Full Length Research Paper

Carbapenem resistance in Turkey: Repeat report on OXA-48 in Klebsiella pneumoniae and first report on IMP-1 beta-lactamase in Escherichia coli

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Meropenem resistant Klebsiella pneumoniae and Escherichia coli isolates were analyzed. A total of 18 non-duplicate meropenem resistant K. pneumoniae and one E. coli isolates from hospitalized patients were analyzed by polymerase chain reaction (PCR) and DNA sequencing to detect the OXA-48, IMP, VIM, and SPM-1 genes. Susceptibilities to antimicrobials were determined using an agar dilution method and E-test; beta-lactamase production was detected by double disk synergy test [Imipenem, imipenem/ethylenediaminetetraacetic acid (EDTA)] (DDST), E-test MBL and E-test ESBL. Homology of the isolates was determined by randomly amplified polymorphic DNA typing (RAPD). Minimum inhibitory concentrations (MICs) of imipenem and meropenem were 2 to 8 and 4 to > 512 mg/L for K. pneumoniae; 2 and 32 mg/L for E. coli, respectively. The isolates were resistant to all beta-lactams. The DDST was positive only for the E. coli. PCR and sequencing identified blaOXA-48 in the E. coli strain and blaOXA-48 in all K. pneumoniae isolates. RAPD-PCR indicated that spread of OXA-48 producing K. pneumoniae in the pediatric intensive care unit (ICU) from June, 2007 to December, 2007 was clonal. This is the first report of IMP-1-producing E. coli in Turkey, while OXA-48 carbapenemases in K. pneumoniae have been reported recently from our country. Carbapenem resistance may spread among Enterobacteriaceae via the transferable enzyme OXA-48, as well as metallo-beta-lactamases.

Key words: OXA-48, IMP-1, carbapenemase, intensive care unit (ICU).

INTRODUCTION

The Ambler class D OXA-48 β-lactamase has been found only in enterobacterial species (Klebsiella pneumoniae, Escherichia coli, Citrobacter freundii, Enterobacter cloacae and Providencia rettgeri) from Turkey (Poirel et al., 2004; Nazic et al., 2005; Aktas et al., 2008; Gulmez et al., 2008; Carrère et al., 2010). Up to now, carbapenemases of the OXA-48 type have seemed to be limited to Turkey but recently also been reported K. pneumoniae isolates from Europe (Cuzon et al., 2008; Carrère et al., 2010; O’Brien et al., 2011), Mediterranean countries such as Lebanon, Tunisia, Morocco and Egypt (Matar et al., 2010; Lahlaoui et al., 2011; Benouda et al., 2010), India (J.M. Bell, D. Mathai, R.N. Jones, J.D. Turnidge, Abstract C2-650, 49th ICAAC) and Argentina (M. Castanheira, A.A. Watters, J. Smayevsky, J.M. Casellas, D. Gür, S. Unal, R.N. Jones, Abstract C2-647, 49th ICAAC). This enzyme possessing significant carbapenemase activity, and blaOXA-48 gene was part of the Tn1999 composite transposon made of two copies of the insertion sequence IS1999 (Aubert et al., 2006). It was weakly related to class D beta-lactamases, with less

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than 46% amino acid (Poirel et al., 2004).

In the present study, we described the first report of IMP-1-producing *E. coli* in Turkey and an outbreak of nosocomial infection in a pediatric intensive care unit (PICU) caused by carbapenem nonsusceptible *K. pneumoniae* strains expressing OXA-48.

**MATERIALS AND METHODS**

**Bacterial strains**

Eighteen nonrepetitive meropenem-resistant *K. pneumoniae* isolates were collected from 12 patients hospitalized at our hospital, between July 2006 and November 2007. Strains were isolated from clinical samples (4 endotracheal aspirate, 3 blood culture, 3 urine, 3 catheter samples, 1 throat and 1 rectal swabs: 2 pus and 1 pleural fluid samples). Most of the patients were hospitalized in pediatric intensive care unit (PICU), and kidney transplantation unit. October and November 2007, 11 patients in PICU at our hospital, were infected or colonized by carbapenem resistant *K. pneumoniae*. *E. coli* isolate 191138 was isolated in January, 2007 at the hematology unit from the urinary tract of a 4-year-old pediatric patient with a urinary tract infection. The strain was found to be resistant to all beta-lactams, including meropenem.

**Antimicrobial susceptibility tests**

The MICs of antibiotics were determined using an agar dilution method and the E-test method (AB BIODISK, Solna, Sweden) and interpreted according to Clinical and Laboratory Standards Institute (CLSI) criteria. Beta-lactamase production were tested by double disk synergy test (DDST), E-test MBL [Imipenem, imipenem/ethylenediaminetetraacetic acid (EDTA)] and ESBL. For DDST; two imipenem disks were placed 20 mm (center to center) away from two blank disks to which 10 μl of 0.1 and 0.5 M EDTA were added (Franklin et al., 2006; Lee et al., 2001). After overnight incubation, enhancement of the inhibition zone in the area between the two disks was considered positive for an MBL. The antimicrobial agents tested were cefotaxime, ceftazidime, cefoxitin, imipenem, meropenem, piperacillin, piperacillin/tazobactam, cefoperazone/sublactam, amikacin, netilmicin and ciprofloxacin, respectively. Quality controls were *E. coli* ATCC 25922, *E. coli* ATCC 35218 and, *Pseudomonas aeruginosa* ATCC 27853, *K. pneumoniae* ATCC 700603.

**Isoelectric focusing (IEF)**

Analytical isoelectric focusing (IEF) was performed using a mini IEF cell system (Bio-Rad, Hercules, CA, USA) (Matthew et al., 1975). The pls of the β-lactamas were estimated from the pls of the previously known enzymes (pls TEM-1:5.4, TEM-8:5.8, SHV-3:7, CMY-1:8, and CMY-2: 9) and commercial pl markers (Bio-Rad).

**Bioassay**

Following IEF, imipenem hydrolyzing activities of the enzymes were investigated by a bioassay performed on the same gel (Bauernfeind et al., 1999).

**Plasmid analysis**

Plasmid DNA was isolated by the method of Kado and Liu and analyzed by agarose gel (Sigma-Aldrich Company, Poole, UK) electrophoresis (Kado and Liu, 1981). All samples were screened on gels containing 0.6% w/v agarose (Sigma) for 5 h at 90 V before staining for 30 min in distilled water containing 5 μg/ml of ethidium bromide; molecular weights were determined in relation with Mws ranging from 54.7 to 2 kb, carried in *E. coli* V517 and reference strain *K. pneumoniae* 11978 carried a 70 kb and a 140 kb plasmids.

**PCR amplification and DNA Sequencing**

DNA templates were prepared by boiling a bacterial suspension for 10 min. PCR amplification for *bla*onA48 was performed by using the following sets of primers: OXA-48A (5'-TTGGTGACGATGATTACGG-3') and OXA-48B (5'-GAGCAGCTTTTGTGATG-3') (743 bp), in a 50 μl volume containing 10XPCR buffer, 2 mM deoxynucleoside triphosphates, 3.4 pmol of each primer, 2.5 mM MgCl2, and 1 U Taq DNA polymerase, 1 μl of genomic DNA. Thermal cycler was used under the following conditions: initial denaturation at 94°C for 5 min; denaturation at 94°C for 60 s; annealing at 56°C for 45 s, and extension at 72°C for 60s, repeated for 35 cycles; final extension at 72°C for 7 min (Aktas et al., 2008).

PCR products of the *bla* genes obtained with oligonucleotides binding to flanking region of the genes (OXA-48-V-CGCTCCACCTAATTGG and OXA-48-R TACAAAGGCGATCGAGCAT; annealing temperature 52°C) were subjected to automated sequencing (ABI 3700; Applied Biosystems, Warrington, UK) using primers OXA-48-V and OXA-48-R (Aktas et al., 2008). The nucleotide sequences were analysed and multiple alignments were performed using the DNAMAN 4.1 Software (Lynnon BioSoft, USA).

To determine the ESBL and metallo-beta-lactamase types, five primer sets for the detection of the *bla*TEM, *bla*SHV, *bla*CTX-M, *bla*AMP, *bla*VIM and *bla*IMP genes were used in the amplification procedure (Gniadkowski et al., 1998; Poirel et al., 2000; Karim et al., 2001; Aktas and Kayacan, 2008). PCR products were separated in 1% agarose gels (1.5% for RAPD-PCR) stained with ethidium bromide and visualized under UV light. ΦX174 replicative-form DNA Haelll fragments were used to assess PCR product size (MBI Fermentas, Germany).

**Random amplification of polymorphic polymerase chain reaction (RAPD-PCR)**

ERIC-2 primer (5'-AAGTAAGTGACTGGGTTGCG-3') was used; initial denaturation at 94°C for 3 min; denaturation at 94°C for 60 s, annealing at 40°C for 60 s, and extension at 72°C for 2 min, repeated for 40 cycles; and a final extension at 72°C for 5 min (Aktas et al., 2008).

**RESULTS AND DISCUSSION**

The Enterobacteriaceae are among the most important causes of serious nosocomial and community-onset bacterial infections in humans and antimicrobial resistance has become a global threat to effective health care delivery (Pitout, 2008). Carbapenems are accepted as the drugs of choice for serious infections caused by ESBL producers.

A lot of number of carbapenemases has been identified in the last years, belonging to either acquired metallo-beta-lactamases (IMP, VIM, SPM, GIM, SIM, KHM, AIM, NDM and DIM types) or classes A (KPC and GES) and class D β-lactamase OXA-48 (Yong et al.,
2009; Kang et al., 2009; Manuel et al., 2010). These enzymes are associated with mobile genetic elements that allow their rapid dissemination in the clinical setting. In addition carbapenem resistance can arise in some organisms by over production of AmpC beta-lactamases associated with loss of outer membrane porins and/or overexpression of efflux pumps (Jacoby, 2009).

In Turkey there are multiple reports of OXA-48, a plasmid-mediated carbapenemase, in K. pneumoniae and, less often, E. coli (Carrër et al., 2008; Gulmez et al., 2008). The first outbreak of carbapenem-resistant K. pneumoniae isolates producing the plasmid-encoded carbapenem-hydrolyzing oxacillinase OXA-48 is reported in our hospital by Carrër et al., 2008. According to their results, the 39 isolates belonged to two different clones and were collected from May, 2006 to February, 2007. Most of the patients were hospitalized in intensive care units (ICUs) or for emergency surgery (Carrër et al., 2008). Our study identified the second outbreak of carbapenem-resistant K. pneumoniae isolates producing the carbapenem-hydrolyzing oxacillinase OXA-48 in PICU patients.

K. pneumoniae and E. coli isolates were found to be nonsusceptible to imipenem or meropenem in routine susceptibility disk diffusion tests. Minimum inhibitory concentrations (MICs) of imipenem and meropenem were 2-8 and 4->512 mg/L for K. pneumoniae; 2 and 32 mg/L for E. coli, respectively (Table 1). These isolates were resistant to all beta-lactams. Each also gave a positive ESBL disk synergy result. Imipenemase activity was not inhibited adequately by EDTA to imply the presence of MBL. The enzyme focusing at pl 7.2 hydrolyzed imipenem or meropenem in all strains. Analysis of plasmid extracts of K. pneumoniae isolates identified a 70-kb plasmid and a 140 kb plasmid. PCR indicated that all K. pneumoniae isolates harboured bla<sub>OXA-48</sub> like genes and, in each case, partial sequencing of a 743 bp PCR product indicated identity with the prototype bla<sub>OXA-48</sub> gene (GenBank accession no. AY236073), described from a K. pneumoniae isolate. They coproduced various beta-lactamases (SHV, TEM and group 1 CTX-M-type). RAPD-PCR indicated that spread of OXA-48 producing K. pneumoniae in the PICU from June to December 2007 was clonal.

The first reports of transferable MBLs reported IMP types at the end of the 1980s in Japan. Nowadays the most common MBL identified worldwide is VIM-2 (Walsh et al., 2005). But only VIM-1 (K. pneumoniae), VIM-2 (Pseudomonas aeruginosa), VIM-5, and IMP-1 (K. pneumoniae, E. cloacae and P. aeruginosa) MBLs have been identified in Turkey to date (Aktas et al., 2006; Yildirim et al., 2007). In our study, E. coli was found to be resistant to all beta-lactams, including meropenem, ertapenem and ciprofloxacin in routine susceptibility tests. A phenotypic double disk synergy test (DDST (imipenem, EDTA) was tentatively interpreted as positive for E. coli. PCR and sequencing identified bla<sub>IMP-1</sub> in this strain.

Three additional beta-lactamase genes (TEM-1, SHV-11 and CTX-M-15) were identified in the same isolate. MICs (mg/L) of antibiotics as follows: ceftazidime: 512, ceftazidime/ clavulanic acid: 32, cefotaxime: 1024, cefoxitin: >256, gentamicin: 64, cefoperazone/ sulbactam: >256, amikacin: 4, piperacillin/ tazobactam: 96, ciprofloxacin: 128, ertapenem: 16.

Carbapenem resistance constitutes a serious threat to the antibiotics available to deal with increasing resistance in Gram negative pathogens infecting neonates, infants, and compromised children with nosocomial infection caused by carbapenemase and ESBL producing bacteria. The emergence and spread of carbapenem-resistant K. pneumoniae is also becoming a serious problem in our hospital (Poirel et al., 2004; Aktas et al., 2008; Carrër et al., 2008). To date, risk factors for carbapenem resistant K. pneumoniae acquisition have not been determined. In PICU patients from whom the OXA-48 positive K. pneumoniae strains were isolated, the age distribution ranged from 2 months to 18 years, with a male to female ratio of 8/3. This phenotype had not been detected in the Pediatrics Hospital previously. All infections were nosocomially acquired, with the patients having been hospitalised from 4 to 292 days. Risk factors for acquisition included prolonged hospitalisation, an ICU stay, ventilator usage, central venous catheterisation, previous use of carbapenem antibiotics and the presence of underlying diseases. Seven of the patients were infected, and 3 of these died. All infected patients were treated with ciprofloxacin or netilmicin. Affected wards were cleaned daily appropriate disinfectant, universal contact precautions were applied and there were three times of education clinical staff in affected wards. Environmental screening was implemented once a week. Rectal swabs or stool specimens were collected from patients within 24 h of admission in PICU. The outbreak was controlled by a combination of intensive infection control measures and physical isolation of these patients.

Conclusion

This is the first report of IMP-1-producing E. coli in Turkey, while OXA-48 carbapenemases in K. pneumoniae have been reported recently from our country. The OXA-48 producers identified here were not clonally related to the previously identified OXA-48 positive K. pneumoniae isolate from our hospital belonging to the year 2001. This outbreak illustrates the possibility of the inter-hospital and the inter-country spread of carbapenem resistance among Enterobacteriaceae via the transferable enzyme OXA-48, as well as metallo-beta-lactamases.

ACKNOWLEDGEMENT

The present work was supported by the Research
### Table 1. Documentation of the OXA-48 positive *K. pneumoniae* outbreak strains.

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Source</th>
<th>Isolation date (day/mo/yr)</th>
<th>Hospitalization unit</th>
<th>Infected or colonized status</th>
<th>MIC (mg/L)</th>
<th>PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>CIP</td>
<td>IMP</td>
</tr>
<tr>
<td>P-1</td>
<td>Catheter</td>
<td>30.06.2007</td>
<td>PICU</td>
<td>C</td>
<td>R 4 8 4/4</td>
<td>+</td>
</tr>
<tr>
<td>P-2</td>
<td>Endotracheal aspirate</td>
<td>13.08.2007</td>
<td>PICU</td>
<td>I</td>
<td>S 2 32 2/1</td>
<td>+</td>
</tr>
<tr>
<td>P-3</td>
<td>Rectal swab</td>
<td>05.10.2007</td>
<td>PICU</td>
<td>C</td>
<td>S &gt;32 2/1</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>S 8 &gt;32 8/4</td>
<td>+</td>
</tr>
<tr>
<td>P-4</td>
<td>Rectal swab</td>
<td>30.10.2007</td>
<td>PICU</td>
<td>I</td>
<td>S 8 &gt;32 8/4</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>S 1.5 32 &lt;4/1</td>
<td>+</td>
</tr>
<tr>
<td>P-5</td>
<td>Blood</td>
<td>21.10.2007</td>
<td>PICU</td>
<td>I</td>
<td>I 1.5 4 1.5/1.5</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>S 4 &gt;32 4/2</td>
<td>+</td>
</tr>
<tr>
<td>P-6</td>
<td>Urine</td>
<td>22.10.2007</td>
<td>PICU</td>
<td>I</td>
<td>S 1.5 8 1.5/1</td>
<td>+</td>
</tr>
<tr>
<td>P-7</td>
<td>Endotracheal aspirate</td>
<td>06.11.2007</td>
<td>PICU</td>
<td>C</td>
<td>R 4 3/3</td>
<td>-</td>
</tr>
<tr>
<td>P-8</td>
<td>Catheter</td>
<td>06.11.2007</td>
<td>PICU</td>
<td>I</td>
<td>S 2 &gt;32 2/1</td>
<td>+</td>
</tr>
<tr>
<td>P-9</td>
<td>Urine</td>
<td>13.11.2007</td>
<td>PICU</td>
<td>I</td>
<td>S 1.5 8 1.5/1</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>C 16 1.5/1</td>
<td>+</td>
</tr>
<tr>
<td>P-10</td>
<td>Blood</td>
<td>19.11.2007</td>
<td>PICU</td>
<td>I</td>
<td>R 8 &gt;512 8/2</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
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<td>R 8 &gt;512 8/2</td>
<td>+</td>
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<tr>
<td>P-11</td>
<td>Blood</td>
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<td>I</td>
<td>R 4 8 4/4</td>
<td>+</td>
</tr>
<tr>
<td>T-12</td>
<td>Urine</td>
<td>30.06.2007 Kidney transplantation</td>
<td>S 8 16 16/3</td>
<td>+</td>
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<td></td>
</tr>
</tbody>
</table>

PICU: Pediatric intensive care unit; I: infected; C: colonized; IMP: imipenem; MEM: meropenem; CIP: ciprofloxacin; IMP/IMP + EDTA: imipenem/ imipenem + EDTA; S: susceptible; I: intermediate susceptible; R: resistant.

**REFERENCES**


