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Prevalence and Characterization of Extended-Spectrum Beta-Lactamase (ESBL)– and CMY-2–Producing *Escherichia coli* Isolates from Healthy Food-Producing Animals in Tunisia

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Abstract

The prevalence of extended-spectrum beta-lactamase (ESBL)– and plasmidic AmpC–beta-lactamase (pAmpC-BL)–producing *Escherichia coli* isolates has been studied in food-producing animals at the farm level in Tunisia, and recovered isolates were characterized for the presence of other resistance genes and integrons. Eighty fecal samples of food-producing animals (23 sheep, 22 chickens, 22 cattle, six horses, five rabbits, and two dromedaries) were obtained from 35 different farms in Tunisia in 2011. Samples were inoculated onto MacConkey agar plates supplemented with cefotaxime (2 mg/L) for cefotaxime-resistant (CTX^R) *E. coli* recovery. CTX^R *E. coli* isolates were detected in 11 out of 80 samples (13.8%), and one isolate per sample was further characterized (10 from chickens and one from a dromedary). The 11 CTX^R isolates were distributed into phylogroups: B1 (five isolates), A (two isolates), D (three isolates), and B2 (one isolate). The following beta-lactamase genes were detected: $bla_{CTX\text{-}M\text{-}1}$ (seven isolates), $bla_{CTX\text{-}M\text{-}1}$+$bla_{TEM\text{-}135}$ (one isolate), $bla_{CTX\text{-}M\text{-}1}$+$bla_{TEM\text{-}1b}$ (one isolate), and $bla_{CMY\text{-}2}$ (two isolates). All ESBL- and pAmpC-BL–producing *E. coli* strains showed unrelated pulsed-field gel electrophoresis patterns. Seven isolates contained class 1 integrons with four gene cassette arrangements: *dfrA17-aadA5* (three isolates), *dfrA1-aadA1* (two isolates), *dfrA15-aadA1* (one isolate), and *aadA1* (one isolate). All isolates showed tetracycline resistance and contained the *tet*(A) +/− *tet*(B) genes. Virulence genes detected were as follows (number of isolates in parentheses): *fimA* (10); *aer* (eight); *papC* (two); and *papGIII*, *hly*, *cnf*, and *bfp* (none). Chicken farms constitute a reservoir of ESBL- and pAmpC-BL–producing *E. coli* isolates of the CTX-M-1 and CMY-2 types that potentially could be transmitted to humans via the food chain or by direct contact.

Introduction

Escherichia coli is a normal inhabitant of the gut microbiota and is also the Gram-negative bacillus most frequently isolated in cases of human infection. Resistance to broad-spectrum cephalosporins has increased among *E. coli* strains from both human and animal sources (Carattoli, 2008), and the mechanism of resistance can be associated with the production of extended-spectrum beta–lactamases (ESBLs) or plasmidic AmpC–beta-lactamases (pAmpC-BL).

Most ESBLs are variants of CTX-M, TEM, or SHV families (Bonnet, 2004) and confer resistance to a variety of beta-lactam antibiotics, including penicillins, 2nd, 3rd, and 4th generation cephalosporins and monobactams (e.g., aztreonam), but usually not carbapenems or cephamycins (e.g., cefoxitin). pAmpC-BL confer resistance to penicillins, 2nd and 3rd generation cephalosporins, including β-lactam/inhibitor combinations, cefamycins (cefoxitin), but usually not 4th generation cephalosporins (cefepime, cefquinome) and carbapenems. The CMY type is the most frequently reported pAmpC-BL in *E. coli* (Jacoby, 2009; EFSA, 2011). ESBL- and CMY-producing *E. coli* have emerged as a community pathogen in many parts of the world (Pitout and Laupland, 2008). Recent reports indicate that food might be a source of human-acquired antimicrobial-resistant *E. coli* (Carattoli, 2008) due to the fact that similar ESBLs and plasmids encoding them have been detected in food-producing animals, food of animal origin, and humans (Leverstein–van Hall *et al.*, 2011).

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Our laboratory recently reported CTX-M-1–producing *E. coli* strains from food samples and healthy humans in Tunisia (Ben Sallem *et al*., 2011; Ben Slama *et al*., 2010; Jouini *et al*., 2007), but this type of beta-lactamase was very infrequent in clinical *E. coli* isolates in this country (Ben Slama *et al*., 2011). The present study was conducted to analyze the prevalence of ESBL- and pAmpC-BL–producing *E. coli* isolates in food-producing animals in Tunisia, to determine the type of enzymes produced by these isolates, to compare the obtained results with previous data on food, healthy human, and clinical samples in this country, and to deepen the knowledge of the routes of transmission of ESBLs in different ecosystems.

Materials and Methods

Isolates and susceptibility testing

Eighty fecal samples of food-producing animals (23 sheep, 22 chickens, 22 cattle, six horses, five rabbits, and two dromedaries) were obtained from 35 different farms in Tunisia from February to May 2011. The farms of chickens and rabbits were of intensive and extensive production, and the farms of the other tested animals were of extensive production (Table 1). In the case of chicken and rabbit farms of intensive production, only one fecal sample of each farm was obtained by mixture of four fecal samples recovered at different areas of the farm (samples were obtained from the floor in chicken farms, when animals were 35 days old). In the case of the farms of extensive production, more than one sample was obtained from each farm from fecal samples of selected animals (Table 1).

Samples were seeded onto MacConkey agar plates supplemented with cefotaxime (CTX, 2 mg/L). After incubation at 37°C for 24 h, colonies showing *E. coli* morphology were recovered, and identified by classical biochemical methods and by species-specific polymerase chain reaction (PCR; amplification of *uidA* gene) (Jouini *et al*., 2007). One CTX-resistant (CTX^R) *E. coli* isolate per sample was selected and screened for ESBL and AmpC phenotypes by double disk test (CLSI, 2010; Tan *et al*., 2009). Susceptibility testing to 18 antibiotics (ampicillin, cefoxitin, ceftazidime, cefotaxime, imipenem, aztreonam, gentamicin, amikacin, tobramycin, kanamycin, streptomycin, nalidixic acid, ciprofloxacin, sulphonamides, trimethoprim-sulfamethoxazole, tetracycline, rifampicin, and chloramphenicol) was carried out by disk-diffusion method (CLSI, 2010). The minimum inhibitory concentrations (MICs) of cefotaxime, ceftazidime, aztreonam, and cefoxitin were determined by agar dilution method (CLSI, 2010). *E. coli* ATCC 25922 was used as a control strain.

Table 1. Characteristics of the Farms and Healthy Food-Producing Animals Tested for ESBL or pAmpC-BL *Escherichia coli* Producers

Type of animal (no. of farms)	*Type of farming (no. of farms)*	*Number of samples*		
		Total tested	*ESBL producers*	*pAmpC-BL producers*
Chicken (17)	Intensive (14)	14	8	2
	Extensive (3)	8	0	0
Sheep (9)	Extensive	23	0	0
Cow (6)	Extensive	22	0	0
Horse (1)	Extensive	6	0	0
Dromedary (1)	Extensive	2	1	0
Rabbit (3)	Intensive (1)	1	0	0
	Extensive (2)	4	0	0
Total (35)		80	9	2

ESBL, extended-spectrum beta-lactamase; pAmpC-BL, plasmidic AmpC–beta-lactamase.

Pulsed-field gel electrophoresis (PFGE) analysis and phylogroup determination of CTX^R E. coli strains

The clonal relationship among CTX^R *E. coli* strains was determined by PFGE using *Xba*I enzyme as previously described (Sáenz *et al*., 2004). Patterns were visually analyzed and interpreted according to previously reported criteria (Tenover *et al*., 1995). The isolates were assigned to the phylogenetic groups A, B1, B2, or D using a PCR strategy with specific primers for *chuA, yjaA,* and *TspE4.C2* determinants (Clermont *et al*., 2000).

Serotyping and virulence genotyping of E. coli isolates

All isolates were screened for O25b and O157 serotypes and for *afa/dra* operon (Blanco *et al*., 2009; Clermont *et al*., 2008). In addition, the *sxt, fimA, papG* allele III, *hlyA*, *cnf1*, *papC*, *aer*, *eae,* and *bfp* genes, encoding virulence factors often found in pathogenic *E. coli* (ExPEC) isolates, were tested by PCR (Ruiz *et al*., 2002).

Detection and characterization of beta-lactamase genes, genetic environment of bla_{CTX-M} and bla_{CMY} genes and other antibiotic resistance genes

The genes encoding TEM, SHV, OXA-1, CTX-M, and CMY type beta-lactamases and the genetic environment of bla_{CTX-M} and bla_{CMY-2} genes were analyzed by PCR and sequencing (Vinué *et al*., 2008). The presence of genes associated with resistance to tetracycline [*tet*(A) and *tet*(B)], sulphonamides [*sul1, sul2,* and *sul3*], gentamicin [*aac*(3)-II, and *aac*(3)-IV], streptomycin [*strA* and *strB*], and quinolones [*qnr*, *qepA,* and *aac*(6′)-Ib-cr] was determined by PCR (Ben Slama *et al*., 2011).

Detection and characterization of integrons

The presence of *intI1* and *intI2* genes (encoding class 1 and class 2 integrases, respectively) and the 3′-conserved segment (*qacEΔ1-sul1* genes) of class 1 integrons was examined by PCR. The variable regions of class 1 and class 2 integrons were characterized by PCR and sequencing in all *intI1*- or *intI2*-positive isolates (Ben Slama *et al*., 2011).

Results

CTX^R *E. coli* isolates were detected in 11 out of 80 fecal samples of healthy food-producing animals analyzed (13.8%) that were recovered in 11 out of the 35 farms tested (31.4%). Nine of these samples contained ESBL-positive *E. coli* isolates, all of them harbored the $bla_{CTX-M-1}$ gene, and two of these strains also harbored the $bla_{TEM-135}$ or bla_{TEM-1b} genes (Table 2). The IS*Ecp1*-$bla_{CTX-M-1}$-*orf477* structure was found in all nine ESBL-positive isolates. The remaining two CTX^R *E. coli* isolates contained the bla_{CMY-2} gene (encoding the beta-lactamase CMY-2). The IS*Ecp1*-bla_{CMY-2}-*blc* structure was identified in these two strains, but the IS*10* sequence was demonstrated in one of them, truncating IS*Ecp1* (Table 2); this

TABLE 2. CHARACTERISTICS OF THE 11 CEFOTAXIME-RESISTANT (CTX^R) *ESCHERICHIA COLI* ISOLATES RECOVERED FROM FECAL SAMPLES OF HEALTHY FOOD-PRODUCING ANIMALS

E. coli isolates (origin)[a]	PFGE	Phylogroup	Beta-lactamase	Genetic environment of bla genes	Resistance phenotype to non-beta-lactam antibiotics[b]	Class 1 integron		Resistance genes detected outside integron	Virulence factors
						intI1 / qacEΔ1 + sul1	Integron structure		
C4329 (C)	P1	B1	CTX-M-1	IS*Ecp1*-$bla_{CTX-M-1}$-*orf477*	SUL-TET	– / –	—	*tet*(A), *sul2*	*fimA*
C4330 (C)	P2	D	CTX-M-1	IS*Ecp1*-$bla_{CTX-M-1}$-*orf477*	SXT-SUL-TET-NAL-STR	+ / +	*dfrA15-aadA1*	*tet*(A), *sul2*	*fimA-aer*
C4331 (C)	P3	D	CTX-M-1	IS*Ecp1*-$bla_{CTX-M-1}$-*orf477*	SXT-SUL-TET-NAL	– / –	—	*tet*(A), *sul2*	*fimA-aer*
C4333 (C)	P4	A	CTX-M-1, TEM-135	IS*Ecp1*-$bla_{CTX-M-1}$-*orf477*	SXT-SUL-TET-NAL-CIP-STR(i)	+ / –	*dfrA17-aadA5*	*tet*(A), *sul2*	*fimA*
C4335 (C)	P5	B1	CTX-M-1	IS*Ecp1*-$bla_{CTX-M-1}$-*orf477*	SUL-TET-NAL-CIP	– / –	—	*tet*(A), *sul2*	*fimA*
C4336 (D)	P6	B1	CTX-M-1	IS*Ecp1*-$bla_{CTX-M-1}$-*orf477*	SUL-TET	– / –	—	*tet*(A), *sul2*	*fimA-aer*
C4337 (C)	P7	B1	CTX-M-1	IS*Ecp1*-$bla_{CTX-M-1}$-*orf477*	SXT-SUL-TET-NAL-STR(i)	+ / +	*dfrA1-aadA1*	*tet*(A), *sul2*, *strA-strB*	*fimA-aer*
C4338 (C)	P8	A	CTX-M-1	IS*Ecp1*-$bla_{CTX-M-1}$-*orf477*	SXT-SUL-TET-STR	+ / –	*dfrA17-aadA5*	*tet*(B), *sul2*	*aer*
C4339 (C)	P9	B1	CTX-M-1, TEM-1b	IS*Ecp1*-$bla_{CTX-M-1}$-*orf477*	SXT-SUL-TET-NAL-STR	+ / –	*dfrA17-aadA5*	*tet*(A), *sul2*, *strA-strB*	*fimA-aer*
C4332 (C)	P10	B2	CMY-2	IS*Ecp1*-bla_{CMY-2}-*blc*	SXT-SUL-TET-NAL-KAN-STR(i)	+ / +	*aadA1*	*tet*(B), *sul2*	*fimA-aer-papC*
C4334 (C)	P11	D	CMY-2	IS*Ecp1*Δ-IS*10*-bla_{CMY-2}-*blc*	SUL-TET-NAL-CIP-STR(i)	+ / +	*dfrA1-aadA1*	*tet*(B), *sul2*	*fimA-aer-papC*

[a]Origin of the samples: C, chicken; D, dromedary.
[b]SXT, trimethoprim-sulfamethoxazole; SUL, sulphonamides; TET, tetracycline; NAL, nalidixic acid; CIP, ciprofloxacin; KAN, kanamycine; STR, streptomycin; (i), intermediate resistance.

last structure is new and has been included in GenBank with the accession number JX440359.

It is of interest that all except one of the CTX^R *E. coli* isolates were recovered from chicken samples. The remaining one was from a dromedary sample. No CTX^R *E. coli* isolates were detected in fecal samples of other farm animals such as sheep, cow, horse, or rabbit. Two types of chicken farms were tested (of intensive and extensive farming), and all CTX^R isolates (ESBL- or pAmpC-BL–producing isolates) were detected in eight out of 14 farms with intensive production, but none in those of extensive production (Table 1).

Seven ESBL-positive isolates contained class 1 integrons with the following gene cassette arrangements: *dfrA17-aadA5* (three isolates), *dfrA1-aadA1* (two isolates), *dfrA15-aadA1* (one isolate), and *aadA1* (one isolate). The *dfrA17-aadA5* was detected inside a class 1 integron lacking the *qacE*Δ1 and *sul1* genes in the three *E. coli* isolates (Table 2). No class 2 integron was detected among the studied strains. The phenotypes of resistance of all CTX^R isolates as well as the MICs of beta-lactams are shown in Tables 2 and 3. As expected, all $bla_{CTX-M-1}$-positive isolates exhibited very high MIC values for cefotaxime (≥ 128 μg/mL) and the two bla_{CMY-2}-positive isolates high MIC values for cefoxitin (64 μg/mL).

PFGE analysis demonstrated unrelated pulsotypes among all 11 CTX^R isolates (Table 2). Phylogenetic analysis revealed that these strains were classified into the following phylogroups: B1 (five strains), A (two strains), D (three strains), and B2 (one strain; Table 2).

A variety of resistance genes located outside integrons were observed among our strains: *tet*(A) or *tet*(B) (in the 11 tetracycline-resistant strains), *strA/B* with/without *aadA1* (in two streptomycin-resistant strains), and *sul2* (in 11 sulphonamide-resistant strains). The virulence genes *fimA*, *aer,* and *papC* were detected in 10, eight, and two isolates, respectively, but none of the ESBL-producing isolates harbored the virulence genes *sxt*, *papG-III, hly*, *cnf1*, *eae*, *afa/dra,* and *bfp*, or were ascribed to the serotypes O25b or O157.

Discussion

To our knowledge, this is the first study of the genetic background of cefotaxime resistance in commensal *E. coli* isolates recovered from food-producing animals in Tunisia and one of the first reports in Africa. There is only one previous report about the detection of ESBL in food-producing animals in the African continent, and it concerned the detection of a CTX-M-15–producing *E. coli* strain among 89 ampicillin-resistant isolates obtained from fecal microbiota of healthy food-producing animals in Nigeria (Fortini *et al.*, 2011). Nonetheless, numerous reports are available from other continents and countries in food-producing animals (Briñas *et al.*, 2005; Carattoli, 2008; EFSA, 2011; Smet *et al.*, 2008).

Our findings show a high percentage of fecal carriage of ESBL-positive *E. coli* isolates from healthy food-producing animals (11.2%) in samples obtained in 2011. If we consider the percentage of carriage in particular animal species, it is of interest that 10 of 22 tested samples of chicken origin carried CTX^R *E. coli* isolates (45.5%): all of them obtained in farms of intensive production and eight of them (36.4%) carrying ESBL-producing isolates. These resistant isolates were not detected in fecal samples of sheep, cattle, horses, or rabbit, in

Table 3. Minimum Inhibitory Concentrations (MICs) of Beta-Lactams for the 11 Cefotaxime-Resistant (CTX^R) Isolates Recovered in This Study

Escherichia coli *isolates*	*Beta-lactamase*	*MICs of beta-lactams (mg/L)*			
		Cefotaxime	*Ceftazidime*	*Aztreonam*	*Cefoxitin*
C4329	CTX-M-1	>256	4	16	4
C4330	CTX-M-1	256	8	16	4
C4331	CTX-M-1	>256	16	64	8
C4333	CTX-M-1, TEM-135	256	4	8	4
C4335	CTX-M-1	>256	32	32	8
C4336	CTX-M-1	256	16	32	4
C4337	CTX-M-1	256	8	32	8
C4338	CTX-M-1	>256	4	16	4
C4339	CTX-M-1, TEM-1b	128	64	16	32
C4332	CMY-2	8	32	4	64
C4334	CMY-2	16	64	16	64

contrast with other studies (Blanc *et al*., 2006; Ho *et al*., 2011; Horton *et al*., 2011; Zhao *et al*., 2001). The number of samples tested in our work was low, but these results could indicate an enrichment of CTX^R *E. coli* isolates (with ESBL or pAmpC-BL) in chicken farms, which might reflect a high antibiotic pressure for selection of resistant bacteria in this ecosystem. These results are in agreement with the high incidence of ESBL-positive *E. coli* carriage on raw chicken meat in Tunisia (Ben Slama *et al*., 2010; Jouini *et al*., 2007). In a previous study performed by our group in 2007 with a low number of fecal samples of food-producing animals at the farm level (Jouini *et al*., 2007), no CTX^R isolates were detected, which could reflect an increase in the prevalence of these resistant microorganisms in past years.

Our results increase the number of hosts of $bla_{CTX\text{-}M\text{-}1}$-producing *E. coli* isolates in Tunisia (food samples and healthy humans) (Ben Sallem *et al*., 2011; Ben Slama *et al*., 2010) and now in farm animals and might reflect the successful spread of an epidemic plasmid. This possibility is also supported by our PFGE result, which showed unrelated patterns among all strains (Table 2). *E. coli* with $bla_{CTX\text{-}M\text{-}1}$ have been identified in food-producing animals in various European countries (Aarestrup *et al*., 2006; Bortolaia *et al*., 2010; Briñas *et al*., 2005; Girlich *et al*., 2007; Moodley *et al*., 2009), and the $bla_{CTX\text{-}M\text{-}1}$ gene has been frequently detected in IncI1 and IncN plasmids (Blanc *et al*., 2006; Girlich *et al*., 2007; EFSA, 2011; Moodley and Guardabassi, 2009; Bortolaia *et al*., 2010).

The IS*Ecp1* insertion sequence has been observed upstream of the ORFs encoding the CTX-M-1 and the CMY-2 enzymes in all CTX^R strains. This IS*Ecp1* element contains typical –35 and −10 putative promoter regions and could mobilize such genes (Eckert *et al*., 2006). Thus, the two different genetic environments detected in this study for the $bla_{CMY\text{-}2}$ gene are of interest.

There are reports of $bla_{CMY\text{-}2}$ genes in *E. coli* isolates from humans, food animals, and companion animals (Ben Slama *et al*., 2010; Briñas *et al*., 2005; Carattoli *et al*., 2005; Mataseje *et al*., 2010; Murphy *et al*., 2009; Yan *et al*., 2004).

Interestingly, one of the strains (C4333) harbored both the $bla_{CTX\text{-}M\text{-}1}$ and the $bla_{TEM\text{-}135}$ genes. This association of genes in the same strain was previously observed in an *E. coli* strain of pet origin in Tunisia (data not shown). The $bla_{TEM\text{-}135}$ gene was first found in *Salmonella enterica* serovar Typhimurium and recently was found in penicillinase-producing *N. gonorrhoeae* isolates (Nakayama *et al*., 2011; Ohnishi *et al*., 2010; Pasquali *et al*., 2005). Although the TEM-135–associated resistance phenotype does not correspond to an ESBL phenotype, its detection in strains of animal origin is worrisome, since it is considered a possible direct precursor of an ESBL (Nakayama *et al*., 2011).

Most ESBL-producing isolates exhibited resistance to antibiotics used in intensive animal production, mainly streptomycin, tetracycline, sulphonamides, and trimethoprim, and these resistances could play an important role in the co-selection of ESBL-producing bacteria (Carattoli, 2008).

In agreement with other studies, integrons were commonly identified, and they corresponded to a few integron types, especially class 1 integrons lacking the *qacE*Δ1 and *sul1* genes (Ben Sallem *et al*., 2011; Ben Slama *et al*., 2010, 2011). Transfer of plasmids with class 1 integrons between bacterial isolates from food-producing animals and humans has been suggested previously (Kang *et al*., 2005; Leverstein-van Hall *et al*., 2002; Singh *et al*., 2005)

Most of our ESBL–producing *E. coli* isolates belonged to phylogroups B1 and A ($n=7$), which are more often associated with animal or human commensal *E. coli* isolates; phylogroups D and B2 were less represented among our isolates ($n=2$). None of our ESBL-positive isolates were ascribed to phylogroup B2, though this group is very frequently detected among clinical ESBL-positive isolates. Nevertheless, one of our CMY-2–producing isolates belonged to phylogroups B2. More studies should be performed in the future in order to determine if the prevalence of the B2 phylogroup is higher among pAmpC-BL–than among ESBL-producing *E. coli* isolates of animal origin.

Conclusion

Chickens have become an important reservoir of CTX^R *E. coli* isolates. Our study reports the dissemination of the genes $bla_{CTX\text{-}M\text{-}1}$ and $bla_{CMY\text{-}2}$ in *E. coli* isolates of fecal samples of chickens at the farm level in Tunisia. Detailed molecular comparison of plasmids and genomes of isolates from various sources will help to better define the transmission dynamics of $bla_{CTX\text{-}M}$ between humans and food-producing animals.

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Disclosure Statement

No competing financial interests exist.

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