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# Characterization of CTX-M and SHV extended-spectrum β-lactamases and associated resistance genes in *Escherichia coli* strains of food samples in Tunisia

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***Objectives*: To assess the occurrence of extended-spectrum β-lactamases (ESBLs) in *Escherichia coli* isolates of faecal samples of animals ($n = 40$) and food samples ($n = 38$) obtained in Tunisia in 2006, and to characterize the type of ESBLs, their genetic environments and the associated resistance genes.**

***Methods*: Samples were inoculated in supplemented media (2 mg/L cefotaxime) for isolation of broad-spectrum cephalosporin-resistant *E. coli* isolates (one isolate/sample). ESBLs and their genetic environments as well as integrons and their gene cassette composition were characterized by PCR and sequencing.**

***Results*: ESBL-producing *E. coli* isolates were detected in 10 of the 38 food samples analysed (26%) and in none of the tested animal faecal samples. Genes found were as follows (number of isolates): $bla_{CTX\text{-}M\text{-}1}$ (5), $bla_{CTX\text{-}M\text{-}1}$ + $bla_{TEM\text{-}1b}$ (1), $bla_{CTX\text{-}M\text{-}14}$ + $bla_{TEM\text{-}1b}$ (2), $bla_{CTX\text{-}M\text{-}8}$ (1) and $bla_{SHV\text{-}5}$ (1). All ESBL-positive isolates showed unrelated PFGE patterns. IS*Ecp1* and IS*903* were detected surrounding $bla_{CTX\text{-}M\text{-}14}$, and IS*Ecp1*/IS*26* and *orf477* surrounding some of the $bla_{CTX\text{-}M\text{-}1}$ genes. Four of the ESBL-positive strains harboured class 1 integrons including different gene cassette combinations.**

***Conclusions*: ESBLs, mainly of the CTX-M class, are detected in *E. coli* of food origin in Tunisia, being the first time that this mechanism has been detected in food *E. coli* strains in Africa.**

Keywords: CTX-M-1, CTX-M-8, CTX-M-14, SHV-5, *E. coli*, animals

## Introduction

The detection of clinical *Escherichia coli* isolates producing extended-spectrum β-lactamases (ESBLs) has been increasingly reported in the last few years in a wide variety of countries, including Tunisia,[1–3] and represents a clinical problem in human medicine.[2] In addition, a new class of ESBL enzymes has emerged, the CTX-M class, which is dramatically spreading among human clinical *E. coli* isolates, mainly in those recovered from the community.[1,4] A few reports have recently appeared referring to the detection of ESBL-containing *E. coli* strains in animal and food samples in different countries of Europe and Asia,[5–8] but to our knowledge, never in Tunisia or in other African countries. The objective of our study was to detect ESBL-containing *E. coli* in faecal samples of food-producing animals as well as in food samples of animal origin in Tunisia and to characterize the type of ESBL enzymes, their genetic environments and the associated resistance genes.

## Materials and methods

### *Samples and E. coli isolates*

A total of 40 faecal samples of healthy animals were recovered either from farm animals (6 chickens, 16 cattle and 14 horses) or from veterinary clinics (2 cats and 2 dogs). In those samples obtained in farms, one lot of animals (50–60 animals for chickens,

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15–20 for cattle and 1–2 for horses) was considered as one unique sample. In addition, 38 food samples of animal origin were also included in this study, and they were obtained either from different supermarkets or from a food laboratory of Tunis (23 samples of beef, 8 of chicken, 2 of turkey, 1 of sheep and 4 of fish). All samples of food and animal origin were obtained in the North of Tunisia during 2006.

Samples were suspended in sterile saline solution and then were seeded on MacConkey agar plates supplemented with 2 mg/L cefotaxime and incubated for 24 h at 37°C. Isolates with typical *E. coli* morphology were selected (one per sample) and identified by classical biochemical methods, as well as by a species-specific PCR of the *uid* gene encoding β-glucuronidase (primers used: *uid*-F, 5′-ATCACCGTGGTGACGCATGTCGC-3′; and *uid*-R, 5′-CACCACGATGCCATGTTCATCTGC-3′).

### *Antimicrobial susceptibility testing*

*E. coli* isolates recovered from the antibiotic-supplemented MacConkey agar plates were submitted to susceptibility testing for 17 antimicrobial agents by the disc diffusion method according to the CLSI criteria.[9] The antimicrobial agents tested were as follows (μg): ampicillin (10), amoxicillin/clavulanic acid (20 + 10), cefoxitin (30), ceftazidime (30), cefotaxime (30), aztreonam (30), imipenem (10), gentamicin (10), amikacin (30), tobramycin (10), streptomycin (10), nalidixic acid (30), ciprofloxacin (5), sulfamethoxazole (300), trimethoprim/sulfamethoxazole (1.25 + 23.75), tetracycline (30) and chloramphenicol (30). The MICs of nalidixic acid and ciprofloxacin were also determined by the agar dilution method in all quinolone-resistant *E. coli* isolates.[9] A screening test for the detection of ESBLs was carried out by the double disc diffusion test (using cefotaxime, ceftazidime and amoxicillin/clavulanic acid discs) according to the CLSI criteria.[9] *E. coli* ATCC 25922 was used as a quality control strain.

### *Characterization of β-lactamase genes and genetic environment of $bla_{CTX\text{-}M}$ genes*

The presence of genes encoding TEM (F, 5′-ATTCTTGAAGACGAAAGGGC-3′; R, 5′-ACGCTCAGTGGAACGAAAAC-3′; amplicon size 1150 bp), SHV (F, 5′-CACTCAAGGATGTATTGTG-3′; R, 5′-TTAGCGTTGCCAGTGCTCG-3′; amplicon size 885 bp), OXA-1 (F, 5′-ACACAATACATATCAACTTCGC-3′; R, 5′-AGTGTGTTTAGAATGGTGATC-3′; amplicon size 813 bp), CTX-M-1 group (F, 5′-GTTACAATGTGTGAGAAGCAG-3′; R, 5′-CCGTTTCCGCTATTACAAAC-3′; amplicon size 1041 bp), CTX-M-8 group (F, 5′-TGATGAGACATCGCGTTAAG-3′; R, 5′-TAACCGTCGGTGACGATTTT-3′; amplicon size 875 bp), CTX-M-9 group (F, 5′-GTGACAAAGAGAGTGCAACGG-3′; R, 5′-ATGATTCTCGCCGCTGAAGCC-3′; amplicon size 857 bp) and CTX-M-10 (F, 5′-CCGCGCTACACTTTGTGGC-3′; R, 5′-TTACAAACCGTTGGTGACG-3′; amplicon size 944 bp) β-lactamases was analysed by PCR and sequencing (on both strands).[5,6] Nucleotide segments and their deduced amino acid sequences were compared with those included in the GenBank database as well as with those deposited at the web site http://www.lahey.org/Studies/, in order to ascribe the specific type of β-lactamase gene. Positive and negative controls were included in all PCR assays. The presence of IS*Ecp1*, IS*26*, *orf477* or IS*903* sequences surrounding the $bla_{CTX\text{-}M}$ genes was analysed by PCR using primers and conditions as previously described;[10] all obtained amplicons were sequenced for confirmation.

The promoter and attenuator region of the chromosomal *ampC* gene was also amplified by PCR, sequenced and compared with the same region of the *E. coli* K12 *ampC* gene, to analyse the mutations associated with the overexpression of this gene.[5]

### *Characterization of integrons and resistance mechanisms to non-β-lactam antimicrobial agents*

The presence of genes associated with tetracycline [*tet*(A) and *tet*(B)], streptomycin [*aadA1* and *aadA2*], sulfamethoxazole [*sul1*, *sul2* and *sul3*] and gentamicin [*aac(3)-I*, *aac(3)-II* and *aac(3)-IV*] resistance was studied by PCR.[11] *gyrA* and *parC* genes were amplified by PCR in all quinolone-resistant *E. coli* isolates, and the obtained sequences were compared with those previously reported for *gyrA* (GenBank accession number X06373) and *parC* (M58408 with the modification included in L22025) genes.[11] The presence of the genes encoding class 1 and class 2 integrases was examined by PCR and the gene cassettes included in the variable region of class 1 integrons were detected by PCR and sequencing.[11]

### *PFGE patterns of E. coli isolates*

The clonal relationship between the strains was studied by PFGE, using *Xba*I as a restriction enzyme.[11]

## Results and discussion

Seventy-eight food and animal faecal samples were analysed to detect the presence of ESBL-producing *E. coli* isolates using cefotaxime-supplemented agar plates. Broad-spectrum cephalosporin-resistant *E. coli* isolates were recovered from 11 of these samples (14%), and ESBL-containing *E. coli* isolates were detected in 10 of them (13%), when the double disc screening test was used (Table 1). All of them were isolated from samples of food origin, representing 26% of the total food samples analysed in this study. No colonization by ESBL-containing *E. coli* isolates was detected among the tested animal faecal samples. The difference observed in the prevalence of ESBL-positive *E. coli* isolates in faecal animal samples in relation to the food animal samples might have different reasons. The animals from which food and faecal samples were obtained were grown in non-related farms. On the other hand, although the *E. coli* contamination of food is expected to be due to faecal *E. coli* at the slaughterhouse level, contamination during the transformation or commercialization processes of food cannot be excluded.

### *Characterization of ESBL genes in E. coli isolates of food origin*

The β-lactamase genes detected by PCR and sequencing in the 10 ESBL-positive *E. coli* isolates were as follows: $bla_{CTX\text{-}M\text{-}1}$ (five isolates, four of beef and one of turkey origin), $bla_{CTX\text{-}M\text{-}1} + bla_{TEM\text{-}1b}$ (one isolate of beef), $bla_{CTX\text{-}M\text{-}14} + bla_{TEM\text{-}1b}$ (two isolates of chicken), $bla_{CTX\text{-}M\text{-}8}$ (one isolate of chicken) and $bla_{SHV\text{-}5}$ (one isolate of chicken) (Table 1). All the ESBL-positive *E. coli* isolates showed unrelated PFGE patterns. It is of interest to underline that these detected CTX-M β-lactamases belong to different groups (groups 1, 8 and 9), including the very unusual CTX-M-8 β-lactamase. The genetic location of $bla_{CTX\text{-}M}$ genes has not been studied in this work,

**Table 1.** Characteristics of the 11 broad-spectrum cephalosporin-resistant *E. coli* strains detected in 11 food samples of animal origin in Tunisia

| *E. coli* strain | Food origin | Screening of ESBL | Type of β-lactamase detected | Resistance phenotype to non-β-lactam antibiotics[a] | Resistance genes to non-β-lactam antibiotics | Class 1 integrons | | MIC (mg/L) | | Amino acid changes | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | *int1* | variable region | NAL | CIP | GyrA | ParC |
| C923 | beef | + | CTX-M-1 | TET-GEN-TOB-STR-CHL-SUL | *tet*(B), *aac(3)-II*, *aadA1*, *aadB*[b], *sul1* | + | (*aadB* + *aadA1*) | ND[c] | ND | ND | ND |
| C924 | beef | + | CTX-M-1 | TET-GEN-TOB-STR-CHL-SUL | *tet*(B), *aac(3)-II*, *aadA1*, *aadB*[b], *sul1* | + | (*aadB* + *aadA1*) | ND | ND | ND | ND |
| C926 | turkey | + | CTX-M-1 | TET-STR[I]-NAL-SUL-SXT | *tet*(B), *aadA1*, *sul1*, *sul2*, *dfrA1*[b] | + | (*dfrA1* + *aadA1*) | 128 | 0.12 | S83A | wild-type |
| C922 | beef | + | CTX-M-1 | TET-STR[I]-SUL-SXT | *tet*(A), *aadA1*, *sul1*, *dfrA1*[b] | + | (*dfrA1* + *aadA1*) | ND | ND | ND | ND |
| C925 | beef | + | CTX-M-1 | susceptible[d] | | − | | ND | ND | ND | ND |
| C920 | beef | + | CTX-M-1 + TEM-1[e] | susceptible[d] | | − | | ND | ND | ND | ND |
| C930 | chicken | + | CTX-M-14 + TEM-1 | GEN-NAL-CIP | *aac(3)-II* | − | | ≥2048 | 128 | S83L +D87N | S80I + E84G |
| C929 | chicken | + | CTX-M-14 + TEM-1 | TET-NAL | *tet*(B) | − | | 512 | 0.5 | S83L | wild-type |
| C921 | chicken | + | CTX-M-8 | TET-NAL | *tet*(B) | − | | 256 | 0.5 | S83L | wild-type |
| C928 | chicken | + | SHV-5 | NAL | | − | | 512 | 0.5 | S83L | wild-type |
| C927[f] | turkey | − | TEM-1 | TET-STR-NAL-SUL-SXT | *tet*(B), *aadA1*, *sul1*, *sul2, dfrA1*[b] | + | (*dfrA1*+*aadA1*) | 512 | 0.5 | D87Y | wild-type |

[a]TET, tetracycline; GEN, gentamicin; TOB, tobramycin; STR, streptomycin; NAL, nalidixic acid; CIP, ciprofloxacin; CHL, chloramphenicol; SUL, sulphonamide; SXT, sulfamethoxazole/trimethoprim; STR[I], intermediate resistance phenotype to STR.
[b]These genes were detected inside the variable region of class 1 integrons.
[c]ND, not determined.
[d]The isolate did not show resistance to the non-β-lactams tested.
[e]The $bla_{\text{TEM-1b}}$ variant was detected in all TEM-1 β-lactamase-producers found in this study.
[f]Three nucleotidic mutations (at positions −28, +52 and +72) were detected in the promoter–attenuator region of the *ampC* gene in this strain in comparison with the reference sequence of *E. coli* K12.

but other authors have demonstrated the plasmidic location of these genetic determinants.[2]

### *Genetic environments of bla$_{CTX-M}$ genes*

Figure 1 shows the genetic environments of *bla*$_{CTX-M}$ genes detected among our ESBL-positive *E. coli* isolates. *orf477* was detected downstream of *bla*$_{CTX-M-1}$ in all six strains that harboured that ESBL gene. The sequence of the fragment obtained by PCR upstream of the *bla*$_{CTX-M-1}$ gene in three of the *E. coli* strains (C920, C924 and C925) revealed the presence of a region of the IS*26* transposase (211 bp) flanking a partially truncated IS*Ecp1* whose size was 214 bp and followed by an intergenic region (32 + 48 bp, named by Eckert *et al.*[10] as X+W sequences, respectively). This whole structure was similar (98%) to that previously described in *E. coli* 5402 with the EMBL accession number AJ416342. The upstream region of the *bla*$_{CTX-M-1}$ gene in the remaining three *E. coli* strains (C922, C923 and C926) could not be identified because all PCRs performed were negative. The presence of IS*Ecp1* and IS*903* was demonstrated upstream and downstream, respectively, of the *bla*$_{CTX-M-14}$ gene in *E. coli* C929 and C930, and an intergenic region of 42 bp (previously named as the Y region[10]) was found between IS*Ecp1* and *bla*$_{CTX-M-14}$ (Figure 1).

All PCRs for detection of IS*Ecp1*, IS*26*, *orf477* or IS*903* sequences surrounding the *bla*$_{CTX-M-8}$ gene were negative in our *E. coli* strain C921. The role of IS*26* and IS*Ecp1* insertion sequences in the mobilization and insertion of *bla*$_{CTX-M}$ genes has been previously described.[10]

### *Mechanism of resistance in a broad-spectrum cephalosporin-resistant and ESBL-negative E. coli strain*

A broad-spectrum cephalosporin-resistant ESBL-negative strain (*E. coli* C927) was recovered in a turkey food sample of this study. *bla*$_{TEM-1b}$ was the unique acquired β-lactamase gene detected in this strain. The promoter/attenuator region of the chromosomal *ampC* gene of this strain was sequenced and three changes in the nucleotide sequence were detected at positions −28, +52 and +72, but no mutations were observed at the important −42 and −32 positions, associated with broad-spectrum cephalosporin resistance.[5]

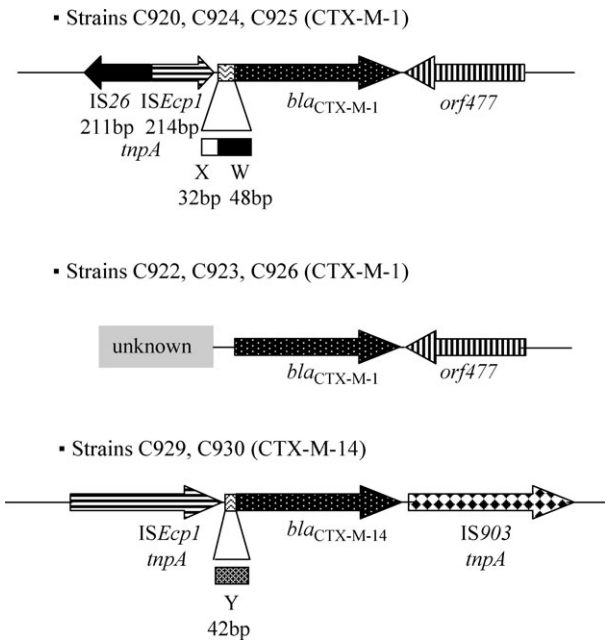


**Figure 1.** Genetic environments of *bla*$_{CTX-M}$ genes detected among our ESBL-positive *E. coli* strains (the intergenic X, Y and W regions have been previously reported[10]).

### *Mechanism of resistance to non-β-lactam antimicrobial agents*

Table 1 shows the pattern of resistance to non-β-lactam antimicrobial agents in all 11 broad-spectrum cephalosporin-resistant *E. coli* strains. Six of the strains showed resistance to at least three of the tested non-β-lactam antimicrobial agents. A variety of resistance genes were observed among our strains: *tet*(A) or *tet*(B) (in the seven tetracycline-resistant strains), *aadA1* with/without *aadB* (in five streptomycin-resistant strains), *sul1* with/without *sul2* (in five sulphonamide-resistant strains), *aac(3)-II* (in three gentamicin-resistant strains) and *dfrA1* (in three trimethoprim/sulfamethoxazole-resistant strains). The presence of class 1 integrons was demonstrated in 5 of the 11 cephalosporin-resistant strains and two different gene cassette combinations were identified (Table 1).

Amino acid changes in GyrA and ParC proteins were observed among our six quinolone-resistant strains. Five of the strains showed nalidixic acid resistance (MICs of 128–512 mg/L), being ciprofloxacin susceptible (MICs of 0.125–0.5 mg/L), and one amino acid change in the GyrA protein (but not in ParC) was observed (Ser-83→Leu or Ser-83→Ala or Asp-87→Tyr). The remaining quinolone-resistant strain (*E. coli* C930) showed a high-level nalidixic acid and ciprofloxacin resistance phenotype (MICs of ≥2048 and 128 mg/L, respectively), and two amino acid changes in both GyrA (Ser-83→Leu + Asp-87→Asn) and ParC (Ser-80→Ile + Glu-84→Gly) proteins were identified (Table 1).

### *Conclusions*

A high percentage of food samples were colonized by ESBL-containing *E. coli* strains (26%), mainly of the CTX-M class. In addition, certain associations between animal species and CTX-M types seem to occur: CTX-M-1 has been detected in strains recovered from food samples of beef origin and the CTX-M-14 or CTX-M-8 enzymes in those of chicken origin. The use of cephalosporins in food-producing animals could be a selective factor for the appearance of ESBL-containing bacteria in animals. Nevertheless, due to the fact that most of the ESBL-containing *E. coli* isolates recovered either from animals or from humans harbour a wide variety of resistance genes to non-β-lactam antimicrobial agents, the possibility of co-selection by the use of non-β-lactams could happen and this fact should be extensively evaluated in the future.

To our knowledge, this is the first report describing ESBL-producing *E. coli* strains detected in samples of food origin in Tunisia and in the African continent. In addition, this is the first time that the unusual CTX-M-8 β-lactamase has been detected in bacteria of non-human origin. It seems that CTX-M β-lactamases are spreading not only among animals and humans in Europe and Asia, but also in Tunisia. More studies should be

carried out in the future to track the evolution of these types of β-lactamases in isolates of different origins in different countries of the African continent.

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## Transparency declarations

None to declare.

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