Diversity of carbapenemases in clinical isolates of *Enterobacteriaceae* in Croatia—the results of a multicentre study

V. Zujić Atalić[1], B. Bedenić[2,3], E. Kocsis[4], A. Mazzariol[4], S. Sardelić[5], M. Barišić[6], V. Plečko[2,3], Z. Bošnjak[3], M. Mijač[3], I. Jajić[7], M. Vranić-Ladavac[8] and G. Cornaglia[4]

1) *Department of Microbiology, Public Health Institute Osijek, Osijek,* 2) *School of Medicine, University of Zagreb, Zagreb,* 3) *Department of Clinical and Molecular Microbiology, University Hospital Centre Zagreb, Zagreb, Croatia,* 4) *Department of Pathology and Diagnostics, University of Verona, Verona, Italy,* 5) *Department of Microbiology, University Hospital Centre Split, Split,* 6) *Elementary School Varaždin, Varaždin,* 7) *Department of Microbiology, Clinical Hospital Centre Sisters of Mercy, Zagreb and* 8) *Department of Microbiology, Public Health Institute of Istria County, Pula, Croatia*

Abstract

Since the first carbapenem-resistant *Klebsiella pneumoniae* strain was isolated in 2008, *Enterobacteriaceae* with reduced susceptibility to one or more carbapenems have emerged sporadically in different geographical regions in Croatia. These observations gave rise to a multicenter study on carbapenem resistance in *Enterobacteriaceae* from Croatia. Fifty-seven carbapenem-non-susceptible strains of *Enterobacteriaceae* were collected during 2011–2012 from four large hospital centres in Croatia. Overall, 36 strains produced VIM-1 β-lactamase, three produced NDM-1, and one produced KPC-2. A high degree of clonal relatedness was observed in *Enterobacter cloacae* and *Citrobacter freundii* strains, in contrast to *K. pneumoniae* strains. Bla_{VIM} genes were located within class1 integron which contained genes encoding resistance to aminoglycosides (aac_{A4}). The study found strong association between bla_{VIM} and qnr_{B6} and between bla_{NDM} and qnr_{A6} genes.

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Corresponding author: B. Bedenić, Department of Clinical and Molecular Microbiology, University Hospital Centre Zagreb, Kišpatićeva 12, 10000 Zagreb, Croatia
E-mails: branka.bedenic@zg.t-com.hr, branka.bedenic@kbc-zagreb.hr

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Carbapenems are often used to treat infections with *Enterobacteriaceae* expressing extended-spectrum β-lactamase (ESBL) and AmpC enzymes. However, β-lactamase-mediated resistance to carbapenems has been reported in *Enterobacteriaceae*, mostly because of expression of class A serin β-lactamases (KPC, SME, IMI, and NMC), class B metallo-β-lactamases (MBLs) of the IMP, VIM or NDM family, or OXA-48 β-lactamase belonging to the class D β-lactamase family. Furthermore, carbapenem resistance can be mediated by hyperproduction of ESBLs or plasmid-mediated AmpC β-lactamases combined with porin loss [1]. The first carbapenem-resistant enterobacteria in Croatia was an NDM-1-producing *Klebsiella pneumoniae* strain isolated in 2008 in the University Hospital Centre Zagreb [2]. Later, in 2011, a KPC-2-positive *K. pneumoniae* strain was isolated from the same institution [3]. A remarkable increase in the number of carbapenem-resistant isolates was observed in 2012. This observation gave rise to a multicentre study on carbapenem resistance in *Enterobacteriaceae* from Croatia.

A total of 57 carbapenem-non-susceptible strains of *Enterobacteriaceae* (32 *Enterobacter cloacae*, 13 *K. pneumoniae*, eight *Citrobacter freundii*, one *Klebsiella oxytoca*, one *Escherichia coli*, one *Serratia marcescens*, and one *Enterobacter amnigenus*) from various clinical specimens were collected during 2011–2012 from four large hospital centres located in different geographical regions of Croatia; the majority were from University Hospital Centre (UHC) Zagreb (52 strains), Clinical Hospital Centre Sisters of Mercy Zagreb (one strain), UHC Split (UHC; three strains), and General Hospital Pula (one strain). The total numbers of enterobacterial strains in the participating centres were 11 891 in UHC Zagreb, 3040 in Sisters of Mercy Hospital, 3828 in UHC Split, and 7707 in General Hospital Pula, with 121 (1%) strains, four (0.1%) strains, 28 (0.7%) strains and one (0.01%) strain with reduced susceptibility to at least one carbapenem, respectively. The strains with reduced susceptibility to either imipenem, meropenem (zone diameter of <23 mm) or ertapenem (zone diameter of <22 mm) were collected from 31 December 2011 until 31 December 2012. Only the strains fully resistant to at least one carbapenem were subjected to molecular analysis of resistance mechanisms.

The total number of microbiology laboratories in Croatia is 39, and four of them participated in this study (the locations of the centres are shown in the map in Fig. S1). The antimicrobial susceptibilities to a wide range of antibiotics were determined with the disk diffusion and broth microdilution method, and interpreted according to CLSI breakpoints [4]. MICs of tigecycline were interpreted according to the FDA breakpoints for *Enterobacteriaceae* (susceptible, ≤2 mg/L; intermediate, 4 mg/L; resistant, ≥8 mg/L). The double-disk synergy test [5] and CLSI combined disk test with the addition of clavulanic acid [4] were performed to detect ESBLs. Plasmid-mediated AmpC β-lactamases were detected with a combined disk test with cephalosporin disks combined with 3-aminophenylboronic acid (PBA). The modified Hodge test was used to screen for the production of carbapenemases [6]. Additionally, the strains were tested with 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 [7].

The strains were uniformly resistant to amoxycillin alone and combined with clavulanate, piperacillin, cefazoline, cefuroxime, cefotaxime, ceftriaxone, ceftazidime, and ertapenem, but uniformly susceptible to colistin. High resistance rates were observed for piperacillin–tazobactam (94.7%), meropenem (79.0%), imipenem (70%) ciprofloxacin (68.4%), gentamicin (70.2%), and cefepime (63.2%). Amikacin and tigecycline maintained good activity, with resistance rates of 14% and 7%, respectively. Susceptibility to tigecycline was variable, with MICs ranging from 0.25 to 8 mg/L. Seventeen strains had negative Hodge test results (seven *K. pneumoniae*, nine *E. cloacae*, and one *Escherichia coli*), and all showed resistance to ertapenem, and variable levels of susceptibility/resistance to imipenem or meropenem. The double-disk synergy test and the combined disk test with clavulanic acid were positive in all but four strains, indicating the production of ESBLs in 53 strains. The results of the modified Hodge test with carbapenems were consistent with the activity of carbapenemases in 40 of 57 strains. The combined disk test with PBA gave a positive result in one strain, as is is typical for KPC, and the rest of the 39 carbapenemase-positive strains showed augmentation of inhibition zone with EDTA, indicating the production of MBLs.

The transferability of meropenem resistance was determined by conjugation (broth mating method), with *Escherichia coli* A15R$^-$, which is resistant to rifampicin, and *Escherichia coli* J65, which is resistant to sodium azide [8]. Transconjugants were selected on combined plates containing meropenem (1 mg/L) to inhibit the growth of the recipient strain, and rifampicin (256 mg/L) or sodium azide (100 mg/L) to inhibit the growth of the donor strain.

Fourteen *E. cloacae*, three *C. freundii* and four *K. pneumoniae* strains transferred carbapenem resistance to the *Escherichia coli* recipient. The frequency of conjugation ranged from 1×10^{-8} to 3×10^{-4}. MICs of carbapenems in transconjugants were one to two dilutions lower than those of their respective donors.

Isoelectrofocusing was performed according to Matthew *et al.* [9]. Thirty-six strains (63%) produced a β-lactamase with a pI of 5.3, corresponding to VIM-1, three produced a β-lactamase with a pI of 5.8, corresponding to NDM-1 (5%), and one produced a β-lactamase with a pI of 6.7, corresponding to KPC-2 (1.8%), as shown in Table 1. Forty-eight strains showed a pI band of 5.4, consistent with TEM-1, 54 showed a pI band of 8.9, consistent with CTX-M-15, and 45 showed a pI band of >9, consistent with AmpC β-lactamase. Fourteen *K. pneumoniae* strains showed a band with a pI of 7.6, corresponding to the intrinsic SHV-1 β-lactamase of this species (Table 1). The presence of genes encoding broad-spectrum β-lactamases and ESBLs (bla_{SHV}, bla_{TEM}, $bla_{CTX\text{-}M}$, and $bla_{PER\text{-}1}$) [10–13], plasmid-mediated AmpC β-lactamases [14], class A carbapenemases (bla_{KPC}, bla_{SME}, bla_{IMI}, and bla_{NDM}), MBLs (bla_{VIM}, bla_{IMP}, and bla_{NDM}) and carbapenem-hydrolysing oxacillinases ($bla_{OXA\text{-}48}$) [15] was determined by PCR with protocols and conditions as described previously. PCR assays with primers 5′-CS and 3′-CS combined with forward and reverse primers for bla_{VIM} were performed to determine the location of bla_{VIM} within the class 1 integron [16]. Amplicons were column-purified with a Qiagen DNA purification kit (Inel, Zagreb, Croatia), and sequenced directly by Macrogene Europe sequencing service (sequenced in South Korea). $Bla_{KPC\text{-}2}$ was carried by only one *K. pneumoniae* strain. Bla_{VIM} was carried by 22 *E. cloacae* strains, seven *C. freundii* strains, four *K. pneumoniae* strains, one *K. oxytoca* strain, one *S. marcescens* strain, and one *E. amnigenus* strain, and was associated with TEM-1 in 32 strains, CTX-M-15 in 34 strains, CMY in ten strains, and DHA-1 in one strain. Three strains were found to produce NDM-1-type MBLs (one *E. cloacae* strain, one *C. freundii* strain, and one *K. pneumoniae* strain), accompanied by CTX-M-15 and CMY-4 in all three. Twenty-one transconjugant strains were subjected to PCR with primers specific for KPC, VIM and NDM β-lactamases, in order to analyse carbapenem resistance in an isogenic *Escherichia coli* strain. Nineteen strains were found to be positive for bla_{VIM}, one for bla_{KPC}, and one for bla_{NDM}, like their respective donors. The strains without true carbapenemases did not transfer carbapenem resistance to the *Escherichia coli* recipient strain. Bla_{VIM} was located within the class 1 integron, which contained genes encoding resistance to aminoglycosides (*aacA4*) as the first gene cassette. The plasmid-borne quinolone resistance genes *qnrA*, *qnrB* and *qnrS* were determined by PCR as described