International Journal of Food Microbiology 231 (2016) 69–75

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

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

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ARTICLE INFO

Article history:
Received 16 December 2015
Received in revised form 29 April 2016
Accepted 2 May 2016
Available online 3 May 2016

Keywords:
Fecal carriage
CMY-2
CTX-M-1
Plasmids

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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http://dx.doi.org/10.1016/j.ijfoodmicro.2016.05.001
0168-1605/

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