CARBAPENEM RESISTANCE DUE TO *BLA*$_{OXA-48}$ AMONG ESBL-PRODUCING *ESCHERICHIA COLI* AND *KLEBSIELLA PNEUMONIAE* ISOLATES IN A UNIVESITY HOSPITAL, TURKEY

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Abstract. Bacterial isolates producing Class D OXA-48 carbapenemase may be missed in routine laboratory testing, allowing them to spread undetected. The purpose of the present study was to detect *bla*$_{OXA-48}$ among ESBL-producing *Klebsiella pneumoniae* and *Escherichia coli* isolates collected from a university hospital, Turkey. Ninety-two ESBL-producing isolates (66 *E. coli*, 26 *K. pneumoniae*) were obtained in 2010. Antibiotic susceptibility tests were performed using the disc diffusion method and VITEK 2 system. Carbapenemase activity was screened using modified Hodge test. Beta-lactamase genes were detected by PCR and *bla*$_{OXA-48}$-positive amplicons were sequenced. Genetic relatedness among *K. pneumoniae* isolates was investigated by pulsed-field gel-electrophoresis (PFGE). Carbapenemase activity was detected in 1 *E. coli* and 9 *K. pneumoniae* isolates and 8 of the *K. pneumoniae* plus the *E. coli* isolates were resistant to ertapenem. Three *K. pneumoniae* and 1 *E. coli* isolates were resistant to imipenem. All 10 isolates were susceptible to meropenem. *bla*$_{OXA-48}$ was present in all 10 isolates. Additionally, 9 isolates contained at least one beta-lactamase gene, including *bla*$_{SHV}$, *bla*$_{CTX-M}$ and *bla*$_{VEB}$ type. PFGE revealed different karyotypes among 9 *K. pneumoniae* isolates suggesting that the dissemination of *bla*$_{OXA-48}$ gene was not spread by a single *K. pneumoniae* clone. Thus OXA-48-producing isolates found in carbapenem-susceptible strains according to CLSI guidelines.

Keywords: *Klebsiella pneumoniae,* carbapenemase, OXA-48, VEB, ESBL, carbapenems

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INTRODUCTION

Carbapenems are valuable antibiotics which are often used as the last choice for treating infections especially due to ESBL-producing strains. The spread of carbapenemase-producing enterobacte-

ria isolates is a significant threat to the management of nosocomial infections, especially in intensive care units (Aktas, *et al*, 2008; Livermore, *et al*, 2009).

According to the Ambler classification, carbapenem-hydrolyzing enzymes can belong to class A, B (metallo-β-lactamases), or D (oxacilinases) (Queenan and Bush, 2007). The resistance of *Enterobacteriaceae* to carbapenems could also be related to a dual mechanism associating an outer membrane permeability defect with beta-lactmases, such as AmpC cephalosporinase and ESBLs, particularly with the presence of CTX-M types (Pasteran *et al*, 2011). The class A carbapenemases, encoded by bla_{KPC}, are present in *Klebsiella pneumoniae* strains and they are increasingly widespread (Pitout, 2008). This type of carbapenemases has been reported in various *Enterobacteriacae* spp from several parts of the world, including USA, Europe, South America, Asia and the Middle East (Pitout, 2008). Plasmid-encoded class B metallo-β-lactamases, such as IMP and VIM types, are also distributed globally (Toraman *et al*, 2004; Queenann and Bush, 2007). Class D OXA-48 was first reported in a *K. pneumoniae* from Turkey in 2004, and since then several enterobacterial isolates producing OXA-48 have occasionally been reported mostly from Turkey. However, in recent years, it has been reported more frequently, not only from Turkey but also countries such as Belgium, Lebanon, United Kingdom, India and Argentina (Poirel *et al*, 2004; Cuzon *et al*, 2008; Livermore, 2009; Carrer *et al*, 2010; Hawser *et al*, 2011; Ktari *et al*, 2011; Lahlaoui *et al*, 2011; Moquet *et al*, 2011).

The undetected spreading of bla_{OXA-48} gene is of concern due to the inability of routine laboratory to detect OXA-48 producing strains. The purpose of the present study was to detect bla_{OXA-48} gene among ESBL-producing *K. pneumoniae* and *Escherichia coli* isolates in Turkey.

MATERIALS AND METHODS

Samples

A total of 92 ESBL-producing isolates (66 *E. coli*, 26 *K. pneumoniae*) were obtained from the Microbiology Laboratories of Istanbul Medical Faculty, Turkey, a 1,750-bed tertiary care teaching hospital, from 1 April to 31 August 2010. The isolates were from clinical specimens and identified by conventional methods (Garcia and Isenberg, 2010) and by PCR for identification of strains positive for bla_{OXA-48} using VITEK 2 System (bioMérieux, Marcy l'Etoile, France).

Antimicrobial susceptibility and synergy tests

The Kirby-Bauer disc diffusion method was employed for susceptibility testing (CLSI, 2011). The double disc synergy test was used for screening the ESBL production. Carbapenemase activity was screened using the modified Hodge test (CLSI, 2009). OXA-48-producing *Citrobacter freundii* Lut strain from the previous study (Nazic *et al*, 2005) and *E. coli* ATCC 25922 were used as control strains. Minimum inhibitory concentration (MIC) of isolates was determined by VITEK 2 System (Pasteran *et al*, 2011). Ten positive isolates by modified Hodge test (CLSI, 2009) were included for further experiments.

Detection of bla_{OXA-48} and related β-lactamase genes

DNA extraction was performed as described previously (Mammeri *et al*, 2005; Nazik *et al*, 2011b). In brief, colonies were boiled in distilled water and supernatants used as DNA templates for polymerase chain reaction (PCR). Supernatants were

stored at -20°C prior to subsequent DNA amplification (Mammeri *et al*, 2005; Nazik *et al,* 2011b). PCR amplification for bla_{OXA-48} was performed using OXA-48A (5′-TTGGTGGCATCGATTATCGG-3′) and OXA-48B (5′-GAGCACTTCTTTTGTGATGGC-3′) producing 743 bp amplicons, in a 50 µl volume containing 10x PCR buffer (5 µl), 2 mM deoxynucleoside triphosphates, 3.5 pmol of each primer, 2.5 mM $MgCl_2$ (5 µl), 1 U *Taq* DNA polymerase and 1 µl of genomic DNA of the test strain. A thermal cycler (Takara Thermal Cycler TP600, Takara Bio, Shiga, Japan) was used under the following conditions: 94°C for 5 minutes; 35 cycles of 94°C for 60 seconds; 55°C for 45 seconds, and 72°C for 60 seconds, and a step of 72°C for 7 minutes (Aktas *et al*, 2008; Nazik *et al,* 2011b). bla_{TEM}, bla_{SHV}, bla_{CTX-M}, bla_{VIM-1}, bla_{VIM-2}, bla_{IMP-1}, bla_{IMP-2} and bla_{KPC} were investigated by PCR as described previously (Poirel *et al*, 2004, 2005; Mammeri *et al*, 2005; Pallecchi *et al*, 2007; Queenan and Bush, 2007). PCR amplicons were separated by 1.5% agarose gel-electrophoresis, stained with ethidium bromide and visualized under UV light. Φ174 *Hae* III fragments were used as DNA size markers (MBI Fermentas; St Leon-Rot, Germany). After OXA-48 PCR amplification, amplicon was purified using High-Pure Purification kit (Roche Diagnostics, Castle Hill, NSW, Australia) and sequenced in both directions in an Applied Biosystems sequencer (ABI 377) (Applied Biosystems, Foster City, CA). The nucleotide and deduced protein sequences were analyzed with sofware at the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov).

Pulsed-field gel electrophoresis (PFGE)

Genetic relatedness of the *K. pneumoniae* isolates was determined by PFGE following extraction of genomic DNA and digestion with *Xba*I (Carrer *et al*, 2010). CHEF DR2 unit (Bio-Rad Laboratories, Nazareth, Belgium) was used for performing PFGE and the macrorestriction patterns were analysed with 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 (Tenover *et al*, 1995). According to the criteria of Tenover *et al* (1995), the strains categorized as being: indistinguishable, closely related, possibly related or different.

RESULTS

Among the 92 strains, carbapenemase activity was detected in 10 isolates (1 *E. coli* and 9 *K. pneumoniae*) using the modified Hodge test and these strains were included for further analysis. bla_{OXA-48} in these isolates were demonstrated by PCR and sequence analysis. Additionally, except for the *E. coli* isolate, all 9 *K.pneumoniae* isolates co-produced at least one ESBL; 3 and 5 of these strains contained bla_{SHV} and bla_{SHV}/bla_{CTX-M}, respectively (Table 1). One *K. pneumoniae* isolate contained bla_{CTX-M} and $bla_{VEB.}$ PFGE revealed 9 different restriction-types among the *K. pneumoniae* isolates (Fig 1).

The modified Hodge test-positive isolates were resistant to ertapenem. Three *K. pneumoniae* isolates and the single *E. coli* isolate were resistant also to imipenem. All 10 isolates were susceptible to meropenem. Additionally, these 10 isolates were resistant to amoxicillin-clavulanic acid, piperacillin-tazobactam and cefazolin. Resistance to the third generation cefalosporins was observed in 8 isolates. However, 6 of the isolates remained susceptible to cefepime. The antibiotic resis-

tance patterns of the strains are presented in Table 1.

The antibiotic resistance rates of the 92 test strains are presented in Fig 2. In general, *E. coli* isolates displayed high rates of resistance to cefotaxime, ampicillin-sulbactam, amoxicillin-clavulanic acid, ceftazidime, gentamicin. High rates of resistance to cefotaxime, ampicillin-sulbactam, amoxicillin-clavulanic acid, ceftazidime, cefoperazone-sulbactam, piperacillin-tazobactam, and gentamicin were detected among *K. pneumoniae* isolates.

DISCUSSION

In addition to class A and class B carbapenemases, the class D carbapenemase, OXA-48 type, might lead significantly to carbapenem resistance in *Enterobacteriacae*. We have recently investigated plasmid-mediated quinolone resistance determinants in 22 OXA-48 producing isolates (Nazik *et al*, 2011b). In this report, among 26 *K. pneumoniae* and 66 *E. coli* clinical isolates we detected bla_{OXA-48} more frequently in *K. pneumoniae* (35%) than in *E. coli* (1.5%).

In our findings, OXA-48-producing isolates can be susceptible to carbapenems, especially imipenem and meropenem according to the current CLSI break-

Table 1
Related beta-lactamases and resistance patterns of OXA-48 producing *K. pneumoniae* and *E. coli* clinical isolates.

Isolate number	Bacteria species	Related *bla* genes	[a]MICs for carbapenem (µg/ml)			Antibiotic resistance pattern
			ETP	IMP	MEM	
1	*E. coli*	-	≥8	4	1	AMC, TZP, CZ, CXM, ETP, IPM, LEV, SXT
2	*K. pneumoniae*	CTX-M, VEB	2	≤1	≤0,25	AMC, TZP, CZ, CXM, CAZ, CRO, ETP
3	*K. pneumoniae*	SHV, CTX-M	≥8	4	1	AMC, TZP, CZ, CXM, CAZ, CRO, ETP, IMP GM, SXT
4	*K. pneumoniae*	SHV, CTX-M	≥8	4	1	AMC, TZP, CZ, CXM, CAZ, CRO, FEP, ETP, IMP, GM, SXT
5	*K. pneumoniae*	SHV	0,5	≤1	≤0,25	AMC, TZP, CZ, SXT
6	*K. pneumoniae*	SHV, CTX-M	≥8	4	1	AMC, TZP, CZ, CXM, CAZ, CRO, FEP, ETP, IMP, GM, SXT
7	*K. pneumoniae*	SHV	2	2	≤0,25	AMC, TZP, CZ, CXM, CAZ, CRO, ETP, GM, SXT
8	*K. pneumoniae*	SHV, CTX-M	≥8	2	1	AMC, TZP, CZ, CXM, CAZ, CRO, FEP, ETP, GM, SXT
9	*K. pneumoniae*	SHV	2	2	≤0,25	AMC, TZP, CZ, CRO, ETP, GM, SXT
10	*K. pneumoniae*	SHV, CTX-M	≥8	≤1	≤0,25	AMC, TZP, CZ, CXM, CAZ, CRO, FEP, ETP, 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, trimethoprim/sulfamethoxazole; GM, gentamicin, TEM, bla_{TEM}, SHV: bla_{SHV}, CTX-M: bla_{CTX-M}; VEB, bla_{VEB}. [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

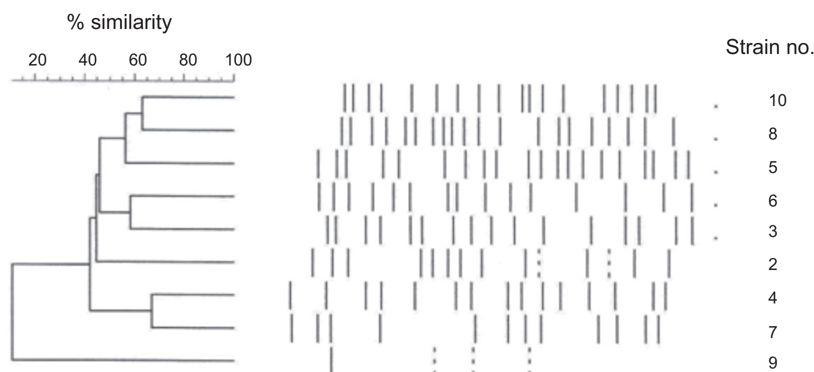

Fig 1–PFGE patterns of 9 OXA-48-producing *K. pneumoniae* isolates. The methods used are described in materials and methods.

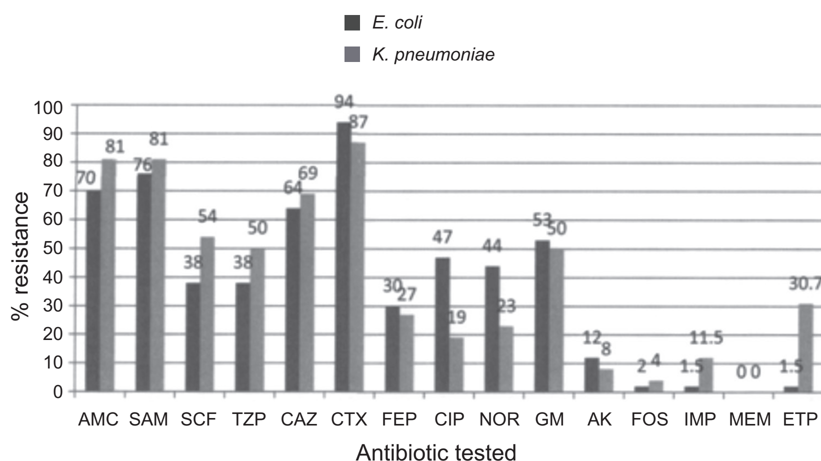

Fig 2–*In vitro* antibiotic resistance rate (%) of 66 *Esherichia coli* and 26 *K. pneumoniae* clinical isolates. Tests were performed using disc diffusion method and VITEK 2 system. AMC, amoxicillin-clavulanic acid; SAM, ampicillin-sulbactam; SCF, cefoperazone-sulbactam; TZP, piperacillin-tazobactam; CAZ, ceftazidime; CTX, cefotaxime; FEP, cefepime; CIP, ciprofloxacin; NOR, norfloxacin; GM, gentamicin; AK, amikacin; FOS, fosfomycin; IMP, imipenem; MEM, meropenem; ETP, ertapenem

points. It is often difficult to detect these strains in clinical laboratories (Cuzon *et al*, 2008), and automated antimicrobial susceptibility testing systems might incorrectly detect the OXA-48 producing isolates (Woodford, 2010). The current CLSI breakpoints for ertapenem and imipenem/meropenem, which define nonsusceptibility by a MIC of ≥0.5 µg/ml and ≥2 µg/ml, respectively, increase the detection of carbapenem resistance. However, these updates, especially for ertapenem, also have enhanced the poor ability of the commercial antimicrobial susceptibility system to distinguish carbapenemase-producing isolates with ESBL and/or AmpC combined with porin loss, resulting in poor specifity (Pasteran *et al*, 2011). The presence of dual mechanisms might be important for the high prevalence of CTX-M-type beta-lactamase especially among the *E. coli* and *K. pneumoniae* in Turkey (Nazik *et al*, 2011c,d). Thus recent studies have focused on finding screening methods based on the use of carbapenems other than ertapenem in order to develeop a more accurate identification of isolates suspected of producing carbapenemase (Ho *et al*, 2011; Pasteran *et al*, 2011).

The co-existance of *bla*$_{\text{OXA-48}}$ together

with the other ESBL types, such as bla_{TEM}, bla_{SHV}, and $bla_{CTX\text{-}M}$, among nosocomial pathogens is very important due to the restriction of the effectiveness of all β-lactams including carbapenems, which are mostly used as a last resort of therapy against ESBL- producing organisms. In the present study, high rates of resistance to different group of antibiotics, such as gentamicin and quinolones, were detected. In addition to TEM, SHV and CTX-M type (Pitout, 2008) which are widespread worldwide, another type of beta-lactamases (VEB) has emerged in recent years (Poirel *et al*, 2005). Here, in addition to the older ones, a VEB type β-lactamase was detected in one *K. pneumoniae* isolate. The presence of $bla_{OXA\text{-}48}$ together with $bla_{CTX\text{-}M}$, bla_{SHV} or bla_{TEM} has been described among *E. coli* and *K. pneumoniae* strains (Martinez-Martinez, *et al*, 2008). In our previous study, a *Citrobacter freundii* isolate producing $bla_{OXA\text{-}48}$ and bla_{VEB} has been reported from same hospital in Istanbul (Nazik *et al*, 2005). This finding showed that VEB type β-lactamase persists in microorganisms in Turkey (Nazik *et al*, 2011a).

Moreover, the present study demonstrated that *K. pneumoniae* isolates were not clonally related. Thus the dissemination of $bla_{OXA\text{-}48}$ was not due to a single *K. pneumoniae* clone although the strains were isolated from same units. This means that several OXA-48-producing clones were distributed in our hospital in Istanbul. This finding has also demonstrated in a few studies previously (Carrer *et al*, 2008, 2010).

The presence and worldwide dissemination of OXA-48 type carbapenemase together with other ESBLs, such as CTX-M and VEB, in *K. pneumoniae* and *E. coli* clinical isolates is of concern by virtue of the extension of the antibiotic resistance spectrum to include carbapenems. Thus, considerable efforts are necessary for the detection of $bla_{OXA\text{-}48}$ in clinical strains of Enterobacteriaceae.

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