Molecular characterization of *Escherichia coli* isolates from patients with urinary tract infections in Palestine

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Antibiotic resistance of *Escherichia coli* isolated from urinary tract infections (UTIs) is increasing worldwide. A total of 41 *E. coli* isolates were obtained from urine samples from hospitalized patients with a UTI in three hospitals in the northern districts of the West Bank, Palestine during March and June 2011. Resistance rates were: erythromycin (95%), trimethoprim–sulfamethoxazole (59%), ciprofloxacin (56%), gentamicin (27%), imipenem (22%), amoxicillin (93%), amoxicillin–clavulanic acid (32%), ceftazidine (66%) and ceftoxime (71%). No meropenem-resistant isolates were identified in this study. Among the isolates, phylogenetic group B2 was observed in 13 isolates, D in 12 isolates, A in 11 isolates and B1 in five isolates. Thirty-five of the isolates were positive for an extended-spectrum β-lactamase phenotype. Among these isolates, the *bla*<sub>CTX-M</sub> gene was detected in 25, and eight harboured the *bla*<sub>TEM</sub> gene. None of the isolates contained the *bla*<sub>SHV</sub> gene. Transformation experiments indicated that some of the β-lactamase genes (i.e. *bla*<sub>CTX-M</sub> and *bla*<sub>TEM</sub>) with co-resistance to erythromycin and gentamicin were plasmid encoded and transmissible. Apart from this, enterobacterial repetitive intergenic consensus-PCR (ERIC-PCR) revealed that the 41 isolates were genetically diverse and comprised a heterogeneous population with 11 ERIC-PCR profiles at a 60% similarity level.

INTRODUCTION

Urinary tract infections (UTIs) represent one of the most common diseases that are encountered in clinical practice and are caused mainly by *Escherichia coli* (Murugan *et al.*, 2012; Chakupurakal *et al.*, 2010; Karlowsky *et al.*, 2011; Baral *et al.*, 2012). Resistance to antibiotic treatment in patients with UTIs is a representative example of the increasing problem of antimicrobial resistance (Mukherjee *et al.*, 2013). The recent emergence and rapid worldwide dissemination of *E. coli* resistant to extended-spectrum cephalosporins due to the production of extended-spectrum β-lactamas (ESBLs) has clearly shown that *E. coli* antibiotic resistance is currently a real public health concern (Mukherjee *et al.*, 2013; Khanna *et al.*, 2011; Vaidya, 2011; Xiao *et al.*, 2011; Zhang *et al.*, 2012). To our knowledge, no epidemiological surveillance studies in Palestine have investigated the molecular nature of *E. coli* strains circulating in the healthcare settings. The objectives of our study were to describe trends in *E. coli* resistance to antibiotics and to molecularly characterize *E. coli* isolates circulating in Palestine. The results of the present study might be valuable to both health professionals and the scientific community, and may aid the current understanding of the situation in UTIs caused by *E. coli*.

METHODS

**Bacterial isolates.** A total of 41 non-repetitive *E. coli* isolates were obtained from urine samples from hospitalized patients with a UTI in three hospitals in the northern districts of the West Bank, Palestine during March and June 2011. The urine samples were inoculated onto MacConkey agar (Oxoid) and blood agar (Oxoid) and incubated at 37 °C overnight. Positive urine cultures were defined by the growth of a single colony morphotype of *E. coli* with counts ≥10⁵ c.f.u. ml⁻¹. *E. coli* was identified by standard laboratory methods involving morphological characteristics and biochemical tests including indole, methyl red, Voges–Proskauer and citrate (IMViC), triple sugar iron, urease, and nitrate reduction (Harley & Freston, 2002).

**Antimicrobial susceptibility testing.** The susceptibilities of the isolates to ten antibiotics were performed by the standard disc diffusion method as recommended by the Clinical and Laboratory

Abbreviations: ERIC, enterobacterial repetitive intergenic consensus; ESBL, extended-spectrum β-lactamase; UPGMA, unweighted pair group method for arithmetic averages; UTI, urinary tract infection.

The GenBank/EMBL/DDBJ accession numbers for three *bla*<sub>CTX-M</sub> gene sequences and one *bla*<sub>TEM</sub> gene sequence of *Escherichia coli* are KF696718, KF696719, KF696720 and KF696717, respectively.
**Fig. 1.** Dendrogram of *E. coli* isolates based on the UPGMA method derived from analysis of the ERIC-PCR-profiles. Phylogenetic group, antibiotic resistance profile and β-lactamase type are also shown. The roman numerals on the dendrogram are ERIC-PCR clusters. Shaded cells in column 1 show the multidrug-resistant isolates. Column 2 shows the distribution of the isolates among the three hospitals studied. Columns 4–13: r, resistant; s, sensitive. Columns 14–15 show the ESBL genes of each isolate tested, each column showing the results for a single ESBL gene: +, gene present; −, gene absent. The vertical line labelled ‘Cut-off point’ indicates 60% similarity level.
Standards Institute (CLSI, 2013). The antibiotic discs (Oxoid) used in the present study are shown in Fig. 1.

**Phenotypic and molecular identification of ESBL producers.** ESBL-producing isolates were screened by the confirmatory double disc combination test using ceftazidime (30 μg) and cefotaxime (30 μg) discs alone and in combination with clavulanic acid (10 μg) according to the CLSI (CLSI, 2013). Results were interpreted on the basis of the CLSI criteria (CLSI, 2013). Isolates confirmed as ESBL producers were examined by multiplex PCR for the presence of bla<sub>CTX-M</sub>, bla<sub>TEM</sub> and bla<sub>SHV</sub> genes as described previously (Akpaka, et al., 2010).

**Transfer of resistance determinants and plasmid analysis.** Plasmids were isolated according to the method of Takahashi & Nagano (1984). Plasmid DNA was separated on a 0.8 % agarose gel in 20 mM Tris/acetate, 0.5 mM EDTA for 1 h at 70 V. DNA digested with HinIII (Promega) was used as a DNA marker. Transformation of plasmids of six ESBL-producing isolates was carried out by electroporation using an EC100 electroporator (EC Apparatus Corporation) at 2800 V, with E. coli DH5α cells as the recipient strain. Transformants were selected on Luria–Bertani agar plates containing 100 μg ampicillin ml<sup>-1</sup> and 30 μg cefotaxime ml<sup>-1</sup> (Sigma Aldrich). Analyses of antibiotic resistance, plasmids and β-lactamase genes of the transformants were performed as described previously (CLSI, 2013; Akpaka, et al., 2010; Takahashi & Nagano, 1984).

**Cluster analysis by enterobacterial repetitive intergenic consensus-PCR (ERIC-PCR) assay.** ERIC-PCR profiles of DNA of the 41 isolates were examined and the size of bands was determined by comparing the migration distance of bands relative to the molecular mass of known size markers; a 100 bp DNA ladder and VisionWorksLS releases 7.0 software (Ultra-Violet Products) were used for this process. A binary matrix of band presence or absence was analysed by the unweighted pair group method for arithmetic averages (UPGMA), using SPSS Statistics version 20 (IBM). A cut-off of 60 % similarity was selected based on the mean of similarity matrix.

**DNA sequencing of selected bla<sub>CTX-M</sub> and bla<sub>TEM</sub>.** PCR amplifications of four β-lactamase genes (three bla<sub>CTX-M</sub> and one bla<sub>TEM</sub>) were sequenced by a dideoxy chain termination method on an ABI PRISM Sequence Instrument (model 3130; Hitachi) at Bethlehem University, Bethlehem, Palestine. Nucleotide sequence analysis and homology searches were carried out using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The nucleotide sequences of the three bla<sub>CTX-M</sub> genes from isolates 14, 32 and 33 and the bla<sub>TEM</sub> gene from isolate 14 reported here were submitted to GenBank.

**RESULTS AND DISCUSSION**

Antibiotic-resistant E. coli isolated from UTIs are increasingly found, and are a serious problem in many areas. Many strains are multiresistant (Ma & Wang, 2013; Al-Assil et al., 2013). In our study, 76 % of the isolates were noted to be multiply resistant, that is, resistant to three or more of the following antibiotics: β-lactam antibiotics, erythromycin, trimethoprim- sulfamethoxazole, ciprofloxacin and gentamicin. Resistance to individual antibiotics among the isolates was as follows: erythromycin (95 %), trimethoprim–sulfamethoxazole (59 %), ciprofloxacin (56 %), gentamicin (27 %), imipenem (22 %), amoxicillin (93 %), amoxicillin–clavulanic acid (32 %), ceftazidime (66 %) and cefotaxime (71 %). No meropenem-resistant isolates were identified (Fig. 1). The high level of resistance (>50 %) seen to erythromycin, trimethoprim–sulfamethoxazole, ciprofloxacin, amoxicillin, ceftazidime and cefotaxime is most likely due to selective pressure resulting from uncontrolled and inappropriate use of these agents in hospitals and in the country as a whole. This is promoted by the lack of an antibiotic policy and the availability of antibiotics sold over the counter in Palestine. The high rate of antimicrobial resistance has major therapeutic implications insofar as 76 % of our E. coli population were noted to be multiply resistant.

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**Fig. 2.** Agarose gel electrophoresis. (a) ERIC-PCR profiles of DNA from E. coli isolates. Lanes: M, molecular sizes marker (100 bp ladder DNA); 1, pattern I; 2, pattern II; 3, pattern III; 4, pattern IV; 5, pattern V; 6, pattern VI; 7, pattern VII; 8, pattern VIII; 9, pattern IX; 10, pattern X; 11, pattern XI. (b) Representative results of transformation. Lanes: M, molecular sizes marker (HindIII-digested λ DNA); 1, isolate 39; 2, transformant 34 kb (isolate 39); 3, isolate 14; 4, transformant 26 kb (isolate 14).
To our knowledge, up to now, no article has documented the molecular characterization of ESBL genes in the circulating *E. coli* population in Palestine. This characterization has a pivotal role in epidemiological studies, management of the outbreaks and implementing control and preventive measures (Wollheim *et al.*, 2011). In this study, β-lactamase genes were found in 25 of the 35 *E. coli* positive for an ESBL phenotype (Fig. 1). Ten isolates with resistance to cefotaxime and/or ceftazidime but with no ESBL gene were found; further studies are clearly needed for detection of other ESBL genes in these isolates. Among the 25 isolates, the most prevalent β-lactamase gene was *bla*<sub>CTX-M</sub>, which was detected in all of the isolates; the *bla*<sub>TEM</sub> gene was found in eight isolates. Eight of the 25 isolates carried both *bla*<sub>CTX-M</sub> and *bla*<sub>TEM</sub> genes. None of the isolates contained the *bla*<sub>SHV</sub> gene. The high predominance of the *bla*<sub>CTX-M</sub> gene has been reported among clinical isolates worldwide and is replacing *bla*<sub>TEM</sub> and *bla*<sub>SHV</sub> genes (Naseer & Sundsfjord, 2011; Livermore & Hawkey, 2005; Peirano & Pitout, 2010; Cantón *et al.*, 2008). The DNA sequencing results of the three *bla*<sub>CTX-M</sub> genes revealed 100% similarity to *bla*<sub>CTX-M-15</sub>, while the sequence of the *bla*<sub>TEM</sub> gene demonstrated *bla*<sub>TEM-1</sub> as the most closely related match.

Genotyping by the ERIC-PCR method showed different DNA banding profiles. A 60% similarity cut-off value analysis indicated that there were a total of 11 unique clusters of ERICs within the 41 *E. coli* isolates (Figs 1 and 2a). Only four clusters represented five or more isolates, while the remaining seven ERICs contained between one and three isolates each. These findings demonstrate that isolates included in our study were genetically diverse, a finding mirrored elsewhere (Leflon-Guibout *et al.*, 2004). This was expected as the isolates in this study were isolated from different hospitals. Although four distinct ERIC clusters were identified (Fig. 1), no direct correlation was observed between ERIC-PCR genotypes, phylogenetic groups and resistance gene determinants, suggesting that multiple subtypes of the species are involved in UTIs.

Phylogenetic grouping revealed that 61% of the isolates clustered mainly in phylogenetic groups B2 and D, which most often denote virulent extra-intestinal strains, 11 isolates belonged to group A and five to group B1 (Fig. 1). Additionally, it should be noted that the majority of the ESBL-producing isolates were derived from phylogenetic groups D, A and B2, as reported previously (Lee *et al.*, 2010; Pitout *et al.*, 2005).

The occurrence of the *bla*<sub>CTX-M</sub> gene along with resistance genes is a matter of concern. Unlike isolates carrying *bla*<sub>TEM</sub> and *bla*<sub>SHV</sub>, *bla*<sub>CTX-M</sub>-containing isolates present a complex situation and are associated with the spread of self-transmissible plasmids carrying transportable antibiotic resistance genes rather than clonal epidemics (Leflon-Guibout *et al.*, 2004; Lee *et al.*, 2010; Rakotonirina *et al.*, 2013; Mendonça *et al.*, 2007; Smet *et al.*, 2010). The results of this study indicate that 19 (76%) of the *bla*<sub>CTX-M</sub>-containing isolates were noted to be multiply resistant and

<table>
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<th>Isolate no.</th>
<th>Resistance profile of donor*</th>
<th>ESBL of donor</th>
<th>ERIC-PCR pattern</th>
<th>Plasmid donor (kb)</th>
<th>Plasmid transferred (kb)</th>
<th>Resistance profile of transformant</th>
<th>ESBL of transformant</th>
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<td>CTX-M, TEM</td>
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*ERM, erythromycin; SXT, trimethoprim-sulfamethoxazole; CIP, ciprofloxacin; GEN, gentamicin; AMX, amoxicillin; AMC, amoxicillin-clavulanic acid; CAZ, ceftazidime; CTX, cefotaxime.*
exhibited diverse ERIC genotypes, indicating that the predominant presence of bla<sub>CTX-M</sub> in the isolates included in our study was not due to the spread of a single <i>E. coli</i> clone. Thus, these data suggest the potential role of the plasmid horizontal transfer of β-lactamase-encoding genes and other antibiotic resistance genes in <i>E. coli</i> isolates in Palestine.

These findings were supported by the fact that successful transfer of β-lactamase carrying plasmids genes (in particular bla<sub>CTX-M</sub>) mediated resistance to β-lactam antibiotics (amoxicillin, amoxicillin–clavulanic acid, cefazidime and cefotaxime) along with gentamicin and erythromycin (Table 1). The size of the transferable plasmids varied from 22 to 38.7 kb (Fig. 2b). These results are in agreement with observations described previously (Leflon-Guibout et al., 2004; Rakotonirina et al., 2013), and emphasize the epidemic potential of multiple-antibiotic resistance mediated by bla-encoding genes, especially bla<sub>CTX-M</sub>, within and among the three study hospitals in Palestine.

In conclusion, this study has described the emergence of ESBLs among <i>E. coli</i> isolates obtained from hospitalized patients in Palestine. In addition, similar to the situation that is occurring in other countries, we demonstrated the spread of <i>E. coli</i>-producing ESBLs (particularly CTX-M), a finding that emphasizes the need for implementation of strict hospital infection control policies, including the review of current therapeutic modalities, control of the use of non-prescribed antibiotics and continuous monitoring of antibiotic sensitivity profiles of <i>E. coli</i> isolates.

REFERENCES


