DETECTION AND SPREAD OF OXA-48-PRODUCING *KLEBSIELLA OXYTOCA* ISOLATES IN ISTANBUL, TURKEY

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Abstract. Five OXA-48 producing *Klebsiella oxytoca* strains isolated in April-July 2010 were analyzed. Antibiotic susceptibility tests were performed using disc diffusion method and VITEK 2 system. Carbapenemase activity was investigated using the Modified Hodge test. Beta-lactamase genes were detected by PCR and $bla_{\text{OXA-48}}$ was sequenced. Genetic relatedness between *K. oxytoca* isolates was investigated by pulse-field gel electrophoresis (PFGE). Carbapenemase activity was detected in 5 isolates by Modified Hodge test. Although all strains were resistant to ertapenem and imipenem, only one strain was also resistant to meropenem. $Bla_{\text{OXA-48}}$ in 4 isolates harbored 2 or 3 other ESBL types, namely, bla_{TEM}, bla_{SHV}, $bla_{\text{CTX-M}}$, or bla_{VEB}. PFGE revealed 3 different pulso-types among the *K. oxytoca* isolates. The presence of OXA-48 carbapenemase in other species of clinical isolates should also be considered.

Keywords: *Klebsiella oxytoca*, beta-lactamase, carbapenem resistance, plasmid, OXA-48 carbapenemase

INTRODUCTION

Emergence and spread of carbapenemase-producing Enterobacteriaceae isolates represent a significant threat to the management of especially nosocomial infections. Carbapenem-hydrolyzing enzymes comprise of molecular classes A, B (metallo-β-lactamases), and D (oxacilinases) (Queenan and Bush, 2007). Ambler class D OXA-48 beta-lactamase was initially identified in *Klebsiella pneumoniae* from Turkey in 2004 (Poirel *et al*, 2004), then outbreaks of OXA-48-producing Enterobacteriaceae have been reported in Turkey (Carrer *et al*, 2008), Tunisia (Mkaouar *et al*, 2008), Senegal (Moquet *et al*, 2011), Spain (Sahagun Pareja *et al*, 2005) and France (Cuzon *et al*, 2011). Additionally, OXA-48 producers belonging to *Enterobacteriaceae* have also been reported in several countries, such as Belgium, Lebanon, Egypt and Morocco (Carrer *et al*, 2008; Cuzon *et al*, 2008; Benouda *et al*, 2010; Carrer *et al*, 2010; Kalpoe *et al*, 2011).

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In this study we describe the detection of *Klebsiella oxytoca* isolates producing OXA-48 in Turkey.

MATERIALS AND METHODS

Isolates

Resistant or reduced susceptible isolates to carbapenems were obtained from clinical specimens in Istanbul Medical Faculty, Turkey a 1750 bed tertiary care teaching hospital, from April-July 2010. Isolates were identified by conventional methods and VITEK 2 System (bioMérieux, Marcy l'Etoile, France). Antimicrobial susceptibility tests were performed according to the recommendations of the Clinical and Laboratory Standards Institute (CLSI). Carbapenemase activity was screened by the Modified Hodge test (CLSI, 2011). OXA-48 producing *Citrobacter freundii* Lut strain from previous study and *E. coli* ATCC 25922 were used as the control strains (Nazik *et al*, 2005). Minimal inhibitory concentrations (MICs) of isolates were determined using VITEK 2 System. Ethical approval was not required where only routine laboratory isolates were used. Consent to use isolates that had been provided from patients was acquired retrospectively from the patients, their legal guardians or their next of kin.

Detection of $bla_{\text{OXA-48}}$

DNA extraction was performed as described previously (Mammeri *et al*, 2005; Nazik *et al*, 2009). PCR amplification of $bla_{\text{OXA-48}}$ was carried out using the following set of primers: OXA-48A (5'-TTGGTGGCATCGATTATCGG-3') and OXA-48B (5'-GAGCACTTCTTTTGTGATGGC-3'), producing 743 bp, in a 50 µl final volume containing 10x PCR buffer (5 µl), 2 mM deoxynucleoside triphosphates, 3.5 pmol of each primer, 2.5 mM MgCl (5 µl), 1 U *Taq* DNA polymerase and 1 µl of genomic DNA of the test strain. PCR was performed in a thermal cycler (Takara Thermal Cycler TP600; Otso, Shiga, Japan) using the following conditions: 94ºC for 5 minutes; 35 cycles of 94ºC for 60 seconds; 55ºC for 45 seconds, and 72ºC for 60 seconds; and a final heating at 72ºC for 7 minutes (Nazik *et al*, 2005; Aktaş *et al*, 2008). The bla_{TEM}, bla_{SHV}, $bla_{\text{CTX-M}}$, $bla_{\text{VIM-1}}$ $bla_{\text{VIM-2}}$, $bla_{\text{IMP-1}}$, $bla_{\text{IMP-2}}$ bla_{KPC}, were screened by PCR as described previously (Poirel *et al*, 2004; Mammeri *et al*, 2005; Poirel *et al*, 2005; Pallecchi *et al*, 2007; Queenan and Bush, 2007). PCR products were separated by 1.5% agarose gel-electrophoresis, stained with ethidium bromide and visualized under UV light. Φ174 *Hae*III fragments (MBI Fermentas; St Leon-Rot, Germany) were used to assess PCR product size. Amplicons were purified (High-Pure Purification kit, Roche Diagnostics, Castle Hill, Australia) and both strands were sequenced in an Applied Biosystems sequencer (ABI 377; Applied Biosystems, Foster City, CA). Nucleotide and deduced protein sequences were analyzed with software from the National Center for Biotechnology Information website (www.ncbi.nlm.nih.gov).

PFGE

Genetic relatedness of the *K. oxytoca* isolates was determined by PFGE. Following extraction of genomic DNA and digestion with *Xba*I, a CHEF DR2 system (Bio-Rad Laboratories, Hercules, CA) were used for performing PFGE, and the macrorestriction patterns were analyzed using GelCompar II software (Version 6.0; Applied Maths, Sint-Martens-Latem, Belgium). Relatedness was calculated using the unweighted pair group method with mathematical averaging (UPGMA). Cluster designation was determined according to criteria described previously and the strains categorized as indistin-

guishable, closely related, possibly related or different (Tenover *et al*, 1995; Durmaz *et al*, 2009).

RESULTS

Three of the five patients were of pediatric age and two were hospitalized in pediatric intensive care unit. Although all patients were treated with beta-lactam antibiotics, only two patients were treated with carbapenems; all patients were cured (Table 1).

Modified Hodge test revealed carbapenemase activity in 5 isolates and $bla_{\text{OXA-48}}$ genes were demonstrated by PCR and sequence analysis. In addition to $bla_{\text{OXA-48,}}$ 4 isolates harbored 2 or 3 other ESBL types, bla_{TEM}, bla_{SHV}, $bla_{\text{CTX-M}}$, or bla_{VEB} (Table 2). PFGE revealed 3 different pulso-types among the *K. oxytoca* isolates (Fig 1). Three strains, two of which were isolated from the same patient hospitalized in pediatric intensive care unit, were clonally related.

Although all strains were resistant to ertapenem and imipenem, only one strain was also resistant to meropenem. Additionally, all 4 isolates were resistant to ampicillin, amoxicillin-clavulanic acid, piperacillin-tazobactam, cefazolin, and cefuroxime. Resistance to third generation cefalosporins, such as ceftriaxone or ceftazidime, was observed in all isolates. However, 4 isolates remained susceptible to cefepime. In addition to beta-lactam resistance, 4, 3 and 1 isolates were also resistant to gentamicin, Co-trimoxazole and levofloxacin, respectively (Table 2).

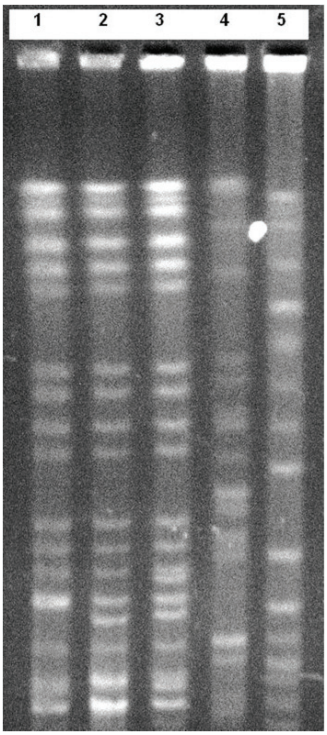

Fig 1–Pulse-field gel electrophoresis patterns of 5 OXA-48-producing *K. oxytoca* isolates. The experimental protocols are described in Materials and Methods.

DISCUSSION

The global spread of ESBLs, particularly CTX-M enzymes, in clinical isolates of *E. coli* and *K. pneumoniae* is a public health problem due to limitation of the effectiveness of all β-lactams except carbapenems, which are mostly a last resort therapy (Livermore *et al*, 2007; Pitout, 2008; Nazik *et al*, 2011c). In addition to class A and class B carbapenemase, class D carbapenemase, OXA-48 type, might lead significantly to carbapenem resistance in *Enterobacteriacae* and are involved in outbreaks in various locations and are increasingly reported in sporadic cases worldwide. Following detection of the first isolate of OXA-48-producing isolate from Turkey (Poirel *et al,* 2004), a number of OXA-48 producing isolates were reported occasionally (Nazik *et al,* 2005; Aktaş *et al,* 2008; Gulmez *et al*, 2008). However, two recent reports indicated

Table 1
Clinical features of patients with OXA-48-producing *K. oxytoca* isolates.

Isolate number[a]	Date (mo/day/yr) of isolation	Sex	Age	Diagnosis	Hospitalization unit	Source	Treatment	Outcome
K. oxytoca 1	05/26/2010	F	1 m	RDS, PDA	Pediatric ICU	Tracheal aspirate	MEM, CTX	Cured
K. oxytoca 2	07/28/2010	F	1 m	RDS, PDA	Pediatric ICU	Tracheal aspirate	MEM, CTX	Cured
K. oxytoca 3	06/25/2010	M	1 m	Prematurity, RDS	Pediatric ICU	Tracheal aspirate	AMP, CTX	Cured
K. oxytoca 4	07/13/2010	M	7 m	Epispadias	Pediatric surgery	Urine	SAM	Cured
K. oxytoca 5	04/24/2010	M	50 y	DM	Emergency unit	Urine	IMP	Cured

ICU, intensive care unit; PDA, patent ductus arteriosus; IPM, imipenem; MEM, meropenem; AMP, ampicillin; SAM, ampicillin-sulbactam; CTX, cefotaxime. [a]Strain 1 and 2 were isolated from the same patient and included in the study due their different colony morphology.

Table 2
Beta-lactamases and resistance patterns of OXA-48-producing *K. oxytoca* clinical isolates.

Isolate number	PFGE	Related *bla*	[a]MIC for carbapenem (µg/ml)			Antibiotic resistance pattern
			ETP	IMP	MEM	
K. oxytoca 1	1	TEM, SHV, VEB	4	4	1	AMP, AMC, TZP, CZ, CXM, CAZ, CRO, GN, SXT
K. oxytoca 2	1	TEM, SHV, VEB	4	8	1	AMP, AMC, TZP, CZ, CXM, CAZ, CRO, GN, SXT
K. oxytoca 3	1	SHV, VEB	2	4	1	AMP, AMC, TZP, CZ, CXM, CAZ, CRO, GN
K. oxytoca 4	2	-	2	4	≤0,25	AMP, AMC, TZP, CZ, CXM, CRO
K. oxytoca 5	3	SHV, CTX-M, VEB	≥8	8	≥16	AMP, AMC, TZP, CZ, CXM, CAZ, CRO, FEP, GN, LEV, SXT

ETP, ertapenem; IPM, imipenem; MEM, meropenem; AMC, amoxicillin-clavulanic acid; TZP, piperacillin-tazobactam; CZ, cefazolin; CXM, cefuroxime; CAZ, ceftazidime; CRO, ceftriaxone; FEP, cefepime; LEV, levofloxacin; SXT, Co-trimoxazole; GN, gentamicin; [a]MIC, range of antibiotics: for ETP: ≤0.25 susceptible, 0.5 intermediate, ≥1 resistant; for IMP and MEM: ≤1 susceptible, 2 intermediate, ≥4 resistant.

that the problem might actually be more serious than has been reported in Turkey. The first report was in 2008, an outbreak including 39 carbapenem-resistant, OXA-48 positive, *K. pneumoniae* strains isolated from mostly adults in intensive care units and emergency surgery in our hospital (Carrer *et al*, 2008). The second report was

in 2010, when 18 carbapenem-resistant, OXA-48-positive enterobacterial isolates were detected in Turkey, Lebanon, Egypt, France and Belgium (Carrer *et al,* 2010). Recently, we reported 22 multiresistant OXA-48 producing *K. pneumoniae* isolates (Nazik *et al*, 2011a) and 10 multiresistant isolates including 9 *K. pneumoniae* and 1 *E. coli* isolates (Nazik *et al*, 2012) from the same hospital. Here, we presented another member of *Enterobacteriaceae*, *K. oxytoca*, with carbapenemase activity.

The co-existence of $bla_{\text{OXA-48}}$ together with other ESBL types, such as bla_{TEM}, bla_{SHV}, and $bla_{\text{CTX-M}}$, is a threat to the use of all beta-lactams including carbapenems (Pitout, 2008; Queenan and Bush, 2007). In addition to TEM, SHV and CTX-M types, which are widespread worldwide, another type, beta-lactamases-VEB, has emerged in recent years (Poirel *et al,* 2005). In this study, in addition to previous beta-lactamases, the VEB type β-lactamase was detected in 4 *K. oxytoca* isolates. In our previous studies, *Citrobacter freundii* and *K. pneumoniae* isolates producing $bla_{\text{OXA-48}}$ and bla_{VEB} have been reported from same hospital in Istanbul (Nazik *et al,* 2005). This finding showed that VEB type β-lactamase persists not only in *C. freundii* and *K. pneumoniae* but also in *K. oxytoca* isolates in Turkey.

Moreover, the present study demonstrated that 3 strains isolated from pediatric intensive care unit were clonally related, but 2 distinct clones were also detected in different units. Thus the dissemination of $bla_{\text{OXA-48}}$ was not spread by a single *K. oxytoca* clone as shown previously for *K. pneumoniae* isolates (Carrer *et al,* 2008, 2010; Nazik *et al,* 2011b), but that several OXA-48-producing clones were distributed in our hospital in Istanbul. The spread of *K. oxytoca* isolates harboring $bla_{\text{OXA-48}}$ but also ESBLs, such as TEM, SHV, CTX-M, and now VEB-1 type, may be a serious problem for treatment. Another concern is the difficulty to detecting these isolates by clinical laboratories as they may appear susceptible to carbapenems especially imipenem and meropenem according to the current CLSI breakpoints. Considerable but necessary efforts will have to be instituted in order to detect $bla_{\text{OXA-48}}$ in enterobacteriaceal clinical isolates.

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