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MOLECULAR CHARACTERIZATION OF CLASS B CARBAPENEMASES IN ADVANCED STAGE OF DISSEMINATION AND EMERGENCE OF CLASS D CARBAPENEMASES IN *ENTEROBACTERIACEAE* FROM CROATIA

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ABSTRACT

Carbapenemases involved in acquired carbapenem resistance in *Enterobacteriaceae* belong to Ambler class A serin β-lactamases, class B metallo-β-lactamases (MBL) or class D OXA-48-like β-lactamases. The aim of the present study was to analyse the molecular epidemiology and the mechanisms and routes of spread of class B and class D carbapenemases in Croatia.

In total 68 isolates were analyzed. Antibiotic susceptibility was determined by broth microdilution method. PCR was used to detect antibiotic-resistance genes. Genotyping was performed by rep-PCR and MLST.

Sixty-five isolates were found to harbour VIM-1 carbapenemase, seven of which were positive also for NDM-1, while two strains harboured only NDM-1. OXA-48 was detected in three isolates, two of which coproduced VIM-1. Thirty-six strains possessed additional CTX-M-15 β-lactamase whereas 64 were positive for TEM-1. CMY was found in 18 *C. freundii* isolates and DHA-1 in one *E. cloacae* isolate. Four different plasmid-incompatibility groups were found: A/C, L/M, N and FIIAs. Unlike *C. freundii* and *E. cloacae*, *K. pneumoniae* showed high diversity of rep-PCR patterns. *E. cloacae* and *C. freundii* predominantly belonged to one large clone which was allocated to ST105 and ST24, respectively.

Three different types of carbapenemases were identified showing the complexity of CRE in Croatia.

Key words: *Enterobacteriaceae,* VIM-1, NDM-1, OXA-48, carbapenems

1. Introduction

Carbapenems are the drugs of choice for the treatment of infections caused by multiresistant Gram-negative bacilli (Queenan and Bush, 2007). Carbapenemases involved in acquired resistance to carbapenems in *Enterobacteriaceae* belong to Ambler class A serin β-lactamases (KPC, GES, SME, IMI, NMC), class B metallo-β-lactamases (MBL) of IMP, VIM or NDM family or OXA-48-like β-lactamases belonging to the class D (Canton et al., 2012). The class B enzymes are the most clinically important carbapenemases, because they are capable of hydrolyzing all β-lactam antibiotics except of aztreonam (Queenan and Bush, 2007).

The first carbapenem–resistant Enterobacteria detected in Croatia was NDM-1 producing *Klebsiella pneumoniae* isolated in 2008 in the University Hospital Center Zagreb (Mazzariol et al., 2012). A remarkable increase in the number of carbapenem-resistant isolates was observed in 2012. This observation gave rise to a multicenter study on carbapenem-resistance in *Enterobacteriaceae* from Croatia, conducted in 2011 to 2012 which revealed the predominance of VIM-1 β-lactamase in two large hospital centers (Zujic-Atalic et al., 2014)). Two years later the clonal outbreak of VIM-1 positive *Enterobacter cloacae* and *Citrobacter freundii* at alarming rate was observed in the largest hospital center in Croatia and emergence of OXA-48 β-lactamase in *Enterobacteriaceae* was reported for the first time in two hospital centers in Croatia. This prompted us to analyse the epidemiology, the mechanisms of antibiotic resistance, and the routes of spread of class B and class D carbapenemases in Croatia.

2. Material and methods

2.1.Bacterial isolates

In total 68 non-duplicate isolates producing class B and D carbapenemase were analyzed: 34 *Enterobacter cloacae,* 18 *Citrobacter freundii,* 15 *Klebsiella pneumoniae* and 1 *Klebsiella oxytoca*.

Isolates were obtained from three hospital centers located in different geographic regions of Croatia (University Hospital Zagreb 60 isolates, University Hospital Split seven isolates and University Hospital Osijek one isolate) were analyzed. KPC positive strains and those with carbapenem non-susceptibility due to porin loss combined with ESBLs were not included in the study. KPC-positive isolates were included in another study. The strains were identified by Vitek2 (Biomerieux) and Maldi-TOFF (Bruker).

Since 2012 surveillance system for detection of carbapenem-resistant *Enterobacteriaceae* from different regional hospitals including phenotypic and molecular detection of resistance genes has been established in University Hospital Center Zagreb.

During the study period (2013-2014) in University Hospital Center Zagreb, decreased susceptibility to carbapenems was detected in 306 strains of *Enterobacteriaceae* belonging to four genera (out of 19938 in total). Sixty strains out of 306 produced class B and class D carbapenemases and all were subjected to molecular analysis. The rest of the strains produced KPC (class A) or had reduced susceptibility to carbapenems due to porin loss in combination with ESBL production and they were not the subject of the study. In University Hospital Center Split there were 86 isolates (out of 5610 in total) showing reduced susceptibility to carbapenems and belonging to three genera. In total, 29 strains (out of 86) were positive for class B and D β-lactamases, seven of which were subjected to molecular analysis.

In University Hospital Osijek there was one carbapenem-resistant isolate in 2013. It produced class B carbapenemase. Data for 2014 are not available. In Children's hospital there were no carbapenemase-producing isolates in 2013-2014. The total number of *Enterobacteriaceae* in Children's Hospital during the study period was 2493.

2.2. Antimicrobial susceptibility testing and phenotypic tests for detection of ESBLs, plasmid-mediated AmpC β-lactamases and carbapenemases

The antimicrobial susceptibility to amoxicillin alone and combined with clavulanate, piperacillin/tazobactam, cefazoline, cefuroxime, ceftazidime, cefotaxime, ceftriaxone, cefepime, cefoxitin, imipenem, meropenem, gentamicin, ciprofloxacin and colistin was determined by disk-diffusion and broth microdilution method according to CLSI standardss (CLSI, 2012) and for colistin according to EUCAST standard (Http://www.eucast.org). The strains were classified as multidrug-resistant, extensively- drug resistant or pan- drug resistant (Magiorakis et al., 2012).

Double disk synergy test (DDST) (Jarlier et al., 1988) and CLSI combined disk test with addition of clavulanic acid to cephalosporin disks were performed to detect ESBLs (CLSI, 2012). Chromosomal or plasmid-mediated AmpC β-lactamases were detected by combined disk test using cephalosporin disks combined with PBA (3-aminophenylboronic acid (Coudron, 2005). Modified Hodge test (MHT) was used to screen for the production of carbapenemases (Lee et al., 2003). Additionally the isolates were tested by combined disk tests with imipenem and meropenem alone and combined with 3-aminophenylboronic acid test (PBA), 0.1 M EDTA or both to screen for KPC, MBLs, or simultaneous production of KPC and MBL, respectively (Pasteran et al 2009, Kim et al., 2007).

2.3. Conjugation

The transferability of ceftazidime resistance was determined by conjugation (broth mating method) at 35°C employing *E. coli* A15R^{-} strain resistant to rifampicin and *E. coli* J65 resistant to sodium azide (Elwell and Falkow, 1986).

2.4. Molecular detection of resistance genes

The genes conferring resistance to β-lactams including broad spectrum and extended-spectrum β-lactamases (*bla*$_{SHV}$, *bla*$_{TEM}$, *bla*$_{CTX-M}$ and *bla*$_{PER-1}$) (Nuesch-Inderbinen et al., 1996; Arlet et al., 1995; Woodford et al., 2004; Pagani et al., 2004; Woodford et al., 2006), plasmid-mediated AmpC β-lactamases (Perez-Perez and Hanson, 2002), class A (*bla*$_{KPC}$, *bla*$_{SME}$, *bla*$_{IMI}$, *bla*$_{NMC}$,) and class B carbapenemases (*bla*$_{VIM,}$ *bla*$_{IMP}$ and *bla*$_{NDM}$) and carbapenem hydrolyzing oxacillinases (*bla*$_{OXA-48-like}$) (Poirel, et al, 2011) and to fluoroquinolones (*qnr*A, *qnr*B, *qnr*S) (Robiscek et al., 2006) were determined by PCR using 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 done to determine the location of *bla*$_{VIM}$ gene within class 1 integron (Jeong et al., 2003).

2.5. Characterization of plasmids

Plasmids were extracted from donor strains and their respective transconjugants with Qiagen Mini kit (Medical Intertrade, Zagreb, Croatia) according to the manufacturer's instructions. After staining with ethidium bromide, the DNA was visualised by ultraviolet light [6].

The sizes of the isolates' plasmids were estimated from the standard curve of migration distance versus logarithm (log10) of the molecular weight of four standard plasmids (148, 64, 36 and 7 kb) from *E. coli* NTCC 50192.

PCR-based replicon typing (PBRT) (Carattoli et al., 2005) was applied to determine the plasmid content of the tested strains. Plasmid extractions obtained from transconjugant strains were subjected to PCR for detection of carbapenemases (MBL and OXA-48) and ESBLs in order to determine the location of *bla* genes and to PBRT to determine if the transconjugants harbour the same plasmid types as their respective donors.

2.6. Genotyping

Thirty-one *E. cloacae*, 16 *C. freundii* and 8 *K. pneumoniae* isolates were subjected to molecular typing by rep-PCR as described previously (Overdest et al, 2011).

The same strains were also genotyped by MLST according to Diancourt (Diancourt et al., 2005). For *C. freundii* and *E. clocacae* analysis was done according to the website developed by Keith Jolley: (http://pubmlst.org/cfreundii/http://pubmlst.org/ecloacae/

3. RESULTS

3.1. Patients and isolates

The patients did not have travel history to the endemic areas. The clinical wards included in the study were: medical, haematology, urology, paediatric haematology, psyhiatrics, medical, surgical, and neurological intensive care unit (ICU), while two strains were obtained from outpatients previously treated in University Hospital Center Zagreb.

3.2. Antimicrobial susceptibility testing and phenotypic tests for detection of β-lactamases

All isolates were multidrug resistant according to Magiorakis *et al* since they displayed resistance to at least one antibiotic in three antimicrobial classes. The strains were uniformly resistant to amoxicillin alone and combined with clavulanic acid, cefazoline, ceftazidime, cefotaxime, ceftriaxone and cefoxitin and all but one (*Enterobacter cloacae*), susceptible to colistin as shown in Table 1a, b and c and Table 2. Amikacin was the second most active antibiotic with 35/67 (52%) susceptible strains. Only three (4%) strains were susceptible to imipenem and 1 (1%) to cefepime and meropenem according to CLSI. MICs of carbapenems ranged from susceptibility to frank resistance, depending on the strain. Heterogenous susceptibility was observed with ertapenem resistance and imipenem and meropenem being susceptible, intermediate or resistant. The high resistance rates were noted also for ciprofloxacin (81%) and for gentamicin (84%). There was no significant difference in resistance rates between different species as shown in Table 2.

All strains were shown to be positive for carbapenemase production by Hodge test and all but one showed significant increase of the inhibition zone around carbapenems in the presence of

EDTA indicating the production of MBL. Sixty-three strains were phenotypically positive for ESBLs and only nine for AmpC as shown in Table 1.

3.3. Transfer of resistance determinants

Ceftazidime resistance was transferred to *E. coli* recipient resistant to sodium azide from 18 *C. freundii* strains (66, 74, 88, 95, 116, 118, 195, 255, 273, 287, 335, 337, 347, 348, 355, 356, 361 and 363) and 16 *Klebsiella* spp (strain number 67,73, 86, 87, 117, 174, 178, 216, 271, 279, 289, 290, 301, 305 4524 and 1755) with the frequency ranging from 10^{-7} to 10^{-5}. *Enterobacter cloacae* strains did not transfer ceftazidime resistance. Resistance to gentamicin, sulphametoxazole, tetracycline, cloramphenicol and fluoroquinolones was not cotransferred alongside with ceftazidime resistance.

3.4. Characterization of β-lactamases and other resistance genes

Sixty-five isolates were found to harbour VIM-1 carbapenemase, seven of which were positive for NDM-1 as well, while two strains harboured only NDM-1 (Table 1). OXA-48 was detected in three isolates, two of which coproduced VIM-1. Thirty-six strains possessed additional CTX-M-15 ESBL whereas 64 were positive for TEM-1. CMY was found in 18 *C. freundii* isolates and DHA-1 in one *E. cloacae* isolate. NDM-1 β-lactamase was carried by six *K. pneumoniae*, two *C. freundii* and one *E. cloacae* isolate. All *C. freundii* strains produced either CMY-2 or CMY-4 and all but one TEM-1 as additional β-lactamases to MBL. $bla_{\text{OXA-48}}$ genes were carried by two identical *E. cloacae* strains from Zagreb and one *K. pneumoniae* from Split as shown in Tables 1a, 1b and 1c. All except one *K. pneumoniae* strains were positive for SHV-1 while one produced SHV-11 which is also intrinsic chromosomal β-lactamase of this species. Other species (*E. cloacae* and *C. freundii*) were negative for SHV β-lactamases. Two *K. pneumoniae* strains tested positive for AmpC in phenotypic tests but failed to yield PCR product with primers specific for AmpC β-lactamases.

C. freundii transconjugant strains were positive for *bla*$_{VIM}$ genes and *bla*$_{NDM}$ genes as their respective donors while *bla*$_{OXA-48}$ gene was found in one *K. pneumoniae* transconjugant.

bla$_{VIM-1}$ genes were embedded in class 1 integrons which contained integrase gene followed by *bla*$_{VIM-1}$ and aminoglycoside resistance determinant aadA1. The same integron content was found in different strain genera.

*Qnr*B determinant was found in 20 strains. It was carried by 14 *E. cloacae* and three *C. freundii* and *K. pneumoniae* strains, respectively, as shown in Table 1.

3.5. Characterization of plasmids

The size of plasmids ranged from 50 to 150 kb in donor strains. Transconjugants harboured only the large plasmid of 80 to 150 kb present also in the respective donor strain. IncL/M was the dominant plasmid type identified in 31 isolates, followed by IncN and Inc A/C found in 21 and 19 strains, respectively. FIIA$_s$ was observed in only one *K. pneumoniae* strain positive for VIM-1 and NDM-1, as shown in Table 1. Combination of IncA/C and IncN incompatibility group was noticed in 15 strains (13 *E. cloacae* and 2 *K. pneumoniae*), followed by combination of IncL/M and IncA/C which was present in five strains (three *K. pneumoniae* and one *C. freundii*). All *C. freundii* strains were positive for IncL/M with one strain, as mentioned, carrying also IncA/C type of plasmid. However, the association of plasmid incompatibility group with carbapenemase production was not confirmed. Thirty- four transconjugant strains were positive for VIM, NDM, OXA-48, CTX-M and TEM β-lactamases as their respective donors confirming plasmid location of carbapenemase genes. The trasconjugant strains harboured the same plasmid types as their respective donors.

3.6. Genotyping

Rep-PCR

Unlike *C. freundii* and *E. cloacae* isolates *K. pneumoniae* showed pronounced diversity of their rep-PCR patterns. Eight isolates were classified into two clusters as shown in Figure 1a.

The cluster KI comprised strains 271 and 290 both belonging to ST 1574 while the second cluster KIV included strains 301 and 305 belonging to ST636. Four strains were singletons: 86, 87, 17555 and 73. Five different STs were found: ST1574, ST859, ST15, S636, ST321 all associated with VIM-1 except of ST37 associated with OXA-48.

Sixteen *C. freundii* isolates subjected to genotyping were allocated into two clusters: one large CI with 11 isolates (88, 361, 347, 355, 273, 287, 95, 356, 348, 66, 195) all belonging to ST24 and one small CIV with only two isolates (116 and 255) (Fig. 1b). Three isolates were singletons: 335, 337 and 363. Two different ST were identified: ST24 linked to the large clone and ST8 found in a singleton strain 337.

The *E. cloacae* isolates from Split showed different rep-PCR patterns compared to those from Zagreb. Eight different STs were observed: ST91, ST92, ST105, ST133, ST134, ST190, ST419 and ST418. ST418 which was associated with a small clone comprising three isolates from Zagreb positive for VIM-1 (281, 299 and 164), ST105 was associated with the large clone with 18 isolates from Zagreb positive for VIM-1 (strains 166, 168, 196, 104, 323, 237, 181, 274, 89, 200, 204, 106, 149, 146, 213, 157, 186 and 372), and ST 92 comprising three isolates from Split positive for VIM-1 (6071, 20080 and 12488) and one from Zagreb (266). One *E. cloacae* isolate from Osijek (3294) had identical rep-PCR pattern as the strain from Zagreb (218) (Fig. 1c). It was obtained from the child that was previously hospitalized at the paediatric unit in University Hospital Centre Zagreb and thus it can be concluded that it was imported from Zagreb.

4. Discussion

Croatia is still a country with relatively low prevalence of carbapenem-resistant *Enterobacteriaceae* (CRE; approximately 1 %), compared to other Mediterranean countries

with up to 50% of CRE such as Greece or Cyprus (Canton et al., 2012). The majority of our MBL-positive *Enterobacteriaceae* carried *bla*$_{\text{VIM-1}}$ gene. The study demonstrated endemic occurrence of VIM-1 β-lactamase among CRE in University Hospital Zagreb. The first VIM-1 entrobacterial isolate was *E. coli* from Greece reported in 2001 (Miriagou et al., 2003). This type of MBL was previously associated with clonal outbreak in Split also in *E. cloacae* (Novak et al., 2014). The majority of VIM positive *E. cloacae* and *K. pneumoniae* isolates in our study harboured additional TEM-1 and CTX-M-15 β-lactamase that are usually carried on the same plasmid as *bla*$_{\text{VIM}}$ genes, whereas *C. freundii* isolates were positive for *bla*$_{\text{CMY}}$ genes which are chromosomally encoded in this species. In contrast to other European countries where *K. pneumoniae* was the dominant host harbouring VIM-1, in our study *bla*$_{\text{VIM-1}}$ gene was carried predominantly by *E. cloacae,* followed by *C. freundii*. The majority of VIM-1 strains originated from University Hospital Center Zagreb, but a few strains were obtained from University Hospital Split and University Hospital Osijek. The strain from Osijek was imported from Zagreb, due to patient transfer (Bedenić et al., 2015b). It had the same β-lactamase content, rep-PCR pattern and PBRT as the strain from the same hospital unit in Zagreb. The VIM carrying *E. cloacae* strains from Split were not related to those from Zagreb suggesting that they arose as independent event. None of our VIM-producing *K. pneumoniae* strains belonged to the widespread ST 147 clone (Canton et al., 2012). However, the plasmids carrying *bla*$_{\text{VIM}}$ genes were similarily as in other European countries allocated most frequenty in IncA/C, IncL/M or IncN type plasmid.

NDM-1 β-lactamase was identified in nine of our isolates. It originated from Indian subcontinent (Yong et al., 2009), but later spread in many European with UK being the most important reservoir of NDM positive CRE in Europe. The first NDM-1 positive isolate in Croatia was imported from Bosnia and Herzegovina in 2008 (Mazzariol et al., 2012) and was probably a representative of a Balkan clone. In our study *K. pneumoniae* was the dominant

species carrying the *bla*$_{\text{NDM-1}}$ which is in concordance with the rest of Europe. *K. pneumoniae* isolates positive for NDM belonged to different STs not reported in other European countries but similarly as in other countries the plasmids probably carrying *bla*$_{\text{NDM}}$ genes belonged to IncA/C or IncL/M although we did not prove with certainty that these plasmids carried *bla*$_{\text{MBL}}$ genes.

The study demonstrated emergence of OXA-48 β-lactamase in two hospital centers located in different geographic regions in Croatia. The strains displayed different rep-PCR patterns leading to conclusion that they occurred as independent events. OXA-48 was first report in Turkey in 2001 (Gulmez et al., 2008; Aktas et al., 2008) but later spread in many European countries (Giani, 2012, O'Brien 2011) and also in Slovenia (Pirs et al., 2011) a country adjacent to Croatia. Croatia was spared from this type of carbapenemase until 2014. OXA-48-producing *K. pneumoniae* originating from hospital in Split belonged to ST37 unlike UK where it belonged to ST221 (Canton et al, 2012). and the Netherland where it was allocated to ST636 (Kalpoe et al., 2011). ST37 was in our previous study associated with *bla*$_{\text{KPC-2}}$ gene (Bedenić et al., 2012,a) indicating that different resistance markers can been acquired by the strains belonging to the same clone. Two *E. cloacae* isolates harbouring OXA-48 were assigned to ST 418 and showed identical rep-PCR patterns. They were both obtained from outpatients previously treated at the haematology unit of hospital in Zagreb in the same time period. Most of the OXA-48 positive strains in Western Europe were imported from Turkey, Morocco, Egypt, Algeria or Lybia (Canton et al., 2012). In our study there was no link to the endemic areas which leads to conclusion that *bla*$_{\text{OXA-48}}$ genes arouse independently and were not imported from elsewhere. PCR for plasmid-incompatibility group was negative in all our strains while in other European studies they were associated with L/M, A/C, P or W type (Canton et al., 2012, Pfeifer et al, 2012) .

Interestingly, all *C. freundii* strains were found to be phenotypically positive for ESBLs but PCR did not reveal any tested ESBL gene. All were found to be positive for CMY β-lactamases which are chromosomally encoded in this species although phenotypic tests for AmpC were negative. This could be due to low expression of bla_{CMY} genes in *C. freundii*. Moreover, *C. freundii* was more uniform in plasmid content compared to other species with IncL/M being found in all strains. Genotyping of this species also revealed high level of relatedness demonstrated by both rep-PCR and MLST indicating that one clone was dominant in different hospital units during the study period in contrast to *K. pneumoniae* which showed high diversity of rep-PCR patterns, STs and plasmid incompatibility group suggesting that different strains and plasmids were involved in the spread of MBLs in this species.

On the other hand six *E. cloacae* strains were phenotypically positive for AmpC but did not demonstrate any plasmid-mediated AmpC by PCR. This could be due to overexpression of chromosomal AmpC β-lactamase of *E. cloacae*. Sixty-three strains were positive for ESBL in inhibitor based test with clavulante but only 36 were positive for CTX-M-15. Other types of ESBLs belonging to TEM or SHV family were not found. The rest of the strains had probably false positive ESBL test or had some rare type of ESBL which was not sought in this study. Laboratory detection of OXA-48 β-lactamase pose a serious problem because of low level carbapenem resistance and sometimes lack of additional ESBL. However, in our study all three strains had elevated carbapenem MICs and possessed additional CTX-M-15 β-lactamase. Production of additional VIM-1 β-lactamase in two strains contributed to carbapenem resistance. Unfortunately, there is no inhibitor-based test for detection of OXA-48 β-lactamase. Coproduction of VIM-1 and NDM-1 was found in seven strains. It was previously reported in Morocco (Barguigua A et al., 2013). Combination of NDM-1 and OXA-48 was identified previously in India (Khajuria et al., 2014). In our study we found combination of VIM-1 with OXA-48. Laboratory detection of MBLs in *Enterobacteriaceae*

may also be difficult because of variable MICs of carbapenems. In our study even 8 strains had MIC of imipenem of 2 mg/L or lower and would be considered susceptible if EUCAST criteria were applied (http://www.eucast.org/). Interestingly, the strains from heamatology unit were obtained mostly from stool as surveillance culture whereas the strains from medical ward and urology originated from urine and were associated with infection.

Since the plasmids encoding carbapenemases often contain *bla*$_{\text{ESBL}}$, *bla*$_{\text{AmpC}}$ and *qnr* genes, we can assume that the therapeutic administration of carbapenems for the treatment of severe infections facilitates not only the spread of carbapenemases, but also ESBLs, plasmid-mediated AmpC β-lactamases and fluoroquinolone resistance determinants leading to extensively drug resistant or pandrug resistant phenotype. Emergence of colistin resistance among carbapenemase-producing *Enterobacteriaceae*, as demonstrated in one study strain, is also worrying.

Unfortunately, hospital hygiene measures and screening for faecal carriage did not prevent the spread of CRE in Croatian hospitals. Three different types of carbapenemases were identified showing the complexity of CRE in Croatia. The diversity of carbapenemases can be attributed to the several different introduction into the hospitals environment, their evolution during clonal dissemination, and transfer of plasmids. Similarly as in our first study VIM-1 was the dominant type of MBLs accompanied by sporadic occurrence of NDM-1 and the appearance of OXA-48 in 2013. *bla*$_{\text{VIM-1}}$ genes were carried by variably transferable plasmids probably belonging to IncA/C or IncL/M with low frequency of conjugation in mating conditions applied in this study. However, we cannot prove with certainty that those plasmids carried carbapenemase genes. In concordance with the previous study, the University Hospital Center Zagreb was shown to be the most important reservoir of CRE with predominance of a single *E. cloacae* clone carrying VIM-1 encoding gene together with CTX-M-15 and TEM-1

encoding genes. The *E. cloacae* isolates from Split were not clonally related to those from Zagreb but showed the same type of MBL (VIM-1) and the same class 1 integron structure.

The fact that two strains coharboured VIM-1 and OXA-48 β-lactamase points out to amazing ability of *Enterobacteriaceae* to accumulate resistance genes as described previously (Chang et al., 2015)

Early identification of class B and class D carbapenemases and isolation of carriers seem to be the mainstone of effective infection control strategy in the hospital setting which also prevents the propagation of this important resistance determinants into the community.

Figure legends

Fig. 1.

Rep-PCR dendrogram of study strains. Date of isolation and specimen type are shown. Similarity treshold of 97% was applied to define a clone (no difference in banding pattern).

Fig. 1a. Rep-PCR dendrogram of *K. pneumoniae* isolates

Fig. 1b. Rep-PCR dendrogram of *C.freundii* isolates

Fig. 1c. Rep-PCR dendrogram of *E. cloacae* isolates

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Conflict of interest declaration

The authors do not have any conflict of interest.

Ethical statement

The study was done according in concordance with ethical standards. The ethical approval was not necessary because the study did not involve human or animal subjects.

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Table 1. Phenotypic tests, MICs, β-lactamase content, rep-PCR clusters, plasmid types and fluoroquinolone resistance genes of carbapenemase producing, *Klebsiella* spp. (Table 1a) , *Citrobacter freundii* (Table 1b) *Enterobacter cloacae* isolates (Table 1c).

Abbreviations: ESBL-combined disk test using cephalosporins alone and combined with clavulanate for detection of ESBLs; AmpC-combined disk test using cephalosporins alone and combined with phenylboronic acid for detection of AmpC β-lactamases; MBL-combined disk test using carbapenems alone and combined with EDTA for detection of metallo-β-lactamases; IPM-imipenem; MEM-meropenem; ETP-ertapenem, GN-gentamicin; CIP-ciprofloxacin; COL-colistin; BL-β-lactamase; PBRT-PCR-based replicon type; ST- sequence type; NT-not tested

Table 1a. *Klebsiella* spp

	CENTRE AND ISOLATE NUMBER	SPECIMEN	DEPARTMENT	DATE	ESBL	AMPC	MBL	IPM	MEM	ETP	GN	CIP	COL	BL	REP PCR-CLUSTER AND/OR ST	PBRT	*Qnr*
1	SPLIT-4524	URINE	HAEMATOLOGY	11.03.2013.	+	-	+	8	8	16	16	0.5	0.12	SHV-1, TEM-1, CTX-M-15,, VIM-1	NT	L/M	NEG.
2	ZAGREB-8399 (67)	STOOL	HEAMATOLOGY	15.01.2013.	+	-	+	32	32	16	2	4	0.12	SHV-1, CTX-M-15, VIM-1	NT	NEG.	NEG.
3	ZAGREB-20157-1 (73)	STOOL	HAEMATOLOGY	01.02.2013.	+	-	+	32	32	16	2	8	0.12	SHV-1, TEM-1, CTX-M-15, VIM-1	KVI ST321	L/M, A/C	NEG.
4	ZAGREB-40840 (86)	SPUTUM	MEDICAL	04.03.2013.	+	-	+	8	16	4	4	32	0.25	SHV-1, TEM-1, CTX-M-15, VIM-1	KII ST859	L/M	NEG.
5	ZAGREB-40647 (87)	STOOL	HEAMATOLOGY	04.03.2013.	+	-	+	1	4	32	16	8	0.25	SHV-1, TEM-1, CTX-M-15, VIM-1	KIII ST15	NEG.	NEG.
6	ZAGREB-79559 (117)	RECTAL SWAB	MEDICAL HAEMATOLOGY	02.05.2013.	-	-	+	8	16	16	16	32	1	SHV-1, VIM-1	NT	NEG.	NEG.
7	ZAGREB-12762 (174)	STOOL		11.07.2013.	+	-	+	4	8	8	64	32	0.25	SHV-1, TEM-1, CTX-M-15, NDM-1	NT	N, A/C	B
8	ZAGREB-123327 (178)	URINE	UROLOGY	16.07.2013.	+	-	+	16	16	8	32	64	0.5	SHV-1, TEM-1, CTX-M-15, VIM-1	NT	N, A/C	NEG.
9	ZAGREB-145846 (216)	RECTAL SWAB	MEDICAL	28.08.2013.	-	-	+	4	8	32	32	32	0.5	SHV-11, TEM-1, NDM-1, VIM-1	NT	FIIAs	B
10	ZAGREB-199305 (271)	STOOL	PAEDIATRIC HAEMATOLOGY	12. 11.2013.	+	-	+	4	8	32	32	32	0.5	SHV-1, TEM-1,VIM-1	KI ST1574	L/M A/C	B
11	ZAGREB-208759 (279)	SPUTUM	MEDICAL ICU	12.12.2013.	+	-	+	4	8	16	128	16	0.5	SHV-1, TEM-1, CTX-M-15, VIM-1	NT	L/M	NEG.
12	ZAGREB-2494 -1(289)	STOOL	HEAMATOLOGY	07.01.2014	+	+	+	16	16	32	16	1	0.5	SHV-1, TEM-1, NDM-1, VIM-1	NT	L/M A/C	NEG.
13	ZAGREB-2494 -2(290)	STOOL	HEAMATOLOGY	07.01.2014	+	+	+	16	16	32	16	1	0.5	SHV-1, TEM-1, NDM-1, VIM-1	KI ST1574	L/M A/C	NEG.
14	ZAGREB-10536 (301)	STOOL	HEAMATOLOGY	21.01.2014.	+	-	+	16	8	16	>128	64	0.25	SHV-1, TEM-1, NDM-1, VIM-1	KIV ST636	L/M	NEG.
15	ZAGREB-10536 (305)	STOOL	HEAMATOLOGY	21.01.2014.	+	-	+	16	8	16	16	64	0.25	SHV-1, TEM-1, NDM-1, VIM-1	KIV ST636	L/M	NEG.
16	SPLIT-17555	URINE	MEDICAL	11.11.2014.	+	-	-	32	32	32	32	16	0.12	SHV-11, TEM-1, CTX-M-15, OXA-48	KV ST37	NEG.	NEG.

Table 1b. *Citrobacter freundii*

	CENTRE AND ISOLATE NUMBER	SPECIMEN	DEPARTMENT	DATE	ESBL	AMPC	MBL	IPM	MEM	ETP	GN	CIP	COL	BL	REP PCR-CLUSTER AND/OR ST	PBRT	*Qnr*
1	ZAGREB- 8767 (66)	STOOL	MEDICAL ICU	15.01.2013.	+	-	+	2	4	8	64	>128	0.25	TEM-1, CMY-4, VIM-1	CI	L/M	NEG.
2	ZAGREB-20157-2 (74)	STOOL	HAEMATOLOGY	01.02.2013.	+	+	+	32	32	16	2	8	0.12	TEM-1, CMY-4, VIM-1	NT	L/M.	NEG.
3	ZAGREB-41 613 (88)	STOOL	HEAMATOLOGY	05.03.2013.	+	-	+	2	2	64	8	>128	0.25	TEM-1, CMY-4, VIM-1	CI	L/M	NEG.
4	ZAGREB-47402 (95)	STOOL	HEAMATOLOGY	13.03.2013	+	-	+	2	16	16	64	>128	0.25	TEM-1, CMY-4, VIM-1	CI	L/M	B
5	ZAGREB-81601 (118)	BRONCHIAL CATHETER	MEDICAL	06.05.2013.	+	-	+	8	16	16	16	32	1	CMY-2, NDM-1	NT	L/M	NEG.
6	ZAGREB-8106 (116)	STOOL	PAEDIATRIC HEAMATOLOGY	30.04. 2013	+	-	+	8	16	16	16	64	1	TEM-1, CMY-4, VIM-1	CIV	L/M	B
7	ZAGREB-132452 (195)	STOOL	HEAMATOLOGY	01.08.2013.	+	-	+	8	8	16	64	32	0.5	TEM-1, CMY-4, NDM-1 VIM-1	CI ST24	L/M	NEG.
8	ZAGREB-186879 (255)	STOOL	PAEDIATRIC HEAMATOLOGY	08.11.2013	+	-	+	4	2	32	1	1	0.5	TEM-1, CMY-2, VIM-1	CIV ST24	L/M A/C	B
9	ZAGREB-207315 (273)	URINE	HEAMATOLOGY	10.12.2013	+	-	+	16	16	32	32	8	0.5	TEM-1, CMY-4, VIM-1	CI	L/M	NEG.
10	ZAGREB-217152 (287)	STOOL	HEAMATOLOGY	30.12.2013.	+	-	+	4	16	32	64	1	0.5	TEM-1, CMY-4, VIM-1	CI	L/M	NEG.
11	ZAGREB-56767 (335)	STOOL	HEAMATOLOGY	08.04.2014.	+	-	+	16	16	32	64	1	0.5	TEM-1, CMY-4, VIM-1	CII ST24	L/M	NEG.
12	ZAGREB-57576 (337)	URINE	MEDICAL	09.04.2014.	+	-	+	16	16	32	32	1	0.5	TEM-1, CMY-4, VIM-1	CV ST8	L/M	NEG.
13	ZAGREB-81336 (347)	STOOL	HEAMATOLOGY	21.05.2014.	+	-	+	16	16	32	>128	>128	0.25	TEM-1, CMY-4, VIM-1	CI	L/M	NEG.
14	ZAGREB-84809 (348)	STOOL	HEAMATOLOGY	27.05.2014.	+	-	+	16	16	32	>128	0.06	0.12	TEM-1, CMY-4, VIM-1	CI	L/M	NEG.
15	ZAGREB-97934 (355)	STOOL	HEAMATOLOGY	17.06.201.4	+	-	+	4	16	32	>128	0.06	0.12	TEM-1, CMY-4, VIM-1	CI	L/M	NEG.
16	ZAGREB-97415 (356)	STOOL	HEAMATOLOGY	27.05.2014.	+	-	+	4	16	32	32	>128	0.12	TEM-1, CMY-4, VIM-1	CI	L/M	NEG.
17	ZAGREB-104 881 (361)	STOOL	HEAMATOLOGY	01.07.2014.	+	-	+	16	16	32	32	>128	0.12	TEM-1, CMY-4, VIM-1	CI ST24	L/M	NEG.
18	ZAGREB-105513 (363)	STOOL	HEAMATOLOGY	07.07.2014.	+	-	+	16	16	32	32	>128	0.12	TEM-1, CMY-4, VIM-1	CIII	L/M	NEG.

Table 1c. *Enterobacter cloacae*

	CENTRE AND ISOLATE NUMBER	SPECIMEN	DEPARTMENT	DATE	ESBL	AmpC	MBL	IMI	MEM	ERT	GM	CIP	COL	BL	REP PCR-CLUSTER AND/OR ST	PBRT	*Qnr*
1	SPLIT-2322	URINE	MEDICAL ICU	16.02.2013	+	-	+	0.5	2	8	1	1	0.5	TEM-1, CTX-M-15, VIM-1	NT	NEG.	NEG.
2	SPLIT-2417	URINE	PSYCHIATRIC	19.02.2013.	+	-	+	16	16	32	32	32	0.06	TEM-1, CTX-M-15, VIM-1	NT	NEG.	NEG.
3	ZAGREB-45735 (89)	URINE	HEAMATOLOGY	11.03.2013.	+	-	+	8	16	16	16	16	0.5	TEM-1, CTX-M-15, VIM-1	EVII ST105	N, A/C	NEG.
4	ZAGREB-65063 (104)	STOOL	HEAMATOLOGY	10.04.2013	+	-	+	8	32	16	64	16	0.5	TEM-1, CTX-M-15, VIM-1	EVII ST105	L/M,	NEG.
5	ZAGREB-68389 (106)	STOOL	HEAMATOLOGY	15.04.2013	+	-	+	8	16	16	4	32	1	TEM-1, CTX-M-15, VIM-1	EVII ST105	N, A/C	NEG.
6	ZAGREB-96822 (146)	URINE	MEDICAL	31.05.2013.	+	-	+	8	4	8	64	16	0.25	TEM-1, CTX-M-15, VIM-1	EVII ST105	N, A/C	NEG.
7	ZAGREB-99954 (149)	STOOL	HAEMATOLOGY	05.06.2013.	+	-	+	8	8	16	4	2	0.5	TEM-1, CTX-M-15, VIM-1	EVII ST105	N, A/C	NEG.
8	ZAGREB-112243 (157)	URINE	MEDICAL	27.06.2013.	+	-	+	8	4	8	64	16	0.25	TEM-1, CTX-M-15, VIM-1	EVII ST105	N, A/C	B
9	ZAGREB-114449 (164)	URINE	MEDICAL	01.07.2013	+	-	+	16	16	8	32	>128	0.5	TEM-1. CTX-M-15, VIM-1	EVI ST418	NT	NEG.
10	ZAGREB-17701 (166)	URINE	UROLOGY	05.07.2013.	+	-	+	8	16	8	32	16	0.5	TEM-1, VIM-1	EVII ST105	N, A/C	B
11	ZAGREB-117238 (168)	URINE	UROLOGY	05.07.2013.	+	-	+	16	16	4	32	16	0.5	TEM-1, CTX-M-15, VIM-1	EIV ST105	N, A/C	B
12	ZAGREB-120844 (175)	THROAT SWAB	HEAMATOLOGY	11.07.2013.	+	-	+	8	4	2	32	64	0.5	TEM-1, CTX-M-15, VIM-1	NT	N, A/C	B
13	ZAGREB-124076 (181)	STOOL	HEAMATOLOGY	17.07.2013.	+	-	+	16	32	16	64	16	0.5	TEM-1, CTX-M-15, VIM-1	EVII ST105	N	B
14	ZAGREB -128982 (186)	STOOL	HEAMATOLOGY	23.07.2013..	+	-	+	128	128	16	128	16	1	TEM-1, CTX-M-15, VIM-1	EVII ST105	N, A/C	B
15	ZAGREB-133913 (196)	URINE	UROLOGY	06.08.2013.	+	+	+	4	8	16	128	32	0.5	TEM-1, CTX-M-15, VIM-1	EVII ST105	N, A/C	NEG.
16	ZAGREB-135007 (200)	URINE	HEAMATOLOGY	07.08.2013	+	-	+	4	32	16	32	16	0.12	TEM-1, CTX-M-15, VIM-1	EVII ST105	N, A/C	B
17	ZAGREB-138213 (204)	STOOL	HEAMATOLOGY	13.08.2013.	+	-	+	128	128	16	>128	16	1	TEM-1, CTX-M-15, VIM-1	EVII ST105	N, A/C	B
18	ZAGREB-143666 (213)	BLOOD	HEAMATOLOGY	25.08.2013	-	-	+	8	16	32	8	16	16	TEM-1, VIM-1	EVII ST105	N	B
19	OSIJEK-3294	BLOOD	PAEDIATRIC HEAMATOLOGY	28.08.2013	-	-	+	1	1	0.25	>128	2	0.12	TEM-1, VIM-1	EI ST91	N	NEG.
20	ZAGREB-147369 (218)	BLOOD	PAEDIATRC HEAMATOLOGY	31.08.2013	-	-	+	8	4	16	8	1	0.12	TEM-1, VIM-1	EI ST91	N	B
21	ZAGREB-169731 (237)	URINE	HEMATOLOGY	09.10.2013.	+	-	+	16	16	16	128	16	1	TEM-1, CTX-M-15, VIM-1	EVII ST105	N	NEG.
22	ZAGREB-193444 (266)	BLOOD	NEUROLOGICAL ICU	19.11.2013	+	+	+	8	4	16	128	64	0.12	TEM-1, CTX-M-15, VIM-1, NDM-1	EVII ST92	NEG.	B
23	ZAGREB-209029 (274)	WOUND SWAB	HEMATOLOGY	12.12. 2013.	+	-	+	128	16	16	128	16	1	TEM-1, VIM-1	EVII ST105	N	B
24	ZAGREB-208601 (276)	URINE	MEDICAL	12.12.2013.	+	-	+	4	8	16	128	128	0.5	TEM-1, CTX-M-15, VIM-1	EII ST133	NEG.	NEG.
25	ZAGREB-209377 (281)	URINE	OUTPATIENT	13. 12.2013.	+	-	+	16	16	32	32	>128	0.5	TEM-1, CTX-M-15, VIM-1, OXA-48	EV ST418	NEG.	NEG.
26	ZAGREB-211 765 (285)	STOOL	HEAMATOLOGY	17.12.2013	+	-	+	16	2	4	32	>128	0.5	TEM-1, VIM-1	EIII ST190	L/M	NEG.
27	ZAGREB-8391(299)	URINE	OUTPATIENT	17. 01.2014.	+	-	+	16	16	32	32	16	0.25	TEM-1, CTX-M-15, VIM-1, OXA-48	EV ST418	NEG.	NEG.
28	ZAGREB-45120 (324)	STOOL	HEAMATOLOGY	19.03.2014.	+	-	+	16	16	32	32	16	0.5	TEM-1, CTX-M-15, VIM-1	EIII ST134	L/M	B
29	ZAGREB-46603 (325)	BLOOD	HEAMATOLOGY	21.03.2014.	+	-	+	16	16	32	64	16	0.25	TEM-1, CTX-M-15, VIM-1	EV ST419	L/M	NEG.
30	ZAGREB-45823 (323)	NASAL SWAB	HEAMATOLOGY	20.03.2014.	+	-	+	128	128	16	128	16	1	TEM-1, CTX-M-15, VIM-1	EVII ST105	N, A/C	NEG.
31	ZAGREB-127 998 (372)	THROAT SWAB	HAEMATOLOGY	13.08.2014.	+	+	+	2	8	4	32	16	0.12	TEM-1, CTX-M-15, VIM-1	EVII ST105	NEG.	NEG.
32	SPLIT-UR12488	URINE	MEDICAL ICU	18.08.2014	+	+	+	32	32	4	64	32	0.12	TEM-1, CTX-M-15, VIM-1	EVIII ST92	NEG.	NEG.
33	SPLIT-HK6071	BLOOD	SURGICAL ICU	06.08.2014.	+	+	+	8	16	32	64	2	0.25	TEM-1, CTX-M-15, VIM-1	EVIII ST92	A/C	NEG.
34	SPLIT-UR20080	URINE	MEDICAL	18.12.2014.	+	+	+	2	8	4	32	16	0.12	VIM-1, DHA-1	EVIII ST92	NEG.	B