Hidden Carbapenem Resistance in OXA-48 and Extended-Spectrum β-Lactamase-Positive

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Hidden carbapenemase resistance in OXA-48 and ESBL positive *Escherichia coli*

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Abstract

The purpose of this study is to report the identification OXA-48 carbapenemase in seven ESBL positive *E. coli* clinical isolates, fully susceptible to all carbapenems by disk - diffusion and E-test method, but with a borderline MIC values of MIC of ertapenem. This report points to the necessity for determination of carbapenem MICs in ESBL positive *E. coli* isolates and additional phenotypic testing for carbapenemases in all isolates with borderline ertapenem MIC defined by EUCAST. The isolates showed a high level of resistance to expanded-spectrum cephalosporins due to the production of an additional ESBL belonging to CTX - M family. All isolates isolates and their respective tranconjugants were found to possess L plasmid. PFGE analysis revealed two clusters containg higly related isolates. The global spread of multiresistant *E. coli* should be monitored closely due to the ability of isolates to rapidly obtain additional antibiotic resistance traits such as plasmid-mediated OXA-48 genes.

Keywords: *Escherichia coli*, ß-lactamases, ESBL, carbapenemases, OXA-48

β-lactams antibiotics are one of the most commonly used groups of anti-infective drugs in clinical practice for treating a variety of infections. Carbapenems are the last resort antibiotic for the treatment of serious infections associated with ESBL producing Enterobacteriaceae. Carbapenem resistance or reduced susceptibility in *E. coli* is becoming a huge therapeutic problem worldwide. *E. coli* produces several types of carbapenemases belonging to class A (KPC, IMI, NMC, SME), metallo-β-lactamases belonging to class B (IMP, VIM, NDM) or class D carbapenem hydrolyzing oxacillinases (OXA-48) [1,2]. OXA-48 is one of the few members of the class D found in Enterobacteriaceae. OXA-48 hydrolyzes penicillins except temocillin and carbapenems but spares expanded-spectrum cephalosporins. Similar to other class D carbapenemases, it is not inhibited by clavulanic acid, sulbactam or tazobactam. Multidrug-resistance in OXA-48 producing organisms results from the coproduction of an ESBL or AmpC beta-lactamase. It was first reported in *K. pneumoniae* originating from Turkey in 2004 [3]. In the following decade, OXA-48 has been reported in other species of Enterobacteriaceae in numerous European countries such as Germany, France, Portugal, Romania, but also in Far East countries [4-9]. Among many other classes of carbapenemases, OXA-48 shows an amazing ability of diffusion among different species and strains due to IS 1999 found within the Tn1999 transposon [5]. First reports of OXA-48 producing isolates of *K. pneumoniae* and *Enterobacter cloacae* in Croatia originated from 2016 but the first isolates were identified in 2010 to 2011 [10,11]. In the first study, three OXA–48 producing *K. pneumonia* isolates were identified in University Hospitals in Zagreb and Split. Two isolates from Zagreb coproduced VIM 1 and NDM 1 [10]. A recently published paper in 2017 revealed that the first OXA-48 producing *K. pneumonia* emerged in 2011 in Northwest Croatia [11]. In a recent multicenter study on carbapenemases in Croatia, three *K. pneumonia* and one *E. cloacae* isolate from Slavonski Brod, located in Slavonia region of Croatia, were shown to possess OXA-48 [12].

During 2016, 102 ESBL producing *E. coli* from various type of specimen were collected from hospitalized and community patients in Northeast part of Croatia, in Slavonia region. The phenotype of these isolates implied a CTX-M-type ß-lactamase with high level of resistance to cefotaxime and ceftriaxone. The isolates were subjected to antimicrobial susceptibility testing, PCR for detection of *bla*$_{\text{ESBL}}$ genes and plasmid characterization, in the frames of another study. Disk diffusion test revealed full susceptibility to all three carbapenems (imipenem, meropenem, ertapenem). However, seven out of 102 ESBL producing organisms showed resistant values of MIC for ertapenem (1 mg/L) and were positive in CIM test and Hodge test. Unexpectedly three of ertapenem non-susceptible isolates were shown to harbour A/C plasmid which was previously associated with VIM or NDM metallo-β-lactamases [13-16]. This finding prompted us to analyze the mechanisms of reduced susceptibility to ertapenem.

In this report, seven ertapenem non-susceptible *E. coli* originated from different anatomic sites of hospitalized patients (5 isolates from blood cultures, one wound swab, and one drainage pus) were analyzed.

The susceptibility to a wide range of antibiotics including amoxicillin, amoxicillin/clavulanate, piperacillin/tazobactam, cefazoline, expanded-spectrum cephalosporins (ESC - ceftazidime, cefotaxime, ceftriaxone), cefepime, imipenem, meropenem, ertapenem, gentamicin, and ciprofloxacin was determined by disk-diffusion and broth microdilution method according to EUCAST standards and protocols. *E. coli* ATCC 25933 and *K. pneumoniae* ATCC 700603 were used as quality control strains. The DDST and E-test ESBL (BioMerieux, France) were performed to detect ESBLs. Plasmid-mediated AmpC ß-lactamases were suspected by reduction of the inhibition zone around cefoxitin disc and confirmed by inhibitor based testing using cephalosporin disc supplemented with PBA (3-

aminophenyl boronic acid). Modified Hodge test and CIM test were performed to screen for carbapenemase activity. Additionally, the isolates were tested by combined disk tests with imipenem and meropenem alone and combined with PBA, 0.1 M EDTA or both to screen for KPC, MBLs, or simultaneous production of KPC and MBL, respectively [13,14,15,17].

The isolates were uniformly resistant to cefotaxime, ceftriaxone, gentamicin and ciprofloxacin and uniformly susceptible to imipenem, meropenem and colistin but demonstrated variable susceptibility/resistance phenotype to ceftazidime and cefepime as shown in Table 1. The results of the disk-diffusion test and gradient E-test (BioMerieux, France), showed susceptibility of all isolates to ertapenem with E-test MIC value ranging from 0,023-0,19 mg/L, but the microdilution method yielded ertapenem MIC of 1 mg/L. The isolates exhibited variable MIC values of amoxicillin/clavulante, ranging from 8 to 32 mg /L. Contrarily all isolates were susceptible to piperacillin/tazobactam with the MIC values ranging from 4-8 mg /L as shown in Table 1.

The isolates were positive in DDST, Hodge, and CIM test indicating the production of an ESBL and carbapenemase.

The transferability of cefotaxime resistance and reduced susceptibility to meropenem was determined by conjugation (broth mating method) at 35°C employing *E. coli* J65 recipient strain resistant to sodium - azide [18]. The transconjugants were selected on MacConkey agar containing cefotaxime (2 mg/L) or meropenem, (0.12 mg/L) and sodium azide (100 mg/L).

All isolates transferred cefotaxime resistance to *E. coli* recipient strains with cefotaxime as selective agent with the frequency ranging from 2.2 x 10^{-5} to 1,2 x$10^{-2.}$ Resistance to ciprofloxacin, gentamicin and trimethroprim/sulfamethoxazole was cotransferred to the recipient strain alongside with ceftotaxime from six isolates. Resistance to tetracycline was

cotransferred from one isolate whereas chloramphenicol resistance was not transferable. When meropenem was used to suppress the growth of donor strains four isolates transferred meropenem resistance to *E. coli* recipient strain with the frequency ranging from 3 $x10^{-5}$ to 10^{-5}.

The presence of bla_{CARB} (*bla* $_{KPC}$, bla_{VIM}, bla_{IMP}, bla_{NDM}, bla_{OXA-48}) and bla_{ESBL} genes (bla_{SHV}, bla_{TEM}, bla_{CTX-M}) was determined by PCR as described previously [19,20,21,22].

All isolates were shown to possess group 1of CTX-M β-lactamases in addition to OXA-48 group. Four isolates additionally possess TEM-1 β-lactamase. Sequencing of PCR amplification products revealed bla_{OXA-48}, $bla_{CTX-M-15}$ and bla_{TEM-1} genes.

Bla_{OXA-48} genes were preceded by IS*1999* whereas IS*Ecp* was found upstream of $bla_{CTX-M-15}$ genes in three isolates, as shown in Table 1. The transconjugants obtained with cefotaxime as selective agents were positive for bla_{CTX-M} genes.whereas those obtained with meropenem as selective agent yielded product with primers specific for bla_{OXA-48} genes.

lasmids were extracted with Qiagen mini kit according to the manufacturer's recommendations, subjected to electrophoresis in 0.7% agarose gel and stained with ethidium bromide. *E. coli* NTCC with four plasmid of known size was used to determine the size of plasmids. PCR-based replicon typing (PBRT) was applied to determine the plasmid content of the tested isolates [16]. Since it was previously observed that PBRT can be inefficient in identifying IncL/M plasmid type, an updated method designated to identify and distinguish between IncL and IncM plasmids was applied [23].

The isolates possessed the plasmid of 60 kb. PBRT showed diversity of plasmids belonging to IncL, IncA/C, IncW, IncB/O, IncFIC, and IncW incompatibility plasmid group. The majority of plasmids were found to belong to group IncL usually associated with bla_{OXA-48} (7/7),

IncB/O (6/7), and IncW (5/7). IncA/C plasmid group, usually associated with *bla*$_{\text{ESBL,}}$ *bla*$_{\text{TEM}}$, *bla*$_{\text{SHV}}$ *bla*$_{\text{CTX-M}}$, *bla*$_{\text{OXA}}$, *bla*$_{\text{NDM,}}$ and *bla*$_{\text{IMP}}$ was found in three isolates as shown in Table 1.

PFGE genotyping of *Xba*I-digested genomic DNA was performed with CHEF-DRIII system (Bio-Rad, Hercules, CA, USA); the images were processed using the Gel-Compar software [24].

PFGE analysis revealed two clusters covering all seven isolates and containing subgroups of highly related isolates. The first cluster comprised isolates 16, 17, 18 and 20 all from blood cultures and all except one from surgical ICU. The isolates 9, 23 and 26 belonged to cluster II and originated from surgical wards but from different types of specimens (blood culture, surgical wound swabs, drainage pus) [Figure 1].

The study found a significant number of OXA-48 producing *E. coli* with hidden resistance to carbapenems which would be missed in routine laboratory testing. Disk diffusion and gradient E-test for ertapenem revealed full susceptibility to carbapenems and the isolates were identified as OXA-48 positive due to ertapenem dilution MIC determination for the purpose of another study. Phenotypic test such as CIM and Hodge test showed high sensitivity in detecting OXA-48 [14,15]. OXA-48 is known to exhibit variable MICs of carbapenems from full susceptibility to frank resistance. It does not hydrolyze expanded-spectrum cephalosporins but the elevated MICs of ceftazidime, ceftriaxone, and cefotaxime were attributed to the additional CTX-M-15 β-lactamase. In contrast to other studies, our isolates showed variable patterns of susceptibillity/resistance to penicillin combinations with inhibitors which is untypical for OXA-48 and could be explained by very low expression of *bla*$_{\text{OXA-48}}$ genes probably attributable to low plasmid or gene copy number but the clarification of this issue is beyond this study. Better activity of piperacillin/tazobactam compared to amoxicillin/clavulanate can be explained by higher intrinsic activity of piperacillin against

Enterobacteriaceae compared to amoxicillin. Our study demonstrated high diversity of plasmid types found in clinical isolates but Southern blotting should be done to confirm plasmid location of *bla*$_{\text{OXA-48}}$ genes.

Since 2015 infections caused by ESBL-producing *E. coli* have become frequent in the area from which the examined isolates originate. The carbapenems are regarded as the drug of choice for the treatment of infection caused by such isolates. High incidence of ESBL-producing isolates and associated resistance to non-beta-lactam antibiotics can lead to an extended use of the carbapenems in hospitalized and community-based patients, ultimately leading to a faster acquisition and spread of carbapenem resistance.

The study pointed out to the necessity of performing dilution methods and confirmatory tests in detecting class D carbapenemases in *E. coli* [8,10]. According to our results disk-diffusion and E-test exhibited false susceptibility to ertapenem. The similar phenomenon was previously reported for colistin testing in *A. baumannii*. In previous studies in Croatia *K. pneumoniae* was the first and the dominant species harboring OXA-48 [10,11]. Similarly, as in the previous studies, there was the polyclonal dissemination of OXA-48 positive *E. coli* and horizontal transmission of L plasmid determinant in *E. coli*. In contrast to the previous study, OXA-48 was the sole carbapenemase in the reported isolates [10]. *E. coli* isolates from this report, similar to the *K. pneumoniae* and *E. cloacae* from Slavonski Brod in the recent report were ESBL positive and resistant to ESC but the previous isolates showed reduced susceptibility to ertapenem in disk-diffusion test [12]. A possible explanation is that the expression of *bla*$_{\text{OXA-48}}$ gene is less pronounced in *E. coli* compared to other members of *Enterobacteriaceae* family. The diversity of plasmids detected in the isolates indicates the ability of *E. coli* to obtain various types of resistance [25].

We suggest additional carbapenems susceptibility tests, particularly in patients with severe clinical infections such as bacteremia, sepsis or CNS infections due to *E. coli* ESBL positive isolates, which demonstrate unsatisfactory therapeutic and clinical response to carbapenem therapy. It is not clarified yet in the clinical studies whether carbapenems are appropriate therapeutic option for infections due to carbapenemase producing organisms demonstrating *in vitro* susceptibility to carbapenems. There is always a risk of developing mutants hyperproducing OXA-48 if the patient is treated with carbapenem. All patients had severe, invasive, life-threatening infections and the appropriate antibiotic choice is of uppermost importance.

The global spread of multiresistant *E. coli* should be monitored closely due to the ability of isolates to rapidly obtain additional antibiotic resistance traits such as plasmid-mediated OXA-48 genes.

CONFLICT OF INTEREST

The authors declarer no conflict of interest

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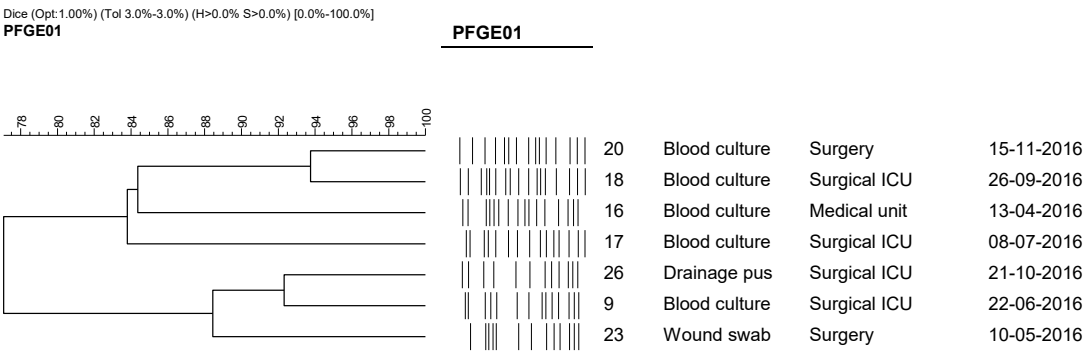

Figure 1. PFGE *E. coli* OXA-48 and ESBL positive isolates

PFGE of chromosomal DNA showing clustering of isolates. A cut-off value of 80 % was applied. Strains were considered to be clonally related if they showed > 80% similarity in the banding pattern. The image was processed with Gel-Compare software, and a dendogram was computed after band intensity correlation by the use of global alignment with 1,5% optimization. Specimen, hospital unit and date of isolation (dd/mm/yyyy) are shown. ICU, intensive-care unit

Isolate No	Date of isolation	Diagnosis	Hospital ward	Specimen	AMC	TZP	CTX	CRO	CAZ	FEP	IPM	MEM	ERT	GM	CIP	β-lactamase content	PBRT	PFGE
9. (19831)	22.06.2016.	UTI	ICU	Blood culture	16	8	>128	>128	32	16	0,06	0,06	1	32	>128	IS1999,*bla*$_{OXA-48,}$ *bla*$_{TEM-1,}$ *bla*$_{CTX-M-1}$	A/C, L, W, B/O	IIB
16. (11822)	14.04.2016.	UTI	Medical unit	Blood culture	8	4	>128	>128	32	64	0,12	0,12	1	32	>128	IS1999 *bla*$_{OXA-48}$ IEScP *blaCTX.M*$_{-15}$	W, L, B/O	IA
17. (21451)	08.07.2016.	UTI	ICU	Blood culture	8	4	>128	>128	8	64	0,25	0,06	1	64	>128	IS1999 *bla*$_{OXA-48}$ IEScP *blaCTX.M*$_{-15}$	X,L, B/O	IA
18. (30550)	26.09.2016.	UTI	ICU	Blood culture	32	8	>128	>128	32	64	0,5	0,5	1	64	>128	IS1999 *bla*$_{OXA-48}$ *blaCTX.M*$_{-1}$	W, L	IA
20. (32344)	15.11.2016.	UTI	Surgery	Blood culture	16	4	>128	>128	8	8	0,25	0,25	1	64	>128	IS1999 *bla*$_{OXA-48}$ *bla*$_{TEM-1}$ ISEcP *bla*$_{CTX.M-15}$	W, L, B/O	IA
23. (15101)	10.05.2016.	Ca colonis	Surgery	Wound swab	32	4	>128	>128	32	32	0,25	0,06	1	64	32	IS1999 *bla*$_{OXA-48}$ *bla*$_{TEM-1}$ *bla*$_{CTX.M-1}$	A/C, L, B/O, FIC	IIB
26. (31911)	21.10.2016.	Ileus	ICU	Drainage content	32	4	>128	>128	32	32	0,25	0,25	1	64	>128	IS1999 *bla*$_{OXA-48}$ *bla*$_{TEM-1}$ *bla*$_{CTX-M-1}$	A/C, L, W, B/O	IIB

Table 1. MIC's, β-lactamase content, frequency of conjugation, plasmid types and pulsed-field gel electrophoresis clusters of OXA-48 and ESBL positive *E. coli*

Abbreviations

MIC – minimal inhibitory concentration(microdillution method); UTI- urinary tract infection; ICU - intensive care unit; AMC - amoxicillin with clavulanate; TZP - piperacillin with tazobactam; CTX - cefotaxime; CAZ - ceftazidime; CRO - ceftriaxone;FEP – cefepime; IPM - imipenem; MEM - meropenem; ERT - ertapenem; GM - gentamicin; CIP – ciprofloxacin; *f* – frequency of conjugation; BL - beta-lactamase content; IS - insertion sequence; PBRT-PCR - based replicon typing; PFGE - pulsed field gel electrophoresis