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High prevalence of extended-spectrum and plasmidic AmpC beta-lactamase-producing *Escherichia coli* from poultry in Tunisia

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ABSTRACT

This study was conducted to detect extended spectrum beta-lactamases (ESBLs) and plasmidic AmpC beta-lactamase (pAmpC-BL)-producing *Escherichia coli* isolates in industrial poultry samples were collected from healthy chickens of the three farms. Samples were inoculated onto desoxycholate-lactose-agar plates supplemented with cefotaxime (2 mg/L). *E. coli* was identified by biochemical and molecular methods and antibiotic susceptibility testing by the disk diffusion method. Genes encoding ESBLs and pAmpC-BL were detected by PCR and sequencing. Phylogenetic groups were determined by triplex PCR. The molecular typing of strains was done by pulsed field gel electrophoresis (PFGE) and Multilocus Sequence Typing (MLST) in those isolates showing different PFGE patterns. Cefotaxime-resistant *E. coli* isolates were recovered in 48 of 137 fecal samples (35%), and one isolate/sample was further studied. The following beta-lactamase genes were detected: $bla_{CTX\text{-}M\text{-}1}$ (29 isolates, isolated in all three farms), $bla_{CTX\text{-}M\text{-}15}$ (5 isolates, confined in farm II), $bla_{CTX\text{-}M\text{-}14}$ and $bla_{CMY\text{-}2}$ (one isolate and 13 isolates, respectively, in farm III). The 48 cefotaxime-resistant isolates were distributed into phylogroups: B1 (n = 21), A (n = 15) and D (n = 12). PFGE analysis revealed 19 unrelated patterns: 15 different profiles among ESBL-positive strains and 4 among the CMY-2-positive isolates. The following sequence types-associated phylogroups were detected: a) CTX-M-1-positive strains: lineages ST542-B1, ST212-B1, ST58-B1, ST155-B1 and ST349-D; b) CTX-M-15-positive strain: lineage ST405-D; c) CTX-M-14-positive strain: lineage ST1056-B1; d) CMY-2-positive strains: lineages ST117-D, ST2197-A, and ST155-B1. Healthy chickens constitute an important reservoir of ESBL- and pAmpC-BL-producing *E. coli* isolates that potentially could be transmitted to humans via the food chain or by direct contact.

1. Introduction

A steady increase in the rates of *Escherichia coli* resistant to third generation cephalosporins (3rd GC) is reported worldwide (Carattoli, 2008). This resistance can be associated with the production of extended spectrum beta-lactamases (ESBLs) and/or plasmidic AmpC beta-lactamases (pAmpC-BL) (Paterson and Bonomo, 2005). Such resistant bacteria can cause severe community or hospital acquired infections. Although person-to-person spread is recognized as the main way of spread of ESBL/pAmpC-BL containing *E. coli* both in hospitals and the community, the primary reservoirs of such organisms are contentious. Also, these microorganisms have been isolated from food-producing animals and derived foods in many countries, which has raised questions about the possible role of animal and food related reservoirs on this phenomenon (Mellata, 2013).The predominant ESBL families are CTX-M, TEM, and SHV (Paterson and Bonomo, 2005). The most common ESBL gene is $bla_{CTX\text{-}M\text{-}1}$ and $bla_{CTX\text{-}M\text{-}15}$ in animals and in humans, respectively, and the most common pAmpC-BL gene is $bla_{CMY\text{-}2}$ (Ben Slama et al., 2011; Ben Sallem et al., 2012). ESBL/pAmpC-BL transmission is mainly driven by mobile genetic elements, some of which are homologous in isolates from both food-producing animals and humans (Liébana et al., 2013). Epidemic plasmids belonging to the Inc groups F, A/C, N, HI2, I1 and K, carrying particular ESBL or pAmpC-BL encoding genes have been detected among farms and companion animals, food products and humans (Carattoli, 2008; Ben Sallem et al., 2014). In Tunisia, only a few studies report the fecal carriage of *E. coli* resistant to 3rd GC in food-producing animals (Ben Sallem et al., 2012; Grami et al., 2013; Kilani et al., 2015).

The present study was conducted to detect ESBL/pAmpC-BL positive *E. coli* isolates from healthy poultry in three Tunisian farms and to

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characterize the mechanisms of antibiotic resistance and the molecular typing of recovered isolates.

2. Materials and methods

2.1. Sampling and bacterial identification

During 2013, 137 fecal samples were collected from healthy chickens at three industrial laying hen farms in North of Tunisia (39 samples in farm I, 62 samples in farm II and 36 samples in farm III). Each farm contained 2 to 4 buildings containing from 5000 to 10,000 birds (only one building was studied in the present work). Chickens were kept in caged (box) systems; each box contained 4 to 6 ones. The approximate area of each building is 1000 m^2, with 10 roof-mounted fans. Water and food intakes were assured by 4 drip-type drinker and ad libitum feeding, respectively, and feces discharge realized at the end of breeding cycles.

Moreover, personal entrance in these farms was very well controlled to avoid contamination. Random boxes were selected and 1 to 3 samples of fresh feces were taken by sterile cotton swabs and transported at 4 °C to the laboratory to be analyzed in the same day. In order to consider each box as a single sample, the 3 cotton swabs were combined and directly rubbed onto desoxycholate lactose agar plates (Biokar Diagnostics, France) supplemented with cefotaxime (CTX, 2 mg/L) to isolate cefotaxime-resistant isolates. After incubation at 37 °C for 24 h, one colony per sample showing *E. coli* morphology were recovered and identified by classical biochemical methods, API 20E system (BioMerieux, Marcy l'Etoile, France), and by species-specific PCR (amplification of *uidA* gene) (Jouini et al., 2007).

2.2. Antimicrobial susceptibility testing and ESBL identification

Antimicrobial susceptibility testing to 17 antibiotics (amoxicillin, amoxicillin-clavulanic acid, cefoxitin, ceftazidime, cefotaxime, imipenem, aztreonam, gentamicin, amikacin, tobramycin, nalidixic acid, ciprofloxacin, trimethoprim-sulfamethoxazole, tetracycline, minocyclin, tigycycline and chloramphenicol) was determined by the agar disk diffusion method on Mueller-Hinton agar plates (Bio-Rad, France) according to the Clinical and Laboratory Standards Institute guidelines (CLSI, 2013). One cefotaxime-resistant *E. coli* isolate per sample was selected and screened for ESBL-phenotype by double-disk synergy test (DDST) with cefotaxime, ceftazidime and amoxicillin-clavulanic acid disks (CLSI, 2013). Those cefotaxime-resistant *E. coli* isolates showing a negative-ESBL-phenotype but showing resistance to amoxicillin-clavulanic acid and cefoxitin, were included in the pAmpC-BL-phenotype.

2.3. Molecular typing of cefotaxime-resistant E. coli strains

In order to determine the clonal relationship among cefotaxime-resistant *E. coli* isolates, agarose plugs containing genomic DNA of the isolates were digested with *Xba*I enzyme (BioLabs, New England). *Xba*I-digested DNA fragments were separated by pulsed-field gel electrophoresis (PFGE) on a 1% agarose gel in 0.5× Tris-Borate-EDTA buffer using a CHEF-DRIII device (Bio-Rad, Marnes-la-Coquette, France). PFGE conditions were as follows: 6 V/cm for 23 h with pulse times ranging from 1 to 30 s at 14 °C (Tenover et al., 1995; Sáenz et al., 2004). A lambda ladder was used as a standard size marker (BioLabs, New England). Patterns were visually compared and analyzed according to previously reported criteria (Tenover et al., 1995; Sáenz et al., 2004).

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).

One cefotaxime-resistant *E. coli* isolate from each of the nineteen different PFGE profiles was characterized by Multilocus Sequence Typing (MLST), by PCR amplification of the standard seven housekeeping loci (Tartof et al., 2005). All the amplicons were sequenced and compared with the sequences deposited in the MLST database (http://mlst.warwick.ac.uk/), to know the specific allele combination and the sequence type (ST).

2.4. Virulence genotyping of cefotaxime-resistant E. coli isolates

All isolates were screened for 18 virulence factors (VFs) found in extra-intestinal pathogenic *E. coli* (ExPEC) using PCR (Johnson and Stell, 2000). A virulence score was calculated as the sum of all VFs for which the isolates tested positive. ExPEC status of the isolates was analyzed based on the operational definition of Johnson et al., that is, presence of ≥2 of the following 5 virulence genes, *papA and/or papC*, *sfa/focDE*, *afa/draBC*, *iutA*, and *kpsM II* (Johnson et al., 2003).

2.5. Molecular analysis of antibiotic resistance genes and the genetic environment of $bla_{CTX\text{-}M}$

DNA extraction was performed for all samples by boiling. The presence of beta-lactamase genes [$bla_{CTX\text{-}M\text{-}consensus}$, $bla_{CTX\text{-}M\text{-}1,\ 2,\ 8,\ 9,\ 25}$-groups, bla_{OXA}, bla_{SHV}, bla_{TEM}, bla_{CMY}, bla_{ACC}, bla_{MOX}, bla_{FOX}, bla_{DHA}, bla_{LAT}, bla_{ACT} and bla_{MIR}] was detected by PCR and sequencing (Pérez-Pérez and Hanson, 2002; Saladin et al., 2002). The genetic environment of $bla_{CTX\text{-}M}$ genes was characterized and determined by PCR and sequencing (Eckert et al., 2006).

2.6. Detection of resistance genes to non-beta-lactam antimicrobial agents

The presence of genes associated with resistance to tetracycline [*tet*(A) and *tet*(B)], sulphonamides [*sul1* and *sul3*], streptomycin [*aadA1* and *aadA2*], chloramphenicol [*cmlA*], and quinolones [*qnrA*, *qnrB*, *qnrS*, *qepA* and *aac*(6′)-*Ib-cr*] was determined by PCR and sequencing (Sáenz et al., 2004).

2.7. Detection and characterization of integrons

The presence of *int1* and *int2* genes (encoding class 1 and class 2 integrases, respectively) was examined by PCR for representative isolates (one isolate per PFGE pattern). The variable regions of class 1 and class 2 integrons were characterized by PCR and sequencing in all *int1*- or *int2*-positive isolates (Ben Slama et al., 2011).

2.8. Plasmid typing

Plasmids carried by selected isolates (one isolate per PFGE pattern) were assigned to the incompatibility groups using PCR-based replicon typing method (Carattoli et al., 2005a).

2.9. Statistical analysis

Virulence score was determined for each strain and calculated as the sum of virulence genes detected, with *papG* alleles counting collectively as a single determinant.

Statistical testing was done using SPSS (version 10.0) and Epi Info (version 6.04) softwares. Comparisons of proportions were determined using the chi-square test or Fisher's exact test. Comparisons of virulence scores were assessed using the Mann-Whitney *U* test and Kruskal–Wallis. Values $P \leq 0.05$ were considered to be significant.

3. Results

3.1. Prevalence of cefotaxime-resistant E. coli isolates and antibiotic susceptibility

Cefotaxime-resistant *E. coli* isolates were recovered in 48 of the 137 fecal samples (35%) of the three poultry industrial farms analyzed [9/39

(23.07%), 13/62 (20.9%), and 26/36 (72.2%) in farm I, II and III, respectively]. Among the 48 cefotaxime-resistant *E. coli* isolates, 35 exhibited a positive ESBL-screening test (72.9%). The remaining isolates (n = 13), all recovered in farm III, showed an pAmpC-BL phenotype characterized by resistance to cefoxitin and amoxicillin-clavulanic acid, in addition to reduced susceptibility to broad-spectrum cephalosporins and a negative ESBL-screening test. Consequently, 25.5% of tested samples carried ESBL positive *E. coli* isolates and 9.4% were carriers of *E. coli* isolates with pAmpC-BL phenotype.

The majority of the 35 ESBL-positive *E. coli* isolates were co-resistant to non-beta-lactam antibiotics (Table 1), being resistant to: trimethoprim-sulfamethoxazole (46%), nalidixic acid (80%), ciprofloxacin (43%), gentamicin and amikacin (3%), tetracycline (94%), and minocyclin (71.4%). In addition, the 13 CMY-2-producing *E. coli* isolates seemed to be more resistant than ESBL-producing isolates. They were resistant to trimethoprim-sulfamethoxazole (100%), nalidixic acid (100%), ciprofloxacin (100%), tetracycline (100%), minocyclin (100%) gentamicin (54%), and amikacin (15%). All cefotaxime-resistant *E. coli* isolates showed susceptibility to carbapenems and tigecycline (Table 1).

3.2. Genes encoding beta-lactamases, clonal relationship and virulence genotyping in cefotaxime-resistant E. coli isolates

The presence of genes encoding beta-lactamases was studied in the 35 ESBL-positive *E. coli* isolates and the following beta-lactamases were identified: CTX-M-1 (29 isolates, recovered in farms I (9 isolates), II (8 isolates) and III (12 isolates)), CTX-M-15 (5 isolates, confined in farm II) and CTX-M-14 (one isolate, in farm III). The bla_{TEM-1} gene was concomitantly present in 8 isolates. All isolates with pAmpC-BL phenotype harbored the bla_{CMY-2} gene (13 isolates, confined in farm III) (Table 1).

Phylogenetic analysis revealed that isolates belonged to the following groups: B1 (n = 21), A (n = 15) and D (n = 12). ESBL-producing isolates were significantly associated with phylogenetic group B1

Table 1
Characteristics of the 48 cefotaxime-resistant *E. coli* recovered from fecal samples of healthy chickens in the three farms.

Strain	*Farm*	*Resistance profile for non-beta-lactam antibiotics*[a]	*Beta-lactamase genes*	Virulence genes profile/virulence score[b]	*Phylogenetic group*	*PFGE pattern*
C6901	I	NAL, SXT, TET, MNO	$bla_{CTX-M-1}$	*iutA/1*	B1	P1
C6902	I	TET, MNO,	$bla_{CTX-M-1}$	*papGII*, *fimH*, *iutA*, *iroN/4*	B1	P2
C6903	I	SXT, TET, MNO	$bla_{CTX-M-1}$	*papGII*, *fimH*, *iutA*, *iroN*, *tratT/5*	B1	P2
C6905	I	SXT, TET, MNO, TOB, AMK	$bla_{CTX-M-1}$	*fimH*, *iroN*, *traT*, *ompT/4*	A	P3
C6906	I	TET, MNO, TOB	$bla_{CTX-M-1}$	*fimH*, *iroN/2*	A	P3
C6908	I	NAL, TET, MNO	$bla_{CTX-M-1}$	*fimH*, *iroN*, *ompT/3*	A	P3
C6904	I	TET	$bla_{CTX-M-1}$	*fimH*, *cnfI*, *kpsM II*, *ompT*, *usp/5*	D	P4
C6907	I	TET	$bla_{CTX-M-1}$	*fimH*, *sat*, *iroN*, *kpsM II*, *ompT/5*	D	P4
C6909	I	No resistance	$bla_{CTX-M-1}$	*fimH*, *sat*, *iroN*, *kpsM II*, *ompT/5*	D	P4
C6911	II	NAL, CIP, SXT, TET, MNO, GEN, TOB	$bla_{CTX-M-1}$, bla_{TEM-1}	*fimH*, *iutA*, *iroN*, *traT*, *ompT/5*	B1	Non typable
C6913	II	NAL, CIP, TET, MNO	$bla_{CTX-M-1}$	*fimH*, *iutA*, *iroN*, *traT*, *ompT/5*	B1	P5
C6915	II	NAL, CIP, TET, MNO	$bla_{CTX-M-1}$	*fimH*, *iutA*, *iroN*, *traT/4*	B1	P6
C6916	II	NAL, CIP, SXT, CHL	$bla_{CTX-M-1}$	*fimH*, *iroN*, *traT/3*	B1	P7
C6922	II	NAL, CIP, SXT, TET, MNO, CHL	$bla_{CTX-M-1}$	*fimH*, *iutA*, *iroN*, *traT/4*	B1	P7
C6920	II	NAL, CIP, TET, MNO	$bla_{CTX-M-1}$	*fimH*, *iutA*, *iroN*, *traT*, *ompT/5*	B1	P8
C6921	II	NAL, CIP, TET, MNO	$bla_{CTX-M-1}$	*fimH*, *iutA*, *iroN*, *traT*, *ompT/5*	B1	P8
C6918	II	NAL, CIP, SXT, TET, MNO	$bla_{CTX-M-1}$	*fimH*, *iroN*, *traT*, *ompT*, *malX/5*	D	P9
C6910	II	NAL, CIP, SXT, TET, MNO, CHL	$bla_{CTX-M-15}$, bla_{TEM-1}	*fimH*, *sat*, *fyuA*, *iroN*, *traT*, *ompT*, *malX/7*	D	P9
C6912	II	NAL, CIP, TET	$bla_{CTX-M-15}$, bla_{TEM-1}	*fimH*, *sat*, *fyuA*, *iutA*,*iroN*, *traT*, *ompT*, *malX/8*	D	P9
C6914	II	NAL, CIP, SXT, TET, MNO, CHL	$bla_{CTX-M-15}$, bla_{TEM-1}	*fimH*, *traT*, *malX/3*	D	P9
C6917	II	NAL, CIP, SXT, TET, MNO, CHL	$bla_{CTX-M-15}$, bla_{TEM-1}	*fimH*, *iroN*, *traT*, *ompT*, *malX/5*	D	P9
C6919	II	NAL, CIP, SXT, TET, MNO, CHL	$bla_{CTX-M-15}$	*fimH*, *iroN*, *traT*, *ompT*, *malX/5*	D	P9
C6927	III	NAL, CIP, SXT, TET, MNO	$bla_{CTX-M-1}$	*fimH*, *iroN*, *traT/3*	B1	P10
C6928	III	NAL, TET, MNO	$bla_{CTX-M-1}$, bla_{TEM-1}	*fimH*, *traT*, *ompT/3*	B1	P11
C6929	III	NAL, TET, MNO	$bla_{CTX-M-1}$	*fimH*, *ompT/2*	B1	P11
C6930	III	NAL, TET, MNO	$bla_{CTX-M-1}$	*fimH*, *ompT/2*	B1	P11
C6931	III	NAL, TET	$bla_{CTX-M-1}$	*fimH*, *ompT/2*	B1	P11
C6933	III	NAL, TET	$bla_{CTX-M-1}$	*fimH*, *ompT/2*	B1	P11
C6923	III	NAL, TET, MNO	$bla_{CTX-M-1}$	*fimH*, *iroN*, *ompT/3*	B1	P11
C6925	III	NAL, TET, MNO	$bla_{CTX-M-1}$	*fimH*, *ompT/2*	B1	P11
C6924[c]	III	NAL, SXT, TET	$bla_{CTX-M-1}$, bla_{TEM-1}	*fimH*, *fyuA*, *iutA*, *iroN*, *kpsM II*, *ompT/6*	A	P12
C6932[c]	III	NAL, SXT, TET	$bla_{CTX-M-1}$, bla_{TEM-1}	*fimH*, *iutA*, *iroN*, *kpsM II*, *ompT/5*	A	P13
C6934[c]	III	NAL, SXT, TET, MNO	$bla_{CTX-M-1}$	*papGII*, *fimH*, *iutA*, *iroN*, *kpsM II/5*	A	P13
C6935	III	NAL, CIP, SXT, TET	$bla_{CTX-M-1}$	*papGII*, *fimH*, *iutA*, *iroN*, *traT*, *ompT/6*	D	P14
C6926	III	NAL, TET, MNO	$bla_{CTX-M-14}$	*papGII*, *fimH*, *iroN*, *iha*, *traT/5*	B1	P15
C6938	III	NAL, CIP, SXT, TET, MNO, AMK, CHL	bla_{CMY-2}	*Sfa/focDE*, *fimH*,*iutA*, *traT*, *ompT*, *usp/6*	D	P16
C6942	III	NAL, CIP, SXT, TOB, TET, MNO, CHL	bla_{CMY-2}	*fimH*,*hlyA*, *iutA*,*iha*, *tratT*, *ompT*, *usp/7*	D	P16
C6943	III	NAL, CIP, SXT, TET, MNO	bla_{CMY-2}	*fimH*, *fyuA*, *iutA*, *iroN*, *traT*, *ompT*, *usp/7*	B1	P17
C6945	III	NAL, CIP, SXT, TET, MNO, AMK, CHL	bla_{CMY-2}	*fimH*, *fyuA*, *iutA*, *iroN*, *iha*, *traT*, *ompT*, *usp/8*	B1	P18
C6939	III	NAL, CIP, SXT, TET, MNO, CHL	bla_{CMY-2}	*Sfa/focDE*, *fimH*,*iroN*,*iha*, *ompT*, *usp/6*	A	P19
C6940	III	NAL, CIP, SXT, TOB, TET, MNO, GEN, AMK, CHL	bla_{CMY-2}	*fimH*, *iroN*, *traT*, *ompT/4*	A	P19
C6941	III	NAL, CIP, SXT, TET, MNO, GEN, TOB, AMK, CHL	bla_{CMY-2}	*fimH*, *iha*, *traT*, *ompT*, *usp/5*	A	P19
C6944	III	NAL, CIP, SXT, TET, MNO, TOB, CHL	bla_{CMY-2}	*fimH*, *traT*, *ompT*, *usp/4*	A	P19
C6936	III	NAL, CIP, SXT, TET, MNO, GEN, TOB, AMK, CHL	bla_{CMY-2}	*Sfa/focDE*, *fimH*, *ompT*, *usp/4*	A	P19
C6937	III	NAL, CIP, SXT, TET, MNO, TOB, AMK, CHL	bla_{CMY-2}	*Sfa/focDE*, *fimH*, *traT*, *ompT*, *usp/5*	A	P19
C6946	III	NAL, CIP, SXT, TET, MNO, GEN, TOB, CHL	bla_{CMY-2}	*fimH*, *ompT*, *usp/3*	A	P19
C6947	III	NAL, CIP, SXT, TET, MNO, GEN, TOB, CHL	bla_{CMY-2}	*fimH*, *ompT*, *usp/3*	A	P19
C6948	III	NAL, CIP, SXT, TET, MNO, GEN, TOB, AMK, CHL	bla_{CMY-2}	*fimH*, *traT*, *ompT*, *usp/4*	A	P19

[a] NAL: nalidixic acid, CIP: ciprofloxacin, SXT: trimethoprim-sulfamethoxazole; TET: tetracycline, MNO: minocyclin; GEN: gentamicin; TOB: tobramycin, AMK: amikacin; CHL: chloramphenicol.
[b] Virulence score was calculated as the sum of all virulence genes for which the isolates tested positive.
[c] Strains qualified as ExPEC (extra-intestinal pathogenic *E. coli*, (defined as the presence of ≥2 of the following 5 virulence genes, *papA and/or papC*, *sfa/focDE*, *afa/draBC*, *iutA*, and *kpsM II*)).

($P = 0.016$), and the majority of CMY-producing isolates (9/13 isolates) were ascribed to phylogenetic group A (Table 1).

PFGE analysis demonstrated 19 unrelated patterns among the 48 cefotaxime-resistant isolates: 15 different profiles among ESBL-positive isolates and 4 among the CMY-2-positive isolates (Fig. 1). More than one cefotaxime-resistant isolate (2–9 isolates) were detected in 10 PFGE profiles, and the remaining 9 PFGE profiles were detected in unique isolates. All CTX-M-15-producing strains belonged to the same PFGE pattern and 9 of the 13 CMY-2-producing ones were clonally related. However, the 29 CTX-M-1-producing strains were assessed onto 13 different PFGE profiles (Table 1).

The presence of virulence genes was analyzed in the whole collection of 48 cefotaxime-resistant strains. Of the 18 VFs sought, *fimH* virulence gene was detected in 47 strains (except in C6901), *iroN* in 30 and *iutA* in 18 strains. Overall, virulence scores were in the range of 1 to 8 (median, 5). *E. coli* strain C6945, typed as ST155-B1 (CMY-2), contained 8 VFs (*fimH*, *fyuA*, *iutA*, *iroN*, *iha*, *traT*, *ompT* and *usp*). Nevertheless, *papGI*, *papGIII* or *afa*/*draBC* virulence genes were not detected in any strain (Table 1). Compared with ESBL-producing isolates, CMY-2-producing isolates were found to be enriched for *usp* virulence gene ($P = 4 \times 10^{-8}$), while no statistical difference was observed in the incidence of other virulence genes. Nonetheless, no statistical difference was observed between the mean virulence score of the ESBL- and CMY-2-producing isolates. High virulence scores were significantly associated with phylogenetic group D ($P = 0.004$). ExPEC status analysis of the isolates showed that 3/35 (8.57%) ESBL-producing isolates were qualified as ExPEC and were typed as ST93-A (CTX-M-1).

3.3. Characterization of PFGE-unrelated *E. coli* isolates producers of ESBL and pAmpC-BL

One isolate corresponding to each of the 19 unrelated PFGE patterns were selected for complete characterization and results are included in Table 2.

Sequence types and associated phylogenetic groups detected among these 19 strains were the following ones: a) CTX-M-1-positive strains: lineages ST542-B1, ST212-B1, ST58-B1, ST4968-B1, ST1431-B1, ST1196-B1, ST93-A, ST350-D, ST155-B1 and ST349-D; b) CTX-M-15-positive strain: lineage ST405-D; c) CTX-M-14-positive strain: lineage ST1056-B1; d) CMY-2-positive strains: lineages ST117-D, ST2197-A, and ST155-B1. Strains showing P1 and P4 PFGE patterns could not be associated with any ST registered, to date, in the Warwick MLST Database. In both cases, point mutations in different alleles were identified by Sanger sequencing.

The genetic environment of the $bla_{\text{CTX-M}}$ gene was analyzed in the strains showing this gene. The IS*Ecp1* insertion sequence was found upstream of $bla_{\text{CTX-M-1}}$ (in 11 of 13 strains), $bla_{\text{CTX-M-15}}$ (in strain C6917) and $bla_{\text{CTX-M-14}}$ genes (in strain C6926). In strain C6924, harboring $bla_{\text{CTX-M-1}}$, IS*Ecp1* was truncated with IS5. The *orf477* sequence was detected downstream of $bla_{\text{CTX-M-1}}$ (in 12 of the 13 selected strains) and $bla_{\text{CTX-M-15}}$ (in strain C6917). IS*903* sequence was found downstream of $bla_{\text{CTX-M-14}}$ in strain C6926 (Table 2).

Over the 19 selected strains, 14 harbored the *int1* gene (associated to *int2* in three strains) and analysis of their variable region (VR) detected the following gene cassette arrangements: *aadA2* (one strain), *dfrA14*

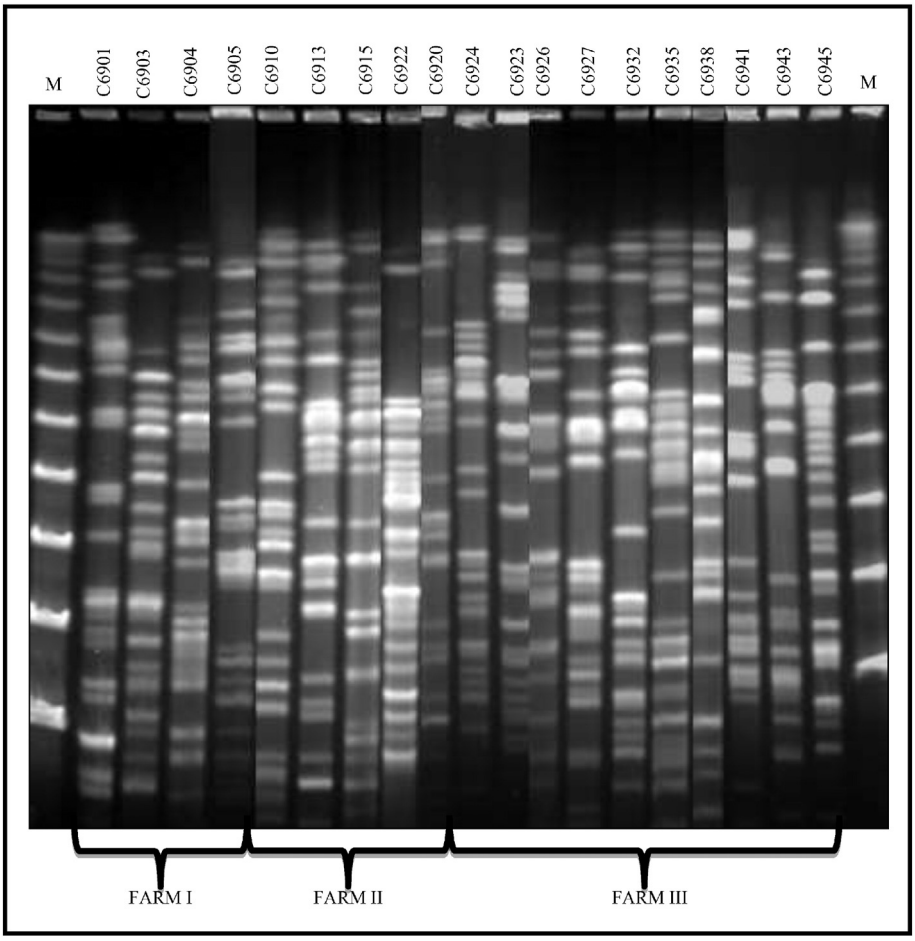

Fig. 1. *Xba*I-PFGE profiles of 19 PFGE-unrelated cefotaxime-resistant *E. coli* isolates recovered from fecal samples of healthy chickens in the three poultry farms (I-III). Lanes M, Lambda Ladder standard.

Table 2
Molecular characteristics of 19 PFGE-unrelated cefotaxime-resistant *E. coli* isolated from fecal samples of healthy chickens in the three industrial farms (I-III).

Strain/Farm	ST	PFGE pattern	β-lactamases genes	Genetic environment Of bla_{CTX-M}	Integron types (structure)	Associated resistance genes	Plasmid replicons
C6901/I	NR	P1	$bla_{CTX-M-1}$	IS*Ecp1*-*orf477*	*int2* (*dfrA12-sat2-aadA1*)	*tetA*	K, I1, FIB, F
C6903/I	ST542	P2	$bla_{CTX-M-1}$	IS*Ecp1*-*orf477*	*int1*	*tetA*, *dfrA1*	I1, N, FIB, F
C6905/I	NR	P3	$bla_{CTX-M-1}$	IS*Ecp1*-*orf477*	*int1*	*tetB*	I1
C6904/I	ST349	P4	$bla_{CTX-M-1}$	IS*Ecp1*-*orf477*	*int1*	–	I1
C6913/II	ST4968	P5	$bla_{CTX-M-1}$	IS*Ecp1*-*orf477*	–	*tetA*	I1, FIB, F
C6915/II	ST1431	P6	$bla_{CTX-M-1}$	IS*Ecp1*-*orf477*	–	*tetA*	I1, N, FIB, F
C6922/II	ST1196	P7	$bla_{CTX-M-1}$	Unknown-*Orf477*	*int1* (*dfrA1-aadA1*)	*tetA*, *sul3*	K, Y, I1, FIB
C6920/II	ST212	P8	$bla_{CTX-M-1}$	IS*Ecp1*-*orf477*	*int1*	*tetA*, *dfrA1*, *sul1*	I1, FIB, F
C6917/II	ST405	P9	$bla_{CTX-M-15}$, bla_{TEM-1}	IS*Ecp1*-*orf477*	*int1*	*tetA*, *tetB*, *dfrA1*	N, FIB, F
C6927/III	ST155	P10	$bla_{CTX-M-1}$	IS*Ecp1*-*orf477*	*int1* (*dfrA1-aadA1*)	*tetA*, *sul3*	Y, I1, F
C6923/III	ST58	P11	$bla_{CTX-M-1}$	IS*Ecp1*-*orf477*	–	*tetA*	K, I1, FIB, F
C6924/III	ST93	P12	$bla_{CTX-M-1}$, bla_{TEM-1}	IS*Ecp1*-**IS5**-*orf477*	*int1*/*int2* (*dfrA12-sat2-aadA1*)	*tetA*	P, I1, FIB, F
C6932/III	ST93	P13	$bla_{CTX-M-1}$, bla_{TEM-1}	Unknown-*orf477*	*int1*/*int2* (*dfrA12-sat2-aadA1*)	*tetA*	P, I1, FIB, F
C6935/III	ST350	P14	$bla_{CTX-M-1}$	IS*Ecp1*-*unknown*	*int1* (*dfrA1-aadA1*)/*int2* (*dfrA12-sat2-aadA1*)	*tetA*, *tetB*, *sul1*	K, FIB, F
C6926/III	ST1056	P15	$bla_{CTX-M-14}$	IS*Ecp1*-IS*903*	–	*tetA*	F
C6938/III	ST117	P16	bla_{CMY-2}	ND	*int1* (*dfrA14-aadA1*)	*tetA*, *sul1*	I1, F
C6943/III	ST155	P17	bla_{CMY-2}	ND	*int1* (*aadA2*)	*tetA*, *dfrA1*	F
C6945/III	ST155	P18	bla_{CMY-2}	ND	*int1*	*tetA*, *dfrA1*	FIB, FIA, F
C6936/III	ST2197	P19	bla_{CMY-2}	ND	*int1*	*tetB*, *dfrA1*	I1

NR: Not registered in the MLST database; ND: not done; ST: sequence type

plus *aadA1* (one strain), and *dfrA1* plus *aadA1* (three strains). Four strains harbored the *int2* gene and the *dfrA12* plus *sat2* plus *aadA1* gene cassettes were detected in all of them. In four strains, neither *int1* nor *int2* gene was detected (Table 2).

Different genes were detected among the antibiotic resistant strains [antibiotic (number of resistant strains)/gene (number of strains)]: tetracycline (19)/*tetA*(16)/*tetB*(4); and trimethoprim-sulfamethoxazole (13)/*sul1* (3)/*sul3* (2).

The IncI1, IncF, IncFIB, IncFIA, IncK, IncY, IncP and IncN replicons were detected among tested *E. coli* strains, and different replicon combinations were identified. The IncI1, IncF and IncFIB replicons were detected combined in eight $bla_{CTX-M-1}$-positive *E. coli* strains (Table 2).

4. Discussion

Antibiotics had been widely used in healthy animals for growth promotion, practice now banned in the European Union and in other countries, but still active in others (Millet and Maertens, 2011). In Tunisia, this restriction on growth promoters exists; although it is not enforced. Under the pressure of antibiotic selectivity, drug-resistant bacteria emerge and disseminate in healthy animals and can spread to humans through consumption of contaminated food, from direct contact with animals, or by environmental spread (Ewers et al., 2007; Horton et al., 2011; Mellata, 2013). The genes coding for antimicrobial resistance can be transferred from microbes carried by animals to microbes that cause disease in humans (Johnson et al., 2012).

In the present study, in all three industrial farms, animals (chickens) were colonized by cefotaxime-resistant *E. coli* isolates. The rate of fecal carriage of these multidrug resistant bacteria in healthy chickens varied from one farm to another (20.9% to 72.2%), which is consistent with global data (Girlich et al., 2007; Reich et al., 2013), including those of Tunisia (42% and 45.5%) (Ben Sallem et al., 2012; Mnif et al., 2012). These results confirm that chicken farms constitute a reservoir of cefotaxime-resistant *E. coli* isolates, which might reflect a high antibiotic pressure for selection of resistant bacteria in this ecosystem (Mellata, 2013).

CTX-M-1 was detected in all three farms; however CTX-M-15 and CMY-2 were confined to farms II and III respectively. In fact, CTX-M-1 enzyme is the most dominant ESBL type in *E. coli* isolates among intestinal microbiota of healthy chickens worldwide (Meunier et al., 2006; Girlich et al., 2007; Ben Sallem et al., 2012; Grami et al., 2013). However, CTX-M-15 enzyme, pandemic in human clinical *E. coli*, appears to be an emerging variant β-lactamase in fecal carriage of healthy animals (Ben Slama et al., 2011; Mnif et al., 2012). CTX-M-14-producing *E. coli*, previously isolated in meat chicken in Tunisia (Jouini et al., 2007), was found in one specimen in farm III. As previously reported, CMY-2 is the most dominant pAmpC-BL in *E. coli* isolates from healthy animals, humans, food samples and companion animals (Brinas et al., 2003; Carattoli et al., 2005b; Ben Slama et al., 2010; Ben Sallem et al., 2012). The high rate occurrence of *E. coli* harboring $bla_{CTX-M-1}$ and bla_{CMY-2} in poultry and their food products can contribute to the transmission of these genes to humans.

In this study, different clones of *E. coli* carried the gene encoding CTX-M-1 in the three tested farms. The CTX-M-15-producing *E. coli* strains were clonally related and typed as ST405-D, being this ESBL-positive clone previously detected in human infections (Ben Slama et al., 2011; Matsumura, et al., 2012). At least three *E. coli* clones carried the gene bla_{CMY-2}, highlighting the detection of lineages ST117 and ST155, previously detected in isolates of animal, human or vegetable origins (Ben Sallem et al., 2014; Ben Said et al., 2015). It seems that clones and genetic variants of ESBLs and pAmpC-BL are not restricted to a unique ecosystem but a continuous flow is occurring. Thus the dissemination of these enzymes is related with the dissemination of plasmids containing their genes. As previously showed, we found that both IncI1 and IncF were the most prevalent plasmid replicon types detected in cefotaxime-resistant *E. coli* strains (Carattoli, 2009; Ben Sallem et al., 2014).

The IS*Ecp1* detected upstream of most of $bla_{CTX-M-1}$, $bla_{CTX-M-15}$ and $bla_{CTX-M-14}$ genes in this study seems to be important for mobilization of ESBL gene (Eckert et al., 2006). The IS5 disruption of IS*Ecp1* in one $bla_{CTX-M-1}$ harboring strain of our study, was previously detected in $bla_{CTX-M-1}$ strains of healthy pets also in Tunisia (Sallem et al., 2013); this disruption might affect the mobilization and/or expression of the gene, fact that should be corroborated in the future. Class 1 integrons were predominant in our study in ESBL-positive isolates in relation to class 2 integrons, what is in agreement to previous studies (Machado et al., 2005; Soufi et al., 2009; Ben Slama et al., 2010; Cergole-Novella et al., 2010; Soufi et al., 2011).

Concerning the distribution of pathogenic *E. coli* strains according to phylogroups, in human it is known that pathogenic strains producing extra-intestinal infections (ExPEC) belong more frequently to the B2 group in relation to other groups. They can be implicated in meningitis, abscess, peritonitis, septicemia and urinary tract infections in humans, among others (Nandanwar et al., 2014); while, groups A and B1 *E. coli* strains are more frequently commensal strains (Ewers et al., 2007). In the present study, the majority of cefotaxime-resistant *E. coli* strains

were found to belong to B1 phylogenetic group (n = 21), followed by A and D (15 and 12, respectively). These results are in agreement with those reported in the literature (Mnif et al., 2012; Huber et al., 2013). Extra-intestinal pathogenic *E. coli* (ExPEC) are part of the intestinal microbiota of a fraction of the healthy population and normally asymptomatically colonize the gut. Once they get access to niches outside of the gut they are however able to efficiently colonize these niches and cause diseases in humans or in many animals (Kohler and Dobrindt, 2011). In fact, several investigations have shown that ExPEC strains may encode widespread VFs that are closely related to colonization, persistence, and pathogenesis of bacteria (Johnson and Stell, 2000). The most important of these factors include adhesins or fimbriae, toxins and iron-acquisition systems (Johnson and Stell, 2000). These, may have been acquired by plasmids or pathogenicity-associated islands (Johnson et al., 2001). In our strain collection, the median VF score is 5. *fimH*, encoding for *fimH* adhesin known for its important role in intestinal colonization and initiation of infection, was the most frequently virulence gene detected.

5. Conclusion

Chickens have become an important reservoir of cefotaxime-resistant *E. coli* isolates which contain virulence determinants, in Tunisia. Our study reports the dissemination of various *E. coli* clones producing CTX-M-1, several CMY-2-*E. coli* clones as well as one *E. coli*-ST405 clone with CTX-M-15 at industrial poultry farms in Tunisia. Detailed molecular comparison of plasmids and genomes of isolates from various sources will help to better define the transmission dynamics of ESBLs between humans and animals.

Conflicts of interest

No competing financial interests exist.

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