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Community fecal carriage of broad-spectrum cephalosporin-resistant *Escherichia coli* in Tunisian children

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ABSTRACT

The spread of extended spectrum β-lactamases (ESBL) and plasmid mediated AmpC β-lactamases (pAmpC) was evaluated in *Escherichia coli* strains collected from the intestinal microbiota of healthy children in Tunisia. The carriage rate of CTX^R *E. coli* was 6.6% (7 of 105 samples) and one strain/sample was further characterized (7 isolates). These isolates harbored $bla_{CTX\text{-}M\text{-}1}$ (n = 4), $bla_{CTX\text{-}M\text{-}15}$ (n = 2), and $bla_{CMY\text{-}2}$ gene (n = 1), which were usually located on FIB replicon type and carried class 1 integrons. The *acc(6′)-Ib-cr* variant was identified in one isolate that harbored $bla_{CTX\text{-}M\text{-}15}$. CTX^R *E. coli* isolates were genetically unrelated and belonged to B1 (n = 3/ST155/ST398/ST58), D (n = 2/ST117/ST493), B2 (n = 1/ST127), and A (n = 1/ST746) phylogroups. Strain virulence scores varied from 3 to 12, and frequently harbored the pathogenicity island PAI IV_{536}.
The intestinal tract of healthy children constitute an important reservoir of ESBL producing *E. coli*. Thus, improvement of hygiene measures mainly in the school environment and rational use of antibiotics would be of great help in preventing selection and diffusion of resistant strains from intestinal microbiota.

1. Introduction

Escherichia coli is typically a commensal bacterium of humans and animals. It can be easily disseminated in different ecosystems through the food chain and water supply where it has been widely used as an indicator of fecal contamination (Van Den Bogaard and Stobberingh, 2000). In humans, *E. coli* can cause a variety of intestinal and extra-intestinal infections, such as diarrhea, urinary tract infection, meningitis, peritonitis, septicemia, pneumonia etc (Russo and Johnson, 2000).

Over the last 2 decades, the emergence and diffusion of multidrug resistant *E. coli* isolates, mainly those producing extended spectrum β-lactamases (ESBLs), have been reported worldwide. These isolates are clinically relevant and remain an important cause for failure of therapy with broad-spectrum antimicrobial agents (Pitout and Laupland, 2008).

The fecal microbiota represents a potential reservoir of antimicrobial resistance acquisition, as well as the site where resistance genes can be transferred from the commensal bacteria to virulent microorganisms (Marshall et al, 2009). Thus, a better knowledge of the frequency of antibiotic-resistant bacteria carried by healthy individuals in the community is needed. Several studies have reported that children tend to be the most exposed to antibiotics and consequently, have the highest risk of carrying resistant commensal bacteria (James Dyar et al, 2012). This study was conducted to evaluate the intestinal carriage of *E. coli* resistant to third generation cephalosporins (3rd GC) among healthy schoolchildren and to determine their resistance encoding genes, phylogenetic groups, and virulence contents.

2. Materials and methods

2.1. Sample collection

This study was conducted during 2012–2013 school year in three elementary schools of different regions of Manouba governorate, located in the North of Tunisia. After explanation of the purpose and methods of the study, a written permission to perform the sampling was issued from the national Ministry of Education as well as from the directors of schools. Healthy children represented the study population. Their parents were asked for verbal consent to participate to the study and fill out a questionnaire on demographic circumstances: age, gender, and origin (urban/rural). Criteria of exclusion were previous hospitalization, diarrhea, and antibiotics use during the six months prior to the sample collection. One hundred and five voluntary students have

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participated in the study and one rectal swab was obtained from each one of them.

2.2. Sample processing and microbial study

Rectal swabs were directly inoculated on desoxycholate lactose agar plates supplemented with cefotaxime (2 μg/ml), as selective screening agar for the isolation of Gram negative bacilli resistant to 3rd GC. After incubation at 37 °C for 18 to 24 hours, growing colonies showing *E. coli* morphology were identified by classical biochemical methods. One cefotaxime resistant (CTX^R) *E. coli* isolate per positive sample was further characterized.

Antimicrobial susceptibility testing was determined using standard disk diffusion method on Mueller Hinton agar according to Clinical and Laboratory Standard Institute guidelines (CLSI, 2011). Extended-spectrum β-lactamase (ESBLs) production was detected by the double-disk synergy-test (DDST) with or without cloxacillin (200 μg/ml) (Drieux et al, 2008).

2.3. β-Lactamase genes detection

Isolates showing positive DDST were screened for $bla_{CTX\text{-}M}$, bla_{SHV}, and bla_{TEM} genes, while those with AmpC phenotype, inactivated by cloxacillin, and characterized by a lack of synergy between third-generation cephalosporins (and/or aztreonam) and clavulanic acid, were screened for plasmid-mediated cephalosporinase genes (bla_{MOX}, bla_{CIT}, bla_{DHA}, bla_{ACC}, bla_{EBC}, and bla_{FOX}), and for the mutations in the promoter region of the chromosomal *ampC* gene by PCR and sequencing (Branger et al, 2005; Cooke et al, 2010; Pérez-Pérez and Hanson, 2002).

2.4. Quinolone resistance characterization

The quinolone resistance determining regions (QRDRs) of *gyrA* and *parC* genes and the following plasmid-mediated quinolone resistance (PMQR) genes (*qnrA*, *qnrB*, *qnrS*, *qnrC*, *qnrD*, *aac (6')-Ib-cr*, *qepA*, and *oqxAB*) detections were performed by PCR and sequencing (Bin Kim et al, 2009; Cattoir et al, 2007; Cavaco et al, 2009; Everett et al, 1996; Jones et al, 2008; Wang et al, 2009; Yamane et al, 2007).

2.5. Integron classes and sul genes characterization

intI1, *intI2,* and *intI3* genes, encoding class 1, class 2 and class 3 integrases, respectively, and sulfonamides resistant genes (*sul1*, *sul2,* and *sul3*) were detected by PCRs. The variable regions of these integrons were further characterized by PCR and sequencing. The presence of *qaqEΔ1-sul1* genes in the conserved region of class1 integrons was also screened in all *intI1* positive isolates (Mazel et al, 2000; Saenz et al., 2004)

2.6. Resistance transfer and plasmid analysis

β-lactam resistance transferability was assayed by conjugation using *E. coli* J53 (azide resistant) as recipient. Transconjugants were selected on Mueller-Hinton agar plates containing azide (100 μg/ml) and cefotaxime (2 μg/ml). Plasmid incompatibility groups were determined by PCR-based replicon typing for the isolates and their transconjugants as previously described (Carattoli et al, 2005).

2.7. Molecular typing of isolates

Phylogenetic groups of *E. coli* isolates were identified by PCR as previously described (Clermont et al, 2000). Clonal relatedness was investigated by pulsed field gel electrophoresis (PFGE), and multilocus sequence typing (MLST) (Saenz et al, 2004; Tartof et al, 2005).

2.8. Virulence factors content and pathogenicity island markers:

Isolates were screened for the presence of 15 virulence genes (*fimH*, *afa*, *sfa/foc*, *papG* (allele I, II, and III), *cnf1*, *sat*, *hlyA*, *iutA*, *iroN*, *fyuA*, *iha*, *kpsMTII*, *ompT*, *traT*, and *usp*) and 8 pathogenicity islands (PAIs) (PAI I536, PAI II536, PAI III536, PAI IV536;PAI ICFT073, PAI IICFT073 PAI IJ96, and PAI IIJ96), using multiplex PCRs (Johnson and Stell, 2000; Ruiz et al, 2002; Sabate et al, 2006; Takahashi et al, 2006).

3. Results

The 105 participants in the study were aged between 6 and 12 years, with a median age of 11 years. The sex-ratio was 0.9 (50 males and 55 females). They were from urban (n = 46) and rural areas (n = 59).

Over the 105 enrolled rectal samples, seven (6.6%) yielded positive growth of CTX^R *E. coli*. All of them were recovered from children of urban area (15.2%; 7/46). Six strains were ESBL producers and one showed AmpC phenotype. Associated resistances were as follows: gentamicin (n = 5), tobramycin (n = 5), nalidixic acid (n = 3), ciprofloxacin (n = 2), tetracycline (n = 3), chloramphenicol (n = 1), and trimethoprim-sulfamethoxazole (n = 4). No resistance to colistin or to carbapenems was detected.

ESBL genes were identified as $bla_{CTX\text{-}M\text{-}1}$ (4 isolates) and $bla_{CTX\text{-}M\text{-}15}$ (2 isolates), and all these isolates also carried the $bla_{TEM\text{-}1}$ gene. The strain with AmpC phenotype harbored the $bla_{CMY\text{-}2}$ gene, and no mutation in the chromosomal promoter region of *amp*C gene was detected.

Mutations in *gyrA* and *parC* genes were identified in the three nalidixic acid resistant *E. coli* strains. Three amino acid changes in GyrA (S83L + D87N) and ParC proteins (S80I) were identified in two strains and a single amino acid change in GyrA (S83L) was found in the remaining strain (Table 1). A PMQR was detected in only one isolate [*acc(6')-Ib-cr* variant], which harbored the $bla_{CTX\text{-}M\text{-}15}$ gene.

The *sul1* and *sul2* genes were detected in one single isolate each, and were associated in three isolates. Class 1 integrons were detected in three $bla_{CTX\text{-}M\text{-}1}$-positive isolates and all of them lacked the conserved *qacEΔ1-sul1* segment (Table 1). Class 2 integron was detected in the $bla_{CMY\text{-}2}$-positive strain. Sequence analysis of the class 2 integron variable regions revealed that the 1900 bp amplicon harbored the *dfrA12-sat2-aadA1* gene cassette encoding resistance to trimethoprim, streptothricin, and streptomycin, respectively. Integrase and *sul* genes were not detected among isolates harboring the $bla_{CTX\text{-}M\text{-}15}$ gene.

Several plasmid replicon types were identified among the 7 CTX^R *E. coli* isolates including type F (7 isolates), K (5 isolates), B/O (1 isolate), I1 (5 isolates), and FIB (5 isolates). Successful transfer of cefotaxime resistance was obtained for 4 strains, and transferred plasmids mainly belonging to IncFIB (Table 2).

PFGE analysis of the CTX^R *E. coli* isolates showed seven unrelated pulsotypes. CTX-M-1 producing isolates belonged mainly to B1 phylogroup (n = 3) (Table 1). The MLST analysis of CTX-M-1 producing strains showed that they belonged to unrelated clones: ST155, ST398, ST58, and ST746. The two CTX-M-15 isolates of phylogroups D and B2 were typed as ST493 and ST127, respectively. The CMY-2 producing isolate was ascribed to phylogroup D and typed as ST117.

Among the seven CTX^R isolates, the most frequent virulence genes detected were *fimH, traT, sat, ompT, fuyA, iutA,* and *iha*. Virulence scores varied from 3 to 12 and the median virulence score was 7 (Table 1). Five of the CTX^R isolates carried PAI markers and PAI IV_{536} was the most frequently found (n = 4). The two CTX-M-15 producing *E. coli* isolates harbored at least two PAIs.

4. Discussion

Few studies have evaluated the intestinal carriage of CTX^R *E. coli* in healthy individuals in the community, especially in children. Overall, using the same selection criteria, the rate of CTX^R *E. coli* found in our study (6.6%) was higher than those reported in other countries