Determination of *Escherichia coli* and *Citrobacter koseri* Isolates with OXA-48 Carbapenemase in İstanbul

İstanbul'da OXA-48 Karbapenamazı Üreten *Escherichia Coli* ve *Citrobacter Koseri* İzolatının Saptanması

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Geliş Tarihi/*Received:* 21.12.2010
Kabul Tarihi/*Accepted:* 20.09.2011

This study was presented as a poster in Thirty-Fourth Turkish Microbiology Congress (7-11 November 2010, Cyprus).

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doi:10.5336/medsci.2010-22148

ABSTRACT Objective: Today, carbapenems are used as almost last choice for the treatment of infections with extended spectrum β-lactamase (ESBL) producing pathogens. However, the prevalence of carbapenamase-producing strains in *Enterobacteriaceae* has been increasing over the last decade. In this study, carbapenem-resistant *Escherichia coli* and *Citrobacter koseri* isolates with OXA-48 carbapenemase obtained from hospitalized patients in different regions of Istanbul were investigated. **Material and Methods:** The strains were identified by conventional methods and VITEK 2 system. Antibiotic susceptibility test was performed using disc diffusion method, and extended-spectrum β-lactamase production was investigated using the double-disc synergy and cefotaxime/boronic acid- cefotaxime/boronic acid/clavulanic acid tests. VITEK 2 system was used for determination of minimum inhibitory concentration (MIC) of the antibiotics. The MICs of imipenem and meropenem for the clinical isolates were also determined by E-tests. The carbapenemase production was investigated with modified Hodge test. β-lactamase genes and plasmid mediated quinolone resistance (PMQR) determinants were screened by polymerase chain reaction (PCR). The amplification products of bla_{OXA-48} genes were sequenced. Conjugation experiment was used for transferring β-lactamase gene. **Results:** The clinical isolates and their transconjugants were positive for bla_{OXA-48}, however, the isolates lacked PMQR and other β-lactamase genes such as bla_{TEM}, bla_{SHV}, bla_{CTX-M}, bla_{VIM}, bla_{IMP} and bla_{KPC}. The bla_{OXA-48} genes detected in both of the isolates were transferred to the recipient strain. The MICs were increased approximately two fold according to recipient strain in transconjugants for carbapenems. Additionally, the transconjugants conferred resistance to ampicillin, amoxicillin-clavulanic acid, piperacillin-tazobactam and cefazolin. **Conclusions:** The carbapenemase-producing isolates may restrict the use of these valuable antibiotics. In Turkey, the bla_{OXA-48} gene not only persists especially in *Klebsiella pneumoniae* but also it has spread among other species. The presence of OXA-48 carbapenemase in rarely encountered species should also be considered.

Key Words: Carbapenemase; *Escherichia coli*; *Citrobacter koseri*; oxacillinase

ÖZET Amaç: Günümüzde, karbapenemler geniş spektrumlu β-laktamaz (ESBL) üreten patojenlerlerin neden olduğu enfeksiyonların tedavisi için genellikle son tercih olarak kullanılmaktadır. Bununla birlikte *Enterobacteriaceae* içinde karbapenemaz üreten türlerin prevalansı son on yılda artış göstermektedir. Bu çalışmada, İstanbul'un farklı bölgelerinde hastaneye yatmış olan hastalardan izole edilen, OXA-48 karbapenamazı üreten karbapenem dirençli *Escherichia coli* ve *Citrobacter koseri* izolatları incelenmiştir. **Gereç ve Yöntemler:** Suşlar, konvansiyonel yöntemler ve VITEK 2 sistemi ile identifiye edilmiştir. Antibiyotik duyarlılık testleri, disk difüzyon yöntemi kullanılarak uygulanmış ve genişlemiş spektrumlu β-laktamaz üretimi, çift disk sinerji ve sefotaksim/boronik asit-sefotaksim/boronik asit/klavulanik asit testleri kullanılarak araştırılmıştır. Antibiyotiklerin minimum inhibitor konsantrasyonunun (MİK) saptanmasında VITEK 2 sistemi kullanılmıştır. Klinik izolatların imipenem ve meropenem için MİK'leri, E-test yöntemi ile de saptanmıştır. Karbapenemaz üretimi, modifiye Hodge testi ile araştırılmıştır. β-laktamaz genleri ve plazmid aracılı kinolon direnci (PMQR) determinanları, polimeraz zincir reaksiyonu ile taranmıştır. bla_{OXA-48} genlerinin amplifikasyon ürünleri sekanslanmıştır. β-laktamaz gen transferi için konjugasyon deneyi kullanılmıştır. **Bulgular:** Klinik izolatlar ve transkonjuganlarında, bla_{OXA-48} geni pozitif bulunmuş ancak PMQR ve bla_{TEM}, bla_{SHV}, bla_{CTX-M}, bla_{VIM}, bla_{IPM} ve bla_{KPC} gibi diğer β-laktamaz genleri saptanmamıştır. Heriki izolatta saptanan bla_{OXA-48} geni, alıcı bakteriye aktarılmıştır. Trankonjuganlarda karbapenemler için saptanan MİK değerleri alıcı bakteriye göre iki kat daha yüksek bulunmuştur. Buna ek olarak transkonjuganlar ampisilin, amoksisilin-klavulanik asid, piperasilin-tazobaktam ve sefazoline direnç gösterdiği saptanmıştır. **Sonuç:** Karbapenemaz üreten suşlar karbapenemlerin kullanınımını sınırlayabilmektedir. Türkiye'de OXA-48 geni özellikle Klebsiella pneumoniae suşlarında bulunmaya devam etmekte, ayrıca diğer suşlar arasında da yayılmaktadır. OXA-48 karbapenemazının varlığı seyrek karşılaşılan türlerde de akla getirilmelidir

Anahtar Kelimeler: Carbapenemase; *Escherichia coli*; *Citrobacter koseri*; oxacillinase

Turkiye Klinikleri J Med Sci 2011;31(6):1502-6

The prevalence of carbapenemase-producing strains in Enterobacteriaceae has been increasing over the last decade. Carbapenem-hydrolyzing enzymes can be classified in molecular Ambler class A, B, or D (oxacillinases). The OXA-48 (Class D) was first reported in *Klebsiella pneumoniae* isolated in Turkey in 2001. The isolate was co-producing TEM-1 and SHV-2a and was resistant to all β-lactams, including carbapenems.[1] Since then, several enterobacterial isolates producing OXA-48 have been reported mostly from Turkey, also from various countries including Belgium, Lebanon, India and Argentina and recently from France and Egypt.[2-4] Extended-spectrum β-lactamase (ESBL) encoding genes located on plasmids are highly transferable and may harbor resistance genes to several different groups of antibiotics. Today, carbapenems are usually the last choice for the treatment of infections with ESBL-producing pathogens. The increasing spread of plasmid mediated quinolone resistance (PMQR) determinants with carbapenem resistance genes considered as a cause of concern.

In this study, carbapenem-resistant *Escherichia coli* and *Citrobacter koseri* isolates with OXA-48 carbapenemase obtained from hospitalized patients in different regions of İstanbul were investigated.

MATERIAL AND METHODS

PATIENT AND BACTERIAL ISOLATE

İstanbul University İstanbul Medical Faculty (1 750 beds) is located in the European part of İstanbul, while the Gülhane Military Medical Academy Haydarpaşa Training Hospital (1000 beds) is located in the Asian part of İstanbul. Clinical *E. coli* 406 strain was isolated from a 43-year-old female patient with renal transplantation in İstanbul University İstanbul Medical Faculty in 2009. The strain was isolated from peritoneal fluid. Clinical *C. koseri* 86 strain was isolated from a patient with peptic ulcer in Gülhane Military Medical Academy Haydarpaşa Training Hospital in 2009. This 77-year-old male patient was hospitalized due to severe gastrointestinal bleeding. After getting worse, the patient was transferred to intensive care unit. One month later, culture of blood samples yielded a multi-drug resistant *C. koseri* isolate. Both of the isolates were identified by the conventional methods and VITEK 2 System (bioMérieux, France).

ANTIMICROBIAL SUSCEPTIBILITY TESTING AND SYNERGY TESTING

Individual strains were tested based on the recommendations of the Clinical and Laboratory Standards Institute (CLSI), by the Kirby–Bauer disc diffusion method for susceptibility.[5] Double disc synergy test with cefotaxime and ceftazidime and cefotaxime/boronic acid- cefotaxime/boronic acid/clavulanic acid test were used for screening the ESBL production. Carbapenemase production was investigated by modified Hodge test.

The following antibiotic discs (Oxoid, Hampshire, UK) were purchased and used as directed by the manufacturer: Amoxicillin-clavulanic acid (AMC, 20/10 mg), cefoperazone-sulbactam (SCF, 75/30 mg), piperacillin-tazobactam (TZP, 100/10 mg), imipenem (IMP, 10 mg), meropenem (MEM, 10 mg), cefotaxime (30 mg), ceftazidime (30 mg), gentamicin (GN, 10 mg), norfloxacin (NOR, 10 mg), ciprofloxacin (CIP, 5 mg), co-trimoxazole (SXT, 1.25/23.75 mg), nitrofurantoin (NIT, 300 mg), fosfomycin (FOS, 200 mg). *E. coli* 25922 was used as a control strain. MICs of ampicillin, amoxicillin-clavulanic acid, piperacillin-clavulanic acid, cefazolin, cefoxitin, ceftazidime, ceftriaxone, ertapenem, imipenem, meropenem, gentamicin, levofloxacin, tigecycline, and trimethoprim/sulfamethoxazole were determined for clinical isolates and their transconjugants by VITEK 2 System. The MICs of imipenem and meropenem for the clinical isolates were also determined by E-test (bioMérieux, France).

TRANSFERABILITY OF BLA_{OXA-48} GENES

Conjugation experiments using an azide-resistant *E. coli* J53 (AzR) as the recipient were performed in liquid culture media as described previously.[6] Transconjugants were selected on trypticase soy agar plates containing sodium azide (100 μg/ml) for counter selection and amoxicillin (100 μg/ml),

cefotaxime (8 µg/ml) for selection of plasmid-encoded resistance. The presence of transferred *bla*$_{OXA-48}$ genes were confirmed by PCR (Figure 1).

CHARACTERIZATION OF BLAOXA-48 GENES AND SEQUENCING

DNA extraction was performed as described previously.[7] Briefly, bacterial colonies were suspended in 2 ml centrifuge tubes and then centrifuged at 12 000 g to obtain pellets. Pellets were washed in 750 ml of TE buffer (10 mM Tris HCl, pH 8.0, 1 mM EDTA) and then boiled for 10 minutes in 500 ml of Tris-EDTA (TE) buffer and centrifuged. Supernatants were stored at -20°C until subsequent DNA amplification. The bla_{TEM}, bla_{SHV}, bla_{CTX-M}, bla_{OXA-48}, bla_{VIM-1}, bla_{VIM-2}, bla_{IPM-1}, bla_{IPM-2}, bla_{KPC} genes were investigated by PCR, as described previously.[8,9] A multiplex PCR was performed to detect qnrA, qnrB and qnrS as described previously by Cattoir et al.[10] PCR amplification of qnrC, qnrD, and qepA and aac (6')-Ib was carried out with specific primers and conditions.[11-13] The amplification products of bla_{OXA-48} genes were sequenced with Applied Biosystem sequencer (ABI PRISM 310 Genetic Analyzer; Applied Biosystems, Foster City, CA, USA). The nucleotide and amino acid sequences were analyzed and compared with the BLAST computer program available in the internet at the National Center for Biotechnology Information website (www.ncbi.nlm.nih.gov).

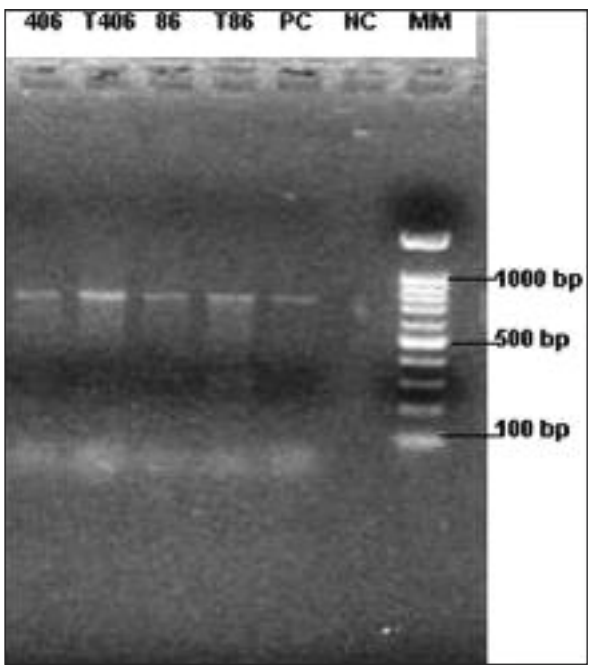

FIGURE 1: PCR gel showing bla_{OXA-48} from *E. coli* and *C. koseri* strains. 406: *E. coli* 406, T406: Transconjugant of *E. coli* 406; 86: *C. koseri* 86, T86: Transconjugant of *C. koseri* 86; NC: negative control; PC: positive control (743 bp); MM-molecular marker (100 bp).

RESULTS

The routine antibiogram with disc diffusion method revealed that both isolates were resistant to ampicillin, amoxicillin-clavulanic acid, cefazolin, and were susceptible to ceftazidime and gentamicin. Additionally, *E. coli* 406 was resistant to levofloxacin and trimethoprim-sulfamethoxazole and *C. koseri* was resistant to ceftriaxone. Although *E. coli* 406 was resistant to all tested carbapenems (imipenem: ≥16 mg/L, meropenem: ≥16 mg/L, ertapenem: ≥8 mg/L), *C. koseri* isolate was resistant to ertapenem (≥8) but presented intermediate susceptibility to imipenem (8 mg/L) and meropenem (8 mg/L) (Table 1).

The MICs for imipenem and meropenem were also detected as ≥32 mg/L and 8 mg/L for *E. coli* and *C. koseri*, respectively (Figure 2). The carbapenemase production was detected in both isolates by modified Hodge test. ESBL production was not observed in both of the strains.

The clinical isolates and their transconjugants were positive for bla_{oxa-48} however, the isolates lacked other β-lactamase genes such as bla_{TEM}, bla_{SHV}, bla_{CTX-M}, bla_{VIM}, bla_{IPM} and bla_{KPC}.

The antibiogram pattern of transconjugants revealed reduced susceptibility to carbapenems. The transconjugants of isolates were susceptible (≤0.25-2 mg/L) to imipenem, meropenem and ertapenem, however MIC for imipenem in transconjugants of *E. coli* 406 and *C. koseri* 86 were increased two fold. Additionally, MIC for ertapenem in transconjugant of *C. koseri* 86 were increased two fold. The transconjugants conferred resistance to ampicillin, amoxicillin-clavulanic acid, piperacillin-tazobactam and cefazolin. The resistance to β-lactams was not reduced by tazobactam and clavulanic acid (Table 1).

TABLE 1: The resistance genes and antibiotic susceptibilities of *Escherichia coli* 406 and *Citrobacter koseri* 86 clinical isolates and their transconjugants.

			MICs (mg/L) for		
	E. coli 406	*E. coli* J53 (p406)	*C. koseri* 86	*E. coli* J53 (p86)	
Antibiotics	bla_{OXA-48}	(bla_{OXA-48})	bla_{OXA-48}	(bla_{OXA-48})	*E. coli* J53
Ampicillin	≥32	≥32	≥32	≥32	≤2
Amoxicillin- clavulanic acid	≥32	≥32	≥32	≥32	4
Piperacillin-tazobactam	≥128	≥128	≥128	≥128	≤4
Cefazolin	≥64	8	≥64	32	≤4
Ceftazidim	≤1	≤1	4	≤1	≤1
Ceftriaxone	2	≤1	8	≤1	≤1
Ertapenem	≥8	≤0.5	≥8	1	≤0.5
Imipenem	≥16	2	8	2	≤1
Meropenem	≥16	≤0.25	8	≤0.25	≤0.25
Gentamicin	4	≤1	≤1	≤1	≤1
Levofloxacin	≥8	≤0.12	4	≤0.12	≤0.12
Tigecycline	≤0.5	≤0.5	1	≤0.5	≤0.5
Trimethoprim/Sulfamethoxazole	≥320	≤20	20	≤20	≤20

*MICs of E.coli 406, C.koseri 86 and their transconjugants, E.coli 406 (p406), C.koseri 86 (p86) and E.coli J53 were indicated.

MIC: minimum inhibitory concentration.

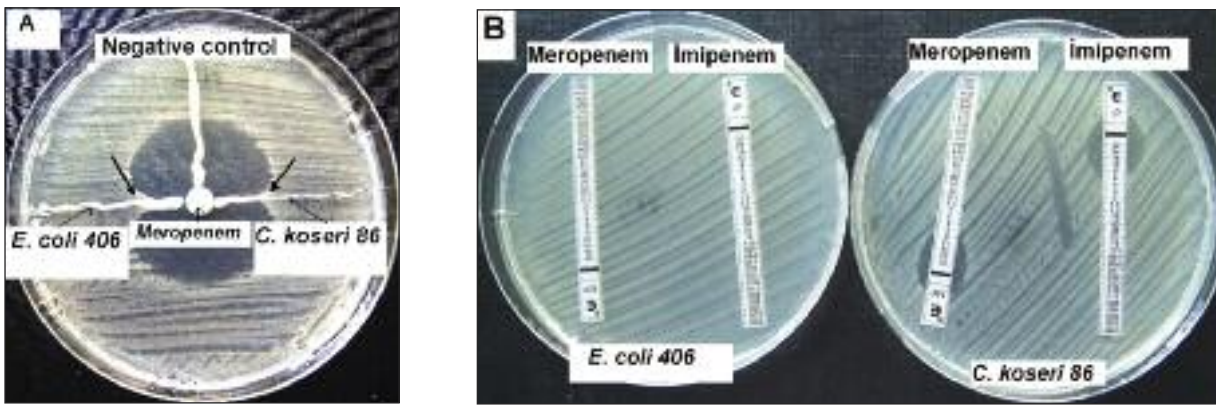

FIGURE 2: The modified Hodge test **(A)** and E-test of the *E. coli* and *C. koseri* isolates **(B)**. The arrow on the Figure 2A shows positive modify Hodge test.

DISCUSSIONS

The spread of carbapenemase-producing *Enterobacteriaceae* isolates is an important threat for the management of nosocomial infections. This carbapenem resistance is extremely important since this drug is a last resort of therapy and highly useful for many types of clinical infections. The class A carbapenemases, like KPC are present in *K. pneumoniae* strains and they are spreading rapidly on a worldwide scale. This type carbapenemases have been reported in various species of *Enterobacteriacae* from several parts of the world including USA, European countries, South America, Asia and Middle East.[14] Plasmid encoded class B metallo-β-lactamases such as IPM and VIM types are also distributed globally.[15] After the detection of first isolate of OXA-48 producing *K. pneumoniae* from Turkey,[1] other OXA-48 producing isolates, mostly *K. pneumoniae* and a few *E. coli*, were reported.[16,17] However, a recent report of an outbreak from İstanbul indicated that it is a real threat for Turkey.[18] The study demonstrated that dissemination of the bla_{OXA-48} gene is not spreading by a single *K. pneumoniae* clone, but several OXA-48-producing clones widely distributed in

İstanbul. In addition to these findings, *bla*$_{OXA-48}$ and ESBLs including *bla*$_{TEM}$, *bla*$_{SHV}$, *bla*$_{CTX}$ and *bla*$_{VEB}$ were not carried on the same plasmids. In the present study, similar to these findings, only *bla*$_{OXA-48}$ gene was detected in both strains/their transconjugants and it has been spreading not only in *E. coli* and *K. pneumoniae*, but also in another species such as *C. koseri.*

This study emphasized that, in addition to class A and class B carbapenemases, the class D carbapenemase OXA-48 type might lead significantly to carbapenem resistance in *E. coli* and *C. koseri. C. koseri* 86 presented intermediate susceptibility to imipenem and meropenem in contrast to ertapenem resistance. However, the transconjugants of the clinical isolates presented only reduced susceptibility to carbapenems. This finding suggested previous studious. Since OXA-48-producing isolates may be found susceptible to carbapenems when tested according to CLSI quidelines, the detection of OXA-48-producing strains are difficult for clinical laboratories. On account of this fact, this resistance genes may be more prevalent than expected.[2] Thus, another increasing concern is that the possibility of undetected spread of *bla*$_{OXA-48}$.

The co-existence of *bla*$_{OXA-48}$ with other resistance genes such as PMQR determinants (aac(6')-Ib-cr, qnr and qepA), together with the OXA-48 gene among nosocomial pathogens may limit alternative agents for the treatment of multi-drug resistant strains. In a previous study, a qnrA-positive-*Citrobacter freundii* isolate producing *bla*$_{VEB-1}$ and *bla*$_{OXA-48}$ has been reported from İstanbul.[19] In the present study, no additional β-lactamase genes and PMQR determinants were detected in contrast to previous studies.

The presence and worldwide dissemination of OXA-48 carbapenemase are worrisome issues according to the fact that carbapenems are often the last choice antibiotics for treating infections especially due to ESBL-producing strains. The presence of OXA-48 carbapenemase in rarely encountered species should also be considered.

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