,30]. In many reports the association of $bla_{CTX-M-15}$/bla_{TEM-1} was common and was related to the spread of clonal plasmids [30]. The bla_{SHV-27} gene was also associated with the $bla_{CTX-M-15}$ gene in three *K. pneumoniae* isolates from wastewater. In Tunisia, this gene has been previously reported in one CTX-M-15-producing *K. pneumoniae* isolate recovered from a neutropenic patient in 2006 [5]. The bla_{SHV-76} gene, found in one $bla_{CTX-M-15}$-producing *K. pneumoniae* isolated from raw milk of goat, first reported in 2009 in *K. pneumoniae* of clinical origin, is not considered as an ESBL genes [31] and has been not reported after. Historically, it appears that the detection of these non-ESBL enzymes, derived from mutations of either bla_{SHV-1} or bla_{TEM-1} genes, are only the result of their chance co-occurrence with important ESBL enzymes; therefore they are rarely reported. Among the 20 ESBL-producing *K. pneumoniae* isolates, 11 did not harbour the bla_{SHV-1} gene or any of its variants. Although the bla_{SHV-1} gene has been reported to be usually present in *K. pneumoniae*, the absence of this gene in *K. pneumoniae* isolates has been previously described not only in Tunisia but also in other countries [29]. It is noteworthy that we report for the first time in Tunisia one *C. freundii* isolate (from wastewater) co-harbouring $bla_{CTX-M-15}$, bla_{TEM-1} and $bla_{OXA-204}$. The $bla_{OXA-204}$ gene, a derivative of the bla_{OXA-48} gene, is an emerging carbapenemase gene that has been sporadically reported from clinical *K. pneumoniae* isolates in Tunisia [32] but never in *C. freundii*.

As reported previously [5,30], the majority of the ESBL-producing isolates were MDR. The MDR trait is common in CTX-M-producing enterobacteria owing to the occurrence of bla_{CTX-M}-containing plasmids inserting various genes encoding antimicrobial resistance [30]. In addition, it is believed that fluoroquinolone resistance or multidrug resistance gives advantage to CTX-M maintenance owing to the co-selection process.

Phylogenetic analysis of the *E. coli* isolates showed the predominance of phylogroup A (37 isolates; 25 A_0 and 12 A_1) and phylogroup B1 (25 isolates), followed by phylogroup D (12 isolates; 11 D_1 and 1 D_2) and phylogroup B2 (7 isolates; all $B2_3$). Interestingly, all 7 $B2_3$ isolates were recovered from wastewater and the 11 D_1 isolates were also from wastewater, and only the D2 isolate was from bovine milk. These results are in accordance with previous reports where the majority of ESBL-producing *E. coli* isolates of animal origin belonged to phylogroups A and B1, whereas clinical isolates were mainly of phylogroups B2 and D. Therefore, the B2 isolates from wastewater samples in the current study might be of human origin that reached the WWTP along with municipal or hospital effluent waters.

The genetic relationships of the *E. coli* and *K. pneumoniae* isolates by PFGE and MLST showed three important findings: (i) occurrence of some clonal *E. coli* isolates from different origins; (ii) detection of the recently emerging O25b-$B2_3$-CTX-M-27-ST131 *E. coli* clone; and (iii) occurrence of particular clones (STs) known as pandemic. Indeed, first, the major CTX-M-1-producing ST224 and CTX-M-15-producing ST5204 *E. coli* clones encompassed isolates from different origins (isolates from faeces of poultry and sheep for ST224, and isolates from faeces of poultry and goat and from the WWTP for ST5204). Some of these strains belonged to different phylogroups and showed various resistance profiles. This was also true for ST69 and ST10 *E. coli* clones. For *K. pneumoniae*, the ST405 clone contained five isolates from WWTPs and one from raw goats milk; however, they displayed different *bla* gene combinations and belonged to two PFGE types (WWTP, PFGE K1/CTX-M–15 + TEM-1; raw goats milk, PFGE K7/CTX-M–15 + SHV-76). It is noteworthy that one *K. pneumoniae* isolate, from a WWTP, was the most resistant isolate amongst the *K. pneumoniae* isolates and belonged to ST147. This ST has been associated in France as well in Tunisia with OXA-204 and CTX-M-15 production [33,34]. These findings highlight the plausible dissemination of particular clonal lineages of ESBL-producing *E. coli* or *K. pneumoniae* within different ecological niches. Second, *E. coli* ST131 containing $bla_{CTX-M-27}$ responsible for human infection has been reported from various continents and is common among ESBL-producing extraintestinal pathogenic *E. coli*, especially in France, Japan and Switzerland [1]. CTX-M-27-producing *E. coli* ST131 isolates have also been reported amongst non-clinical and non-human *E. coli* isolates [35]. Actually, the most common ESBL among *E. coli* ST131 in non-human samples is CTX-M-27 [36]. Indeed, $bla_{CTX-M-15}$-harboring ST131 isolates, found in four of the isolates in the current study, are rare among animal and environmental isolates [2]. Globally, *E. coli* ST131 was mainly serotyped as O25b and belonged to phylogroup B2 and harboured the $bla_{CTX-M-15}$ gene, and CTX-M-27-producing O16 were also reported [37]. However, O25b-B2 *E. coli* ST131 containing $bla_{CTX-M-27}$ has just recently emerged in *E. coli* of human origin [36,37]. To the best of our knowledge, this is the first evidence of this emerging clone in municipal wastewater in Tunisia. Third, in *E. coli* isolates, apart from ST131, some pandemic ST clonal complexes were detected, such as ST224, ST69 and ST10. These clones appeared to be pandemic and are associated not only with CTX-M production but also with carbapenemase production in Europe [38]. This finding was also observed in *K. pneumoniae* isolates where ST405 and ST307 have been also reported as major clones producing ESBL (CTX-M-15) and carbapenemase (KPC-2) [39,40].

5. Conclusion

This study showed a high occurrence of ESBL-E in samples from poultry and WWTPs in Tunisia as well as the specific distribution of CTX-M-1 and CTX-M-15 in *E. coli* of animal and WWTP origins, respectively. In addition, we report for the first time in Tunisia the emergence of O25b-$B2_3$-CTX-M-27-ST131 *E. coli* clone and the presence of OXA-204 in *C. freundii*.

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Competing interests

None declared.

Ethical approval

Not required.

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