Epidemic spread of OXA-48 beta-lactamase in Croatia

Bedenić, Branka; Slade, Mia; Starčević, Lidija Žele; Sardelić, Sanda; Vranić-Ladavac, Mirna; Benčić, Ana; Zujić Atalić, Vlasta; Bogdan, Maja; Bubonja-Šonje, Marina; Tomić-Paradžik, Maja; ...

Source / Izvornik: **Journal of Medical Microbiology, 2018, 67, 1031 - 1041**

Journal article, Accepted version
Rad u časopisu, Završna verzija rukopisa prihvaćena za objavljivanje (postprint)

https://doi.org/10.1099/jmm.0.000777

Permanent link / Trajna poveznica: https://urn.nsk.hr/urn:nbn:hr:105:243037

Bedenić B., Slade M., Žele Starčević L., Sardelić S., Vranić Ladavac M., Benčić A., Zujić Atalić V., Bogdan M., Bubonja-Šonje M., Tomić-Paradžik M., Tot T., Lukić Grlić A., Drenjančević D., Varda Brkić D., Bandić-Pavlović D., Mihaljević S., Zarfel G., Gužvinec M., Conzemius R., Barišić I., Tambić Andrašević A. (2018) ***Epidemic spread of OXA-48 beta-lactamase in Croatia.*** **Journal of Medical Microbiology, 67(8). pp. 1031-1041. ISSN 0022-2615**

https://jmm.microbiologyresearch.org/content/journal/jmm

http://dx.doi.org/10.1099/jmm.0.000777

EPIDEMIC SPREAD OF OXA-48 BETA-LACTAMASE IN CROATIA

Branka Bedenić[1,2], Mia Slade[3], Lidija Žele Starčević[2], Sanda Sardelić[4], Mirna Vranić-Ladavac[5], Ana Benčić[1], Vlasta Zujić Atalić[6,11], Maja Bogdan[6,11], Marina Bubonja-Šonje[7], Maja Tomić-Paradžik[8], Tatjana Tot[9] Amarela Lukić-Grlić[1],[10], Domagoj Drenjančević[11], Dijana Varda-Brkić[2], Daniela Bandić-Pavlović[1,2], Slobodan Mihaljević,[1,2] Gernot Zarfel[12], Marija Gužvinec[13], Rick Conzemius[14], Ivan Barišić[14], Arjana Tambić-Andrašević[13]

[1]School of Medicine, University of Zagreb, Croatia

[2] University Hospital Center Zagreb, Croatia

[3] Croatian Institute of Transfusion Medicine (CITM)

[4]University Hospital Center Split, Croatia

[5]Public Health Institute of Istria County, Pula, Croatia

[6] Public Health Institute of Osijek-Baranja County, Osijek, Croatia

[7] University of Rijeka, Rijeka, Croatia

[8]Public Health Institute Slavonski Brod

[9]Department for Microbiology, General Hospital Karlovac

[10]Children's Hospital Zagreb, Croatia

[11]School of Medicine, University of Osijek, University Hospital Center Osijek

[12]Institute for Microbiology, Hygiene and Environmental Medicine, Medical University of Graz, Austria

[13]University Hospital for Infectious Diseases, Zagreb, Croatia

[14] AIT, Austrian Institute for Technology, Vienna, Austria

Type of article: research article

Correspondence: Branka Bedenić, Clinical Department for Clinical and Molecular Microbiology, School of Medicine, University of Zagreb, University Hospital Center Zagreb, tel: +385 1 23 67 304, fax: +385 1 23 67 393, e-mail: Branka.bedenic@kbc-zagreb.hr; bbedenic@mef.hr

Key words: *Klebsiella pneumoniae*, *Enterobacter cloacae*, carbapenems, resistance, carbapenemase

ABSTRACT

Purpose: Recently, a dramatic increase of OXA-48 β-lactamase, was observed not only in large hospital centers, but also in smaller suburban hospital centers in bordering geographic areas of Croatia. The aim of the study was to analyse the epidemiology, the mechanisms of antibiotic resistance, and the routes of spread of OXA-48 carbapenemase in Croatia.

Methods: Carbapenemase, other β-lactamase and fluoroquinolone resistance genes were detected by PCR and sequencing. Whole genome sequencing (WGS) was performed on five representative isolates. The isolates were genotyped by PFGE.

Results: Forty-eight isolates positive for OXA-48, collected in seven hospital centers in Croatia from May 2016 to May 2017 were analyzed (forty *Klebsiella pneumoniae*, five *Enterobacter cloacae*, two *Escherichia coli* and one *Citrobacter freundii*). Thirty-three isolates were ESBL positive and harboured CTX-M group 1 β-lactamase. In addition to the β-lactam- resistance genes detected by PCR ($bla_{\text{SHV-1}}$, $bla_{\text{OXA-48}}$ and $bla_{\text{OXA-1}}$), WGS of five representative isolates revealed the presence of genes encoding aminoglycoside resistance *aadA2* and *aph3-Ia*, fluoroquinolone resistance determinants *aac(6)Ib-c*, *oqxA* and *oqxB*, the sulfonamide resistance gene *sul1*, and *fosA (*fosfomycin-resistance*)*. IncL plasmid was found in all isolates. Two *K. pneumoniae* isolates belonged to ST16, two *E. cloacae* to ST66 and *E.*

coli to ST354. *K. pneumoniae* isolates were allocated to five clusters by PFGE which occured in different hospitals indicating epidemic spread.

Conclusions: The OXA-48 positive organisms from this study showed high variability in antibiotic susceptibilities, β-lactamase content and PFGE banding patterns. This study revealed switch from predominance of VIM-1 in the 2012 to 2013 to OXA-48 in the last two years.

INTRODUCTION

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 the IMP, VIM or NDM family and OXA-48-like β-lactamases belonging to the class D [1-2]. OXA-48 β-lactamase was reported for the first time in Turkey in 2004 [3]. In the last decade a remarkable increase of OXA- 48 producing organisms was reported in many countries all over the world with the highest rates observed in Turkey [4-7]. OXA-48 is the dominant carbapenemase in Germany [8], Portugal [9], Romania [10] and Far East [11]. The fast dissemination of the OXA-48 β-lactamase is mediated by insertion sequence IS*1999* embedded in transposon Tn*1999* [12]. Hospital outbreaks associated with OXA-48 producing *Klebsiella pneumoniae* have been reported as well [13].

The first carbapenem–resistant enterobacterial strain detected in Croatia was NDM-1 producing *K. pneumoniae* isolated in the University Hospital Center Zagreb in 2008 [14]. In 2012 the first KPC-positive *K. pneumoniae* was reported [15]. 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 the VIM-1- metallo-β-lactamase in two large hospital centers [16]. There was 36 VIM-1 positive isolates (90%) among 40 carbapenemase producing isolates). The remaining three isolates were positive for NDM-1 and one for KPC-2. The rate of VIM-1 producing isolates compared to total number of carbapenem nonsuceptible isolates was 63% (36/57). Two years later the clonal outbreak of VIM-1 positive *Enterobacter cloacae* and *Citrobacter freundii* was observed in the largest hospital center in Croatia. In the same study, the emergence of OXA-48 β-lactamase in

Enterobacteriaceae was reported for the first time in two hospital centers in Croatia [17]. In total thirty-four *E. cloacae* isolates were found to possess VIM-1 and were allocated to eight clusters with one large clone comprising eighteen identical isolates. Seventeen *C. freundii* isolates were identified as VIM-1 producers and they belonged into two clusters with one containing eleven identical isolates. In total 65 patients were infected or colonized with VIM-1 producing organism (14 with *K. pneumoniae*, 34 with *E. cloacae* and 17 with *C. freundii*). In that study coexistance of VIM-1, NDM-1 and OXA-48 was reported for the first time in Croatia, similarly as previously in India [18]. Four *K. pneumoniae* and one *C. freundii* were found to harbour VIM-1 and NDM-1 whereas two *E. cloacae* isolates were positive for VIM-1 and OXA-48.Morevoer, monoclonal outbreak associated with VIM-1 *E. cloacae* (6 isolates) was reported in University Hospital Split in 2012 [19]. VIM-1 was the sole carbapenemase in this hospital center.

The first OXA-48 producing organisms in Croatia, originating from 2011 to 2012 from Nortwest areas of Croatia were reported in the multicenter study performed from 2010 to 2012 but published recently [20].

After sporadic cases during 2010 to 2013 [17,20], an increase of OXA-48 β-lactamase was observed by clinical microbiologists in the participating centers, not only in large hospital centers, but also in the smaller hospitals in the peripheral, bordering areas of Croatia in the last two years. Since all carbapenem resistant Enterobacteriaceae are sent to the reference laboratory for the identification of carbapenemase type, the clinical microbiologist are notified about the results of the carbapenemase identification. They sent only the OXA-48 producing organisms to the University Hospital Center Zagreb to analyse the epidemiology, the mechanisms of antibiotic resistance, and routes of dissemination of OXA-48 carbapenemase in Croatia.

MATERIAL AND METHODS

2.1. Bacterial isolates

In total 48 enterobacterial isolates were collected from May 2015 to May 2017, from ten centers in Croatia which participated in the study: University Hospital Center "Sestre Milosrdnice", University Hospital Center Zagreb, University Hospital Center Split, University Hospital Center Osijek, University Hospital Center Rijeka, Children's Hospital Zagreb, General Hospital Slavonski Brod, General Hospital Pula, General Hospital Gospić and General Hospital Dubrovnik as shown in Fig S1. The isolates were identified to the species level by conventional biochemical testing Vitek 2 or MALDI-TOF, depending on the routine laboratory where they were isolated. Only the isolates confirmed as *bla*$_{\text{OXA-48}}$ positive in the Reference center were included in the study. All enterobacterial isolates with reduced susceptibility to at least one carbapenem by disk diffusion test, in routine diagnostic laboratories in Croatia, are obliged to be sent to the Reference Centre for Antibiotic Resistance Surveillance in the University Hospital for Infectious Diseases in Zagreb. The inhouse Carba NP and commercial test "mastdiscs combi Carba plus (Enterobacteriaceae)" were performed to phenotypically detect carbapenemases. PCRs targeting the *bla*$_{\text{VIM}}$, *bla*$_{\text{NDM}}$, *bla*$_{\text{OXA-48}}$ and *bla*$_{\text{KPC}}$ genes was conducted for the isolates positive in the phenotypic tests. The clinical microbiologists in the routine laboratories are notified by the reference laboratory about the type of carbapenemase and the isolates confirmed to possess OXA-48 were sent to the Clinical Department for Clinical and Molecular Microbiology of the University Hospital Center Zagreb for further analysis.

All other analysis of isolates (sections 2.2, 2.3, 2.4, 2.6, 2.7. and 2.8) including antimicrobial susceptibility testing, phenotypic detection of β-lactamases, molecular detection of other β-lactamases, *qnr* genes, and insertion sequence, plasmid characterization and genotyping was done in the Clinical Department for Clinical and Molecular Microbiology, University

Hospital Center Zagreb. Whole genome sequencing was done at the Austrian Institute for Technology.

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, expanded- spectrum cephalosporins or ESC (ceftazidime, cefotaxime, ceftriaxone), cefepime, imipenem, meropenem, ertapenem, gentamicin, ciprofloxacin and colistin was determined by the broth microdilution method according to CLSI standards [21] and for colistin according to the EUCAST standard (Http://www.eucast.org). The susceptibility to fosfomycin was determined by agar dilution. *E. coli* ATCC 25922 and *K. pneumoniae* 700603 were used as quality control strains for minimum inhibitory concentration (MIC) determination. The susceptibility to sulphametoxazole/trimethoprim, tetracycline and chloramphenicol was determined by disk-diffusion test. The isolates were classified as multidrug-resistant (MDR), extensively drug-resistant (XDR) or pandrug-resistant (PDR) as described previously by Magiorakos et al [22]. The double disk synergy test (DDST) [23] and the CLSI combined disk test with addition of clavulanic acid were performed to detect ESBLs [21]. Chromosomal or plasmid-mediated AmpC β-lactamases were detected by combined disk test using cephalosporin disks with 3-aminophenylboronic acid (PBA) [24]. A modified Hodge test (MHT) and the carbapenem-inactivation method (CIM) were used to screen for the presence of carbapenemases [25-26]. 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 [27, 28].

2.3. 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*$_{CTX-M}$, *bla*$_{OXA-9}$, *bla*$_{OXA-1}$ and *bla*$_{PER-1}$), plasmid-mediated AmpC β-lactamases, class A (*bla*$_{KPC}$, *bla*$_{SME}$, *bla*$_{IMI}$, *bla*$_{NMC}$), class B carbapenemases (*bla*$_{VIM}$, *bla*$_{IMP}$ and *bla*$_{NDM}$), carbapenem hydrolyzing oxacillinases (*bla*$_{OXA-48-like}$) and fluoroquinolone resistance genes (*qnr*A, *qnr*B, *qnr*S) were determined by PCR using protocols and conditions as described previously [29-36]. Group of CTX-M β-lactamases was detected by multiplex PCR [33]. The inactivation of *mgrB* genes and plasmid encoded colistin resistance genes *mcr-1* were analyzed in two isolates with reduced susceptibility to colistin by PCR as described previously [37-38]. β-lactamase encoding PCR amplification products from nine representative isolates from each center (*K. pneumoniae* 16780 from Pula, VG-8166 from Zagreb, OS2 and OS8 from Osijek, UHC 1900807 from Zagreb, UR 22272 from Split, *E. cloacae* 17504 from Pula, *E. cloacae* 30676 from Slavonski brod, and *E. coli* 18464 from Pula) were subjected to sequencing to determine the allelic gene variant of the TEM, SHV and CTX-M β-lactamases. The positive control strains producing TEM-1, TEM-2 and SHV-1 and SHV-2 were kindly provided by Prof. Adolf Bauernfeind (Max von Pettenkofer Institute, Munich, Germany), CTX-M-15 by Prof. Neil Woodford (Health Protection Agency, London, UK) and OXA-48 by Dr. Yvonne Pfeifer (Robert Koch Institute, Wernigerode, Germany). PCR mapping was performed with primers for IS*1999* combined with forward and reverse primers for *bla*$_{OXA-48}$ [12]. The size of the product was determine by the gel electrophoresis, after staining with ethidium bromide. The amplification products from selected strains (*E. cloacae* 17504, *K. pneumoniae* 7210, *K. pneumoniae* 22272, *K. pneumoniae* 17068, *K. pneumoniae* 24889 and *K. pneumoniae* 332-1 were sequenced by a commercial supplier (Eurofin Germany) in order to analyse the genetic context of the *bla*$_{OXA-48}$ genes and the position of genes flanking *bla*$_{OXA-48}$. Genetic context of

bla$_{CTX-M}$ genes was determined by PCR mapping with forward primer for IS*Ecp1* and IS26 combined with primer MA-2 (reverse for *bla*$_{CTX-M}$ genes) [39].

2.4. Conjugation and transformation

The transferability of meropenem and cefotaxime resistance was determined by conjugation (broth mating method) at 35°C employing *E. coli* J65 recipient strain resistant to sodium-azide [40]. The transconjugants were selected on MacConkey agar containing either meropenem (0.5 mg/L) or cefotaxime (2 mg/L) and sodium azide (100 mg/L). The frequency of conjugation was determined relatively to the number of donor cells. Cotransfer of resistance to gentamicin, tetracycline, sulfamethoxazole/trimethoprim, chloramphenicol and ciprofloxacin was determined. The isolates which did not yield transconjugants were subjected to transformation experiment as described previously [41]. Plasmids were extracted with Macherey Nagel- Nucleospin kit (Macherey-Nagel, Gmbh, Germany) and transferred to $CaCl_2$ treated *E. coli* A15R$^-$ recipient strain. Transformants were selected on MacConcey medium containing 1 mg/L of meropenem.

2.5.Whole genome sequencing (WGS)

WGS was done in Austrian Institute for Techology. Five representative OXA-48 producing isolates belonging to different species and hospital centers were selected for further WGS. *K pneumoniae* OS2 (ESBL negative), *K. pneumoniae* OS5 (ESBL positive), both from Osijek, *E. cloacae* 30676 from Slavonski Brod (ESBL positive), *E. cloacae* 17604 from Pula (ESBL negative) and *E. coli* 18464 (ESBL negative) from Pula. The bacterial genomes were sequenced using the IonTorrent PGM platform (Life Technologies, Carlsbad, USA) according to the manufacturer's instructions. The Ion Xpress Plus Fragment Library Kit was used to enzymatically shear 100 ng of the genomic DNA. The target fragment size was 400 bp. Subsequently, the fragmented DNA was processed using the Ion DNA Barcoding kit (Life Technologies) and its size selected using the E-Gel SizeSelect 2% Agarose kit (Life

Technologies). The size distribution of the DNA fragments was analysed using the High Sensitivity Kit (Agilent, Santa Clara, USA). Further sample processing was performed using the Ion OneTouch Kit (Life Technologies). Finally, the amplified DNA was sequenced using the 318 chip (Life Technologies). The single reads obtained were *de novo* assembled using MIRA 3.9.9, which is part of the Assembler plug in on the Ion Torrent server. Subsequently, the contigs were analyzed using the RAST analysis platform and the ResFinder web-service to screen for antibiotic resistance genes and their genetical context [42].

2.6. Characterization of plasmids

Plasmids were extracted from donor strains and their respective transconjugants with Machery Nagel nucleospin kit according to the manufacturer's instructions. After staining with ethidium bromide, the DNA was visualised by ultraviolet light. PCR-based replicon typing (PBRT) [43] was applied to determine the plasmid content of the tested strains. Since it was observed previously that PBRT can be inefficient in identifying L/M plasmid type, an updated method designated to identify and distinguish between IncL and IncM plasmids was applied [44]. Plasmid extractions obtained from transconjugant strains were subjected to PCR for the detection of OXA-48 and ESBLs in order to determine the resistance gene content of the transconjugants. PBRT was also applied on transconjugants to identify incompatibility groups such as in their respective donors. Positive control strains for PBRT were kindly provided by dr. A. Carattoli (Insituto Superiore di Sanita, Rome, Italy).

2.7. Pulsed-field gel electrophoresis (PFGE)

Thirty-nine *K. pneumoniae*, and all *E. cloacae* and *E. coli* isolates were subjected to genotyping by PFGE. One *K. pneumoniae* isolate died before PFGE was finished. PFGE genotyping of *Xba*I-digested genomic DNA was performed with a CHEF-DRIII system (Bio-Rad); the images were processed using the Gel-Compar software. The dendrogram was computed after band intensity correlation using global alignment with 1.5 % optimization and

1% tolerance and unweighted pair-group method using arithmetical averages (UPGMA) clustering. PFGE cluster analysis was carried out with Gel Compare II (Applied Maths, Belgium) using Dice similarity coefficient and clustering by the UPGMA [45]. Band patterns were visually compared to define indistinguishable and closely related subtyped differing by two or three bands, in accordance with the criteria proposed by Tenover [46]. Each PFGE cluster was assigned a roman number followed by a letter indicating closely related isolates. The *K. pneumoniae* clusters have been designated as K and *E. cloacae* as E.

RESULTS

2.1. Bacterial isolates

Thirty-six patients had an infection with an isolate comprising OXA-48, whereas twelve of the patients were only colonized. The type of infection, antibiotic treatment and outcome are shown in Table 1a and b. Urinary tract infection was the predominant type of infections (14 patients), followed by pneumonia (eight patients), septicaemia (four patients), wound infections (three patients), osteomyelitis (two patients), peritonitis (two patients) and otitis media (one patient). For two patients the data were not available.

In total 48 isolates were analysed in the study: twenty-five from University Hospital Center Osijek, eight from University Hospital Center Split, six from General Hospital Pula, four from General Hospital Slavonski Brod, two from University Hospital Center Zagreb and University Hospital Center "Sestre Milosrdnice", respectively and one from University Hospital Center Rijeka as shown in Fig. S 1 (Supplementary material). Out of 48 isolates, 40 were *K. pneumoniae*, five *E. cloacae*, two *E. coli* and one *C. freundii*.

The Children's Hospital in Zagreb, General Hospital Gospić and General Hospital Dubrovnik did not detect any OXA-48 positive organisms in the study period. Twenty-one isolates were obtained from the patients with the infection and the rest from colonized patients. The Children's Hospital in Zagreb, General Hospital Gospić and General Hospital Dubrovnik did not have any OXA-48 positive organisms in the study period (May 2015 to May 2017). The rate of carbapenem resistant Enterobacteriaceae varied from 0,03% in Dubrovnik to 4% in University Hospital Center Zagreb in the period May 2015 to May 2017. The rates were as follows: 0,03 % (4/12361) in Dubrovnik, - 0.04% (6/13 937) in Pula, - 0.09% (8/8151) in University Hospital Rijeka, -0.4% (16/3834) in Children's Hospital Zagreb, -0.68% (48/7053) in University Hospital Center Osijek, -0.9% (29/3181), in General Hospital Slavonski Brod - 1% (93/8958) in University Hospital Center Split and - 4% (355/8806) in University Hospital

Zagreb. The University Hospital „Sestre Milosrdnice“ does not have a surveillance system and the data on the total number of Enterobacteriaceae are not available. An association with the resistance rate and hospital size was observed. The large hospitals such as University Hospital Center Zagreb and University Hospital Split with bone marrow and kidney transplantation wards had higher resistance rates.

The rate of OXA-48 producing organisms among carbapenem- resistant Enterobacteriaceae was as follows: 0.01% (1/8) in University Hospital Center Rijeka, 13% (47/355) in University Hospital Center Zagreb, 14% (4/29) General Hospital Slavonski Brod, 15% (14/93) in University Hospital Center Split, 50% (24/48) in University Hospital Center Osijek and 100% (6/6) in General Hospital Pula. Fifteen patients died and the other twenty-one recovered from infection. Various antibiotic combinations, including meropenem, colistin and amikacin were used for the treatment (Table 1 and and 1b). Meropenem was the most frequently prescribed antibiotic, administered in fifteen cases.

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

All isolates were uniformly resistant to amoxicillin, amoxicillin/clavulanate, piperacillin/tazobactam and cefazoline. *K. pneumoniae* isolates showed high resistance rates to ertapenem and ciprofloxacin (82.5%), expanded-spectrum cephalosporins (ESC) (67.5%), meropenem (47.5%), cefepime (45%), and imipenem (37.5%). Gentamicin and colistin preserved good activity with 85% and 98% susceptible *K. pneumoniae* isolates, respectively, as shown in Table 1a. Eleven out of 40 isolates (27%) were resistant to fosfomycin. Twenty-four isolates (60%) were resistant to sulfamethoxazole/trimethoprim and eight (20%) to tetracycline. *E. cloacae* were uniformly resistant to amoxicillin alone and combined with clavulanate, piperacillin/tazobactam, cefazoline, expanded-spectrum cephalosporins and cefepime (Table 1b). Four out of five isolates (80%) were resistant to ciprofloxacin and

gentamicin. Three (60%) isolates were resistant to imipenem and meropenem. One isolate (2.5%) displayed resistance to fosfomycin. Resistance to sulfamethoxazole/trimethoprim, tetracycline and chloramphenicol was recorded in three isolates.

The two *E. coli* isolates were susceptible to ESC, cefepime and colistin and resistant to ciprofloxacin as shown in Table 1b. They exhibited variable MICs of carbapenems. One isolate was resistant to sulfamethoxazole/trimethoprim and tetracycline. A *C. freundii* isolate was susceptible to all tested antibiotics except of amoxicillin alone and combined with clavulanate and piperacillin (Table 1b). In summary, thirty- one isolates were MDR and two were XDR (OS21 and 24889) since they were resistant to colistin as well (MIC values of 64 and 128 mg/L, respectively). The MDR phenotype was associated with the production of an additional ESBL.

Twenty-eight out of 40 *K. pneumoniae* and all *E. cloacae* isolates were phenotypically positive for ESBL (Table 1a and b). Inhibitor- based test with PBA for detection of AmpC- β-lactamases was positive in four *E. cloacae*, one *C. freundii* and four *K. pneumoniae* isolates. Hodge- test for the detection of carbapenemase activity was positive in all isolates whereas CIM yielded negative results in six OXA-48 producing *K. pneumoniae* isolates (12%) (Table 1a). Two (VG 8166 and OS5) of the six CIM negative isolates were resistant only to ertapenem with MIC value of 16 mg/L and 4 mg/L, respectively. One isolate (VG 16/3885) was susceptible only to imipenem with a MIC value of 0.5 mg/L, whereas the other two (OS4, 158889) were resistant to all three carbapenems with MIC values ranging between 32 mg/L and 64 mg/L. One isolate (OS 13) was fully susceptible to all three carbapenems with MIC values equal or below 1 mg/L as shown in Table 1 a.

2.3. Molecular detection of resistance genes

All twenty- eight ESBL and OXA-48 producing *K. pneumoniae* harboured group 1-CTX-M β-lactamase. Sequencing of representative amplication products from each center revealed the

presence of the *bla*$_{CTX\text{-}M\text{-}15}$ (Table 1a). *bla*$_{CTX\text{-}M\text{-}15}$ genes were preceded by an IS*Ecp* insertion sequence. Twelve CTX-M producing isolates harboured in addition to OXA-48 also TEM-1 and three OXA-1 (Table 1a). All OXA-48 and ESBL positive *E. cloacae* produced group 1-CTX-M- ESBL plus an additional TEM-1 and OXA-1 in two isolates, respectively (Table 1b). The *E. coli* isolates possessed only OXA-48 combined with the broad spectrum TEM-1 β-lactamase in one isolate (Table 1b). A *C. freundii* isolate possessed chromosomal CMY in addition to OXA-48 (Table 1b). Genes *Qnr*A and *Qnr*B, contributing to fluoroquinolone resistance, were found in two and twenty isolates, respectively. *K. pneumoniae* was the dominant species carrying *qnr* genes (19 out of 20). The *mcr*-1 gene, contributing to colistin resistance, was not found in the colistin-resistant *K. pneumoniae* isolates. Other species did not show colistin resistance.

PCR analysis detected wildtype *mgr*B genes. PCR mapping revealed augmentation of the PCR product obtained with forward primer for IS*1999* and reverse for *bla*$_{OXA\text{-}48}$ gene, compared to the size of the PCR product obtained with the primers for IS*1999.* An analysis of the genetic context of selected strains revealed the presence of IS*1999,* IS1R and *tnp*A upstream of the *bla*$_{OXA\text{-}48}$ gene and *lys*R, IS*1999* and *tnp*A downstream of the *bla*$_{OXA\text{-}48}$ gene.

2.4. *Conjugation and transformation*

Reduced susceptibility to meropenem of thirty-one isolates was transferred to *E. coli* recipient strain with the frequency ranging from 1.2 to 8.4 x 10^{-6} . Sixteen out of 33 ESC resistant isolates transferred cefotaxime resistance to *E. coli* recipient with the frequency ranging from 8x10^{-8} to 4 x10^{-4}. The transconjugants obtained with cefotaxime as selective agent showed similar resistance patterns to ESC as their respective donors. Resistance to sulphametoxazole was cotransferred alongside with cefotaxime resistance in six and to tetracycline in two tested isolates which transferred cefotaxime resistance. The transconjugants obtained with meropenem as selective agent did not harbour resistance genes

to sulphonamides, tetracyclines, chloramphenicol or gentamicin. The sixteen tranconjugants obtained with cefotaxime as selective agent harboured *bla*$_{\text{CTX-M}}$ genes as their respective donors. Thirty-one transconjugants obtained with meropenem as selective agent possessed *bla*$_{\text{OXA-48}}$ genes. The remaining 17 isolates which did not transfer meropenem resistance in conjugation experiments were subjected to transformation but transformants were not obtained.

2.5. Whole genome sequencing

WGS of five representative isolates (*K. pneumoniae* n=2, *E. cloacae* n=2 and *E. coli,* n=1) revealed the presence of genes encoding aminoglycoside resistance *aadA2* and *aph3-Ia*, fluoroquinolone resistance determinants *aac(6)Ib-c (*The aminoglycoside acetyltransferase *Aac(6')-Ib-cr* variant, an enzyme usually encoded by a plasmid-borne gene, extends its drug targets to include fluoroquinolones in addition to aminoglycosides), *oqxA* and *oqxB* sulfonamide resistance gene *sul1*, *fosA* (fosfomycin modifying enzymes) encoded fosfomycin resistance and the β-lactamase genes *bla*$_{\text{SHV-1}}$, *bla*$_{\text{OXA-48}}$ and *bla*$_{\text{OXA-1}}$ in the *K. pneumoniae* isolates OS2 and OS8 from Osijek. *E. cloacae* 30676 from Slavonski Brod and *E. cloacae* 17504 from Pula possessed *aac(6')Ib-cr*, *aph(6)-Id, aph(3'')-Ib, aph(3'')-Ib* and *aac(3)-Iia* in addition to the *bla*$_{\text{ACT-7}}$ and *bla*$_{\text{TEM-1b}}$ gene.

The *E. coli* 18464 from Pula possessed the aminoglycoside resistance genes *aac(3-)Iid*, *aph(3'')-Ib*, *aph(6)-Id* and *aph3-Ib*, tetracycline resistance gene *tet(B)*, trimethoprim resistance gene *drfA17* and sulfonamide resistance gene *sul2*, *fosA* encoded fosfomycin resistance and the β-lactamase genes *bla*$_{\text{OXA-48}}$ and *bla*$_{\text{TEM-1b}}$. The presence of *bla*$_{\text{OXA-48}}$ gene flanked with IS1999 sequences was confirmed. BLAST analyses of the contigs comprising the *bla*$_{\text{OXA-48}}$ gene with 100% sequence similarity resulted in hits from plasmid associated NCBI entries.

Two *K. pneumoniae* isolates (OS2 and OS 8) belonged to ST16, two *E. cloacae* (30676 and 17604) to ST66 and the *E. coli* 18464 isolate could not be assigned precisely to a known sequence type. The two most closely related were ST354 and ST39.

2.6. Plasmid characterization

A plasmid of 60 to 70 kb was visible in donor and tranconjugant strains. All plasmid extractions from donor strains yielded L plasmids by PCR with the modified method according to Carattoli [45]. The PCR for L/M plasmid was negative. The transconjugants obtained with meropenem as selective agent harboured L plasmid as their respective donors.

2.7.Pulsed-field gel electrophoresis (PFGE)

Genotyping revealed the existence of five clusters among thiry-nine *K. pneumoniae* isolates which contained subclusters with highly similar isolates. The largest cluster was the clone I which contained 19 isolates allocated to eight subclones, each comprising isolates originating predominantly from Osijek and Split, with one strain isolated in Pula (Fig. S 2a-supplementary material) . Isolates originating from different hospital wards in the same center and with different β-lactamase content, including ESBL positive and ESBL negative, clustered together. The second cluster contained eight isolates, arranged in three subclusters with isolates obtained from five hospital centers (Split, Osijek, Pula, Slavonski Brod and University Hospital "Sestre Milosrdnice" in Zagreb), from different specimens and hospital wards, whereas the third cluster comprised five isolates; from four different hospital centers (University Hospital "Sestre Milosrdnice" , Rijeka, University hospital Center Zagreb, Osijek and Rijeka) . Cluster IV and cluster V comprised only two isolates, respectively. Three isolates (OS 9, OS 13 and KK3602) had unique banding patterns and were designated as singletons (Fig. S 2a-supplementary material). The isolates belonging to one cluster in one hospital were usually from the same period but included different hospital wards indicating cross infection.

All five *E. cloacae* isolates from different centers (Pula, Slavonski Brod, Osijek and Split) were allocated to cluster E I and showed >85% similarity but some diversification was observed within the clones and thus four subclusters were identified (Fig. S 2b-supplementary material).

Two *E. coli* isolates showed different banding patterns.

DISCUSSION

After the first report of OXA-48 in the large hospital centers in Croatia in 2012, the clinical microbiologist in smaller hospitals in peripheral, bordering geographic areas of Croatia have noticed a dramatic increase of OXA-48 among carbapenem-resistant Enterobacteriacae according to the data from the Reference Centre for Antibiotic Resistance Surveillance in the University Hospital for Infectious Diseases in Zagreb, from 0% until 2014 to almost 100% in the last two years, depending on the center .

Croatia has a national surveillance system and specific guidelines for the management of carbapenemase- producing Enterobacteriaceae and obligation to report them to the health authorities, but in spite of these measures there is an increase of OXA-48 producing organisms, similarly as in other European countries. The production of ESBL, predominantly belonging to the CTX-M family, was associated with resistance to ESC. Gentamicin and fluoroqinolones exhibited resistance in ESBL positive isolates due to the additional *qnr* genes usually located at the same plasmid. All isolates showed a high level of resistance to amoxycillin/clavulanate and piperacillin/tazobactam which is typical for OXA-48 β-lactamase since it hydrolyzes penicillins and similarly as other OXA β-lactamases is not inhibited by clavulanic acid or tazobactam. The production of additional OXA-1 β-lactamase in some of the isolates may have contributed to the resistance to β-lactam combinations with inhibitors.

The isolates exhibited variable MICs of carbapenems, probably due to variable levels expression of *bla*$_{\text{OXA-48}}$ genes which could be attributed to different gene copy or plasmid copy numbers. All isolates showed reduced susceptibility to ertapenem in disk-diffusion test and this was used to screen for carbapenemase production. The low level of carbapenem-resistance in some OXA-48 producing organisms pose a serious problem for detection of this increasingly important carbapenem resistance determinant. For that reason microbiologists rely on phenotypic tests. Hodge test showed high sensitivity of 100% in detection of OXA-48 β-lactamase in contrast to CIM test which showed false negative results in 12% isolates. The isolates negative in CIM test exhibited variable carbapenem MICs. The Carba-NP test is recommended as a sensitive test for the detection of carbapenemases, but the drawback of this method is a high cost [47].

Colistin resistance observed in two *K. pneumoniae* isolates was not associated with the inactivation of *mgrB* gene or acquisition of *mcr- 1* or *mcr- 2* genes, and thus, is probably caused by the adaptive mechanisms such as porin loss or upregulation of efflux pumps. Adaptive colistin resistance mechanisms were previously reported in *Enterobacter aerogenes* but there are no reports so far for *K. pneumoniae* [48,49]. Mutations in the two- component signalling transduction system *phoP/phoQ* or *pmrA/pmrB,* regulating genes necessary for lipopolysaccharide modifications may play a role in the colistin resistance [50], but investigation of outer membrane lipopolysaccharides was beyond the scope of the present study. Our results are in disagreement to the previous studies which found the inactivation of *mgrB* genes or acquisition of *mcr* genes as a causative agent for colistin resistance in Europe [38, 51]. Emergence of colistin resistance in OXA-48 producing *K. pneumoniae* was previously reported in Tunisia [52].

Meropenem reduced susceptibility was transferable in the majority of isolates indicating plasmid location of *bla*$_{\text{OXA-48}}$ genes. Furthermore, *bla*$_{\text{OXA-48}}$ carrying transconjugants were

shown to possess L plasmid as their respective donors. However, we did not prove with certainty the plasmid location of the *bla*$_{\text{OXA-48}}$ genes because Southern blotting was not performed although the BLAST analyses of the WGS contigs suggest the presence of *bla*$_{\text{OXA-48}}$ on plasmids. Moreover, *bla*$_{\text{OXA-48}}$ carrying transconjugants obtained in our study did not harbour any additional resistance genes similarily as reported in the previous study [20]. IS*Ecp1*-like element detected in all CTX-M positive isolates was shown previously to play a key role in the mobilization of *bla*$_{\text{CTX-M}}$ genes [39].

The majority of the isolates were positive for IS*1999* upstream of the *bla*$_{\text{OXA-48}}$ gene which is responsible for the mobilization of *bla*$_{\text{OXA-48}}$ genes and enhances the expression of the gene. Analysis of the flanking regions of *bla*$_{\text{OXA-48}}$ gene revealed similar structure as previously reported by Gianni et al with IS1R element between I*S1999* and the OXA-48 encoding gene [12].

PFGE showed the existence of five different *K. pneumoniae* clusters with isolates from different centers and with different β-lactamase content belonging to the same clusters indicating transfer of the related strains by patient or staff transfer. The isolates from the same center but different clinical wards showed high similarity in the PFGE banding patterns pointing out to the cross infections. PFGE patterns did not correlate with resistance gene content and the highly related isolates showed different β-lactamase genes. This indicates that resistance genes were acquired after the spread of the related isolates in the hospital wards.

Unlike sixty-seven MBL producing organisms (65 positive for VIM-1, seven of which were positive also for NDM-1 and two isolates positive only for NDM-1) from the previous study conducted in 2013-2014 in four hospital centers in Croatia [17] which demonstrated almost identical resistance phenotype, β-lactamase content and PFGE profiles, OXA-48 positive organisms from this study showed high variability in antibiotic susceptibilities, β-lactamase content and PFGE banding patterns. In the earlier study (2013-2014) OXA-48 was found in

combination with VIM-1 and NDM-1 in two *K. pneumoniae* isolates from University Hospital Center Zagreb and as a sole carbapenemase in one *K. pneumoniae* isolate from Split [17]. Similarily as in recently published nationwide study on early OXA-48 positive *K. pneumoniae* collected in 2011 to 2012 in Croatia [20] the dissemination of OXA-48 positive isolates was polyclonal. The results points out to the fact the vertical transmission of related isolates by patient or staff transfer and probably dissemination of L plasmids carrying $bla_{\text{OXA-48}}$ genes between different isolates which was previously reported by other authors [12] but not confirmed in our study. In contrast to earlier study in which all OXA-48 strains coproduced CTX-M-15 [20], in our investigation only the part of the isolates harboured ESBL. Although *K. pneumoniae* was the dominant species harbouring $bla_{\text{OXA-48}}$ genes in this study, OXA-48 β-lactamase was described for the first time in *C. freundii* in Croatia.

Significant proportion of isolates originated from colonization, raising the concern that colonization if unobserved, can act as the potential source of dissemination of OXA-48 producing organisms within the hospitals. These finding warrants for continous surveillance in order to prevent the spread of these isolates in our healthcare system.

Further studies are necessary to elucidate if meropenem and imipenem can be administered for treatment of infections with isolates showing susceptibility to them.

The study demonstrated dynamic changes of carbapenem-resistance mechanisms of important hospital pathogens like *K. pneumoniae* or *E. cloacae* with VIM and NDM as dominant carbapenem-resistance mechanism in 2012-2014 and OXA-48 becoming predominant in the period 2015 to 2017. We have also shown the complex epidemiology of OXA-48 producing Enterobacteriaceae including cross infections, transmissions of isolates between hospitals and polyclonal outbreaks in smaller hospitals.

Interestingly, two isolates from Pula were obtained from the residents of a nursing home and one from the ointment used in the long-term care facility indicating the possibility of

dissemination of carbapenem-resistance determinants into the community. The large differences in the prevalence of OXA-48 β-lactamase among the participating centers may suggest that Croatia is not yet in an advanced stage of dissemination of OXA-48 or may reflect variations in the success of infection control in particular sites.

FUNDING

Grant BM013 from the University of Zagreb, FAPIC (Fast assay for Pathogen identification and characterization, D: 634137 (funded under H2020-EU.3.1.3). This project has received funding from the European Union's Horizon 2020 research and innovation program under GA 634137

COMPETINING INTERESTS

None to declare

ETHICAL PERMISSION

Not necessary. The experiments were not done on human or animal subjects

ACKNOWLEDGEMENT

We thank Stjepan Katić and Dubravko Šijak for technical assistance with PFGE.

LIST OF ABBREVIATIONS

MIC-minimum inhibitory concentration, EDTA- Ethylenediaminetetraacetic acid; PCR-polymerase chain reaction; CAZ-ceftazidime; CTX-cefotaxime; CRO-ceftriaxone; FEP-cefepime; IMI-imipenem; MEM-meropenem; ERT-ertapenem; GM-gentamicin; CIP-ciprofloxacin; AMI – amikacin; TOB-tobramycin; AZI- azitromycin; LEV-levofloxacin; CXM-cefuroxime; AMC-amoxicillin-clav.acid; COL-colistin; VAN-vancomycin; TIG-tigecycline; FOS-fosfomycine; CZ-

cephazoline; ESBL-inhibitor based test with clavulanic acid for detection of extended-spectrum beta-lactamases; Amp-C-inhibitor based test with phenyloboronic acid for detection of AmpC beta-lactamases; BL-beta-lactamase content; CIM-carbapenem- inactivation method; PBRT-PCR based replicon typing; R- released from the hospital; I-improved; D-died, NA-not applicable

REFERENCES:

1. **Queenan AM, Bush K.** Carbapenemases: the versatile β-lactamases. *Clin Microbiol Rev* 2007;20(3):440-458.

2. **Canton R, Akova M, Carmeli Y, Giske CG, Glupczynski Y et al.** The European Network on carbapenemases, Rapid evolution and spread of carbapenemases among *Enterobacteriaceae* in Europe. *Clin Microbiol Infect* 2012;18:413-431.

3. **Poirel L, Heritier C, Tolun V, Nordmann P.** Emergence of oxacillinases-mediated resistance to imipenem in *Klebsiella pneumoniae*. *Antimicrob Agents Chemother* 2004;48(1):15-22.

4. **Gulmez D, Woodford N, Palepou MF.** Carbapenem resistant *Escherichia coli* and *Klebsiella pneumoniae* isolates from Turkey with OXA-48 like carbapenemase and outer membrane protein loss. *Int J Antimicrob Agents* 2008;31:523-526.

5. **Aktas Z, Kayacan CB, Schneider I, Can B, Midilli K et al.** Carbapenem-hydrolyzing oxacillinase, OXA-48 persists in *Klebsiella pneumoniae* in Istanbul, Turkey. *Chemotherapy* 2008;54:101-106.

6. **Carrer A, Poirel L, Eraksoy H, Cagatay A, Badur S et al.** Spread of OXA-48-positive carbapenem-resistant *Klebsiella pneumoniae* isolates in Istanbul, Turkey. *Antimicrob Agents Chemother* 2008;52(8):2950-2954.

7. **Nazik H, Aydin S, Albayrak R, Bligi EA, Yildiz I et al.** Detection and spread of OXA-48 producing Klebsiella oxytoca isolates in Istanbul, Turkey. *Southeast Asian J Trop Med Public Health* 2014;45(1):123-9.

8. **Pfeifer Y, Schlatterrer K, Engelmann E, Schiller RA, Frangenberg HR et al.** Emergence of OXA-48-type carbapenemase-producing *Enterobacteriaceae* in German Hospitals. *Antimicrob Agents Chemother* 2012;56(4):2125-2128.

9. **Manageiro V, Ferreira E, Pinto M, Canica M.** First description of OXA-48 carbapenemase harbored by *Escherichia coli* and *Enterobacter cloacae* from a single patient in Portugal. *Antimicrobial Agents Chemother* 2014;58(12):7613-7614.

10. **Timofte D, Panzaru CV, Maciuca IE, Dan M, Mare AD et al.** Active surveillance scheme in three Romanian hospitals, reveals a high prevalence and variety of carbapenemase producing Gram-negative bacteria: a pilot study, December 2014 to May 2015. *Euro Surveill.* 2016;21(25):pii:30262.

11. **Tada T, Tsuchiya M, Shimada K, Nga TT, Thu LT et al**. Dissemination of carbapenem-resistant *Klebsiella pneumoniae* clinical isolates with various combinations of carbapenemases (KPC-2, NDM-1, NDM-4, and OXA-48) and 16S rRNA methylases (RmtB and RmtC) in Vietnam. *BMC Infect Dis*, 2017;17(1):467.

12. **Gianni T, Conte V, Di Pilato V, Aschbacher R, Weber C et al.** *Escherichia coli* from Italy producing OXA-48 carbapenemase encoded by a novel Tn1999 Transposon derivative. *Antimicrob Agents Chemother* 2012;56(4):2211-2213.

13. **Cuzon G, Quanich J, Gondret R, Naas T, Nordmann P.** Outbreak of OXA-48 positive carbapenem-resistant *Klebsiella pneumoniae* isolates in France. *Antimicrob Agents Chemother* 2011;55(5):2420-2423.

14. **Mazzariol A, Bošnjak Z, Ballarini P, Budimir A, Bedenić B et al.** NDM-1 producing *Klebsiella pneumoniae*, Croatia. *Emerg Infect Dis*. 2012;8(3):532-534.

15. Bedenić B, Mazzariol A, Plečko V, Bošnjak Z, Barl P, Vraneš J, Cornaglia G. First report of KPC-producing *Klebsiella pneumoniae* in Croatia. J Chemother 2012; ;24(4):237-239.

16. **Zujić Atalić V, Bedenić B, Kocsis E, Mazzariol A, Sardelić S et al.** Diversity of carbapenemases in clinical isolates of Enterobacteriaceae in Croatia-the results of the multicenter study. *Clin Microbiol and Infect* 2014;20(11):O894-903.

17. **Bedenić B, Sardelić S, Luxner J, Bošnjak Z, Varda-Brkić D et al.** Molecular characterization of clas B carbapenemases in advanced stage of dissemination and emergence of class D carbapenemases in Enterobacteriaceae from Croatia. *Infect Genetic Evol* 2016;43:74-82.

18. **Khajuria A, Prahajar AK, Kumar M, Grover N.** Emergence *of Escherichia coli*, coproducing NDM-1 and OXA-48 carbapenemases, in urinary isolates, at a Tertiary Care Centre at Central India. *J Clin Diagn Res*. 2014;8(6):DC01-4.

19. **Novak A, Goic-Barisic I, Andrašević AT, Butic I, Radic M, Jelic M, Rubic Z, Tonkic M.** Monoclonal outbreak of VIM-1-carbapenemase-producing *Enterobacter cloacae* in intensive care unit, University Hospital Centre Split, Croatia. Microb Drug Resist. 2014 20(5):399-403.

20. **Jelić M, Škrlin J, Bejuk D, Košćak I, Butić I, et al.** Characterization of Isolates Associated with Emergence of OXA-48-Producing *Klebsiella pneumoniae* in Croatia. *Microb Drug Resist*. Dec 21. doi:10.1089/mdr.2017.0168. [Epub ahead of print]

21. **Clinical and Laboratory Standards Institute**. Performance Standards for Antimicrobial Susceptibility Testing. 26th ed. CLSI supplement. M100-S. Wayne, PA: CLSI, 2016.

22. **Magiorakos AP, Srinivasan A, Carey RB, Carmeli Y, Falagas ME et al.** Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. *Clin Microbiol Infect* 2012;18:268-281.

23. **Jarlier V, Nicolas MH, Fournier G, Philippon A.** Extended broad-spectrum beta-lactamases conferring transferable resistance to newer beta-lactam agents in

Enterobacteriaceae: hospital prevalence and susceptibility patterns. *Rev Infect Dis.* 1988;10:867-878.

24. **Coudron PE.** Inhibitor-based methods for detection of plasmid-mediated AmpC β-lactamases in *Klebsiella* spp., *Escherichia coli* and *Proteus mirabilis*. *J Clin Microbiol* 2005;43:416-7.

25. **Lee K, Lim YS, Yong D, Yum JH, Chong Y.** Evaluation of the Hodge test and the imipenem-EDTA-double-disk synergy test for differentiating metallo-β-lactamase-producing isolates of *Pseudomonas* spp. and *Acinetobacter* spp. *J Clin Microbiol* 2005;41:4623-4629.

26. **van der Zwaluw K, de Haan A, Pluister GN; Bootsma HJ, de Neeling AJ et al.** The carbapenem-inacitvation method (CIM) a simple and low-cost alternative for the Carba NP test to assess phenotypic carbapenemase activity in Gram-negative rods. *Plos One*. 2015 Mar 23;10(3):e0123690. doi: 10.1371/journal.pone.0123690. eCollection 2015.

27. **Pasteran F, Mendez T, Guerriero L, Rapoport M, Corso A.** A sensitive screening test for suspected class A carbapenemase production in species of *Enterobacteriaceae*. *J Clin Microbiol* 2009;47:1631-1639.

28. **Kim YK, Geung-Hong S, Moland ES, Thomson KS.** Convenient test using combination of chelating agents for detection of metallo-β--lactamases in clinical laboratory. *J Clin Microbiol* 2007Sep;45(9):2798-2801.

29. **Nüesch-Inderbinen MT, Hächler H, Kayser FH.** Detection of genes coding for extended-spectrum SHV β-lactamases in clinical isolates by a molecular genetic method,

and comparison with the E test. *Eur J Clin Microbiol Infect Dis* 1996 May;15(5):398-402.

30**. Arlet G, Brami G, Decre D, Flippo A, Gaillot O et al.** Molecular characterization by PCR restriction fragment polymorphism of TEM β-lactamases. *FEMS Microbiol Lett* 1995;134:203-208.

31. **Woodford N, Ward ME, Kaufmann ME, Turton J, Fagan EJ et al.** Community and hospital spread of *Escherichia coli* producing CTX-M extended-spectrum β-lactamases in the UK. *J Antimicrob Chemother* 2004 Oct;54(4):735-743.

32**. Pagani L, Mantengoli E, Migliavacca R, Nucleo E, Pollini S et al.** Multifocal detection of multidrug-resistant *Pseudomonas aeruginosa* producing PER-1 extended-spectrum β-lactamase in Northern Italy. *J Clin Microbiol* 2004 Jun;42(6):2523-2529.

33. **Woodford N, Fagan EJ, Ellington MJ.** Multiplex PCR for rapid detection of genes encoding CTX-M extended-spectrum β-lactamases. *J Antimicrob Chemother* 2006 Jan;57(1):154-155.

34. **Perez-Perez FJ, Hanson ND.** Detection of plasmid-mediated AmpC β-lactamase genes in clinical isolates by using multiplex PCR. *J Clin Microbiol* 2002 Jun;40(6), 2153-2162.

35. **Poirel L, Walsh TR, Cuveiller V, Nordman P.** Multiplex PCR for detection of acquired carbapenemases genes. *Diagn Microbiol Infect Dis* 2011 May;70(1):119-125.

36. **Robicsek A, Jacoby GA, Hooper DC.** The worldwide emergence of plasmid-

mediated quinolone resistance. *Lancet Infect Dis* 2006 Oct ;6(10):629-640.

37. **Liu Liu YY, Wang Y, Walsh T, Yi LX, Zhang R et al.** Emergence of plasmid-mediated colistin resistance mechanism MCR-1 in animals and human beings in China: a microbiological and molecular biological study. *Lancet Infect Dis.* 2016 Feb;16(2):161-168.

38. **Cannateli A, Giani T, D'Andrea MM, Di Pilato V, Arena F, Conte V, Tryfininopoulo K.** MgrB inactivation is a common mechanism of colistin resistance in KPC-producing *Klebsiella pneumoniae* of clinical origin. *Antimicrob Agents Chemother* 2014 Oct;58(10):5696-5703.

39. **Saladin, M., Cao, V. T., Lambert, T., Donay, J. L., Herrmann, J. L.,Ould-Hocine, Z., Verdet, C., Delisle, F., Philippon, A. & Arlet, G**.(2002). Diversity of CTX-M beta-lactamases and their promoterregions from Enterobacteriaceae isolated in three Parisian hospitals.FEMS Microbiol Lett 209, 161–168.

40. **Elwell LP, Falkow S.** The characterization of R plasmids and the detection of plasmid-specified genes. In: Lorian V, ed. Antibiotics in Laboratory Medicine. 2nd edn. Baltimore MD: Williams and Wilkins, 1986:683-721.

41**. Choi KH, Kumar A, Schweizer HP** A 10-min method for preparation of highly electrocompetent *Pseudomonas aeruginosa* cells: Application for DNA fragment transfer between chromosomes and plasmid transformation. Journal of Microbiological Methods 2006: 64: 391-397 .

42. **Zankari E, Hasman H, Cosentino S, Vestergaard M, Rasmussen S et al.** Identification of acquired antimicrobial resistance genes. J Antimicrob Chemother. 2012 Nov;67(11), 2640-2644.

43. **Carattoli A, Bertini A, Villa L, Falbo V, Hopkins KL et al.** Identification of plasmids by PCR-based replicon typing. *J Microbiol Methods* 2005;63(3):219-28.

44. Carattoli A, Sieffert S, Schwendener S, Perreten V, Endimiani A. Differentiation of IncL and IncM plasmids associated with the spread of clinically relevant antimicrobial resistance. *Plos one* 2015 May 1;10(5):e0123063. DOI: 10.1371 /journal.pone.0123063. eCollection 2015.

45. **Kaufman ME**. Pulsed-Field Gel Electrophoresis. In: Woodfor N and Johnsons A, eds. Molecular bacteriology. Protocols and clinical applications. 1st edn. New York: Humana Press Inc. Totowa; 1998:33-51.

46. **Tenover FC, Arbeit RD, Goering RV, Mickelsen PA, Murray BE et al.** Interprinting chromosomal DNA restriction patterns produced by pulsed-filed gel electrophoresis; criteria for bacterial strain typing. *J Clin Microbiol* 1995 Sep;33(9):2233-2239.

47.**Osterblad M, Hakanen A, Jalava J.** Evaluation of the Carba NP test for carbapenemase detection. *Antimicrob Agent Chemother* 2014;58 (12):7553-6.

48. **Thiolas A, Bollet C, La Scola B, Raoult D, Pages JM**. Successive Emergence of *Enterobacter aerogenes* strains resistant to imipenem and colistin in a patient. Antimicrob Agents Chemother 2005;1354-1358.

49. **Bedenić B, Vranić-Ladavac M, Venditti C, Tambić-Andrašević A, Barišić N, Gužvinec M, Karčić N, Petrosillo N, Ladavac R, di Caro A.** Emergence of colistin resistance in *Enterobacter aerogenes* from Croatia. J Chemother 2017doi: 10.1080/1120009X.2017.1382121.

50. **Cannatelli A, D'Andrea MM, Giani TZ, Di Pilato V, Arena F, Ambretti S, et al**. In vivo emergence of colistin resistance in *Klebsiella pneumoniae* producing KPC-type of carbapenemases mediated by insertional inactivation of the PhoQ/PhoP mrg regulator. Antimicrob Agents Chemother; 2013;57:5521-6.

50. Huang TD, Bogaets P. Berhin M, Bauraing C, Glupczyinski Y et al. Increasing proportion of carbapenemase-producing Enterobacteriaceae and emergence of MCR-1 producer through a multicenter study among hospital based and private laboratories in Belgium from September to November 2015. *Euro Surveill.* 2017 May;11:22(19). pii: 30530. doi: 10.2807/1560-7917.ES.2017.22.19.30530.

51. **Mansour W, Haenni M, Saras E, Grami R, Mani Y et al.** Outbreak of colistin-resistant carbapenemase producing *Klebsiella pneumoniae* in Tunisia. *J Glob Antimicrob Resist* 2017 Sep;10:88-94

FIGURE LEGEND

Fig. S 1. (supplementary material) Map of Croatia with the location of participating centers.

Fig S 2a. (supplementary material) PFGE dendrogram of OXA-48-producing *K. pneumoniae* isolates. The strain number, protocol number, PFGE cluster, date of isolation, hospital ward and specimen are shown. The cut of value of 80% was used to define a clone. The subclones differed in one or two bands. Abbreviations: NT-not tested, S- singleton

Fig. 2b . PFGE dendrogram of OXA-48 producing *E. cloacae* isolates. The strain number, protocol number, PFGE cluster, date of isolation, hospital ward and specimen are shown. The cut of value of 80% was used to define a clone. The subclones differed in one or two bands. Abbreviations: NT-not tested, S- singleton

No	CENTRE AND ISOLATE NUMBER	SPECIMEN	DEPARTMENT	DATE	ESBL	AMPC	Hodge	CIM	CAZ	CTX	CRO	FEP	IPM	MEM	ERT	GM	CIP	COL	Other β-lactamases and *qnr* genes	PFGE cluster	Type of infection	Antibiotic treatment	Clinical outcome
	Klebsiella pneumoniae																						
1	Osijek OS 5 (2977)	Catheter urine	Neurology	26.04.2016	+	+	+	-	64	>128	>128	>128	0.5	1	4	0.25	>128	1	SHV OXA-1 CTX-M TEM *Qnr* A	KIa	Colonization	No	R
2	Osijek OS 3 (4568)	Pharyngeal swab	Haematology	14.03.2016.	-	-	+	+	0.5	0.25	0.25	0.12	0.06	4	4	0.5	>128	1	SHV	KIa	Colonization	COL	R
3	Osijek OS 17 (16859)	Tracheal aspirate	ICU	17.10.2016.	+	-	+	+	>128	>128	>128	8	0.5	0.25	4	0.5	>128	0.25	SHV CTX-M *Qnr* B	KIb	Pneumonia	MEM	D
4	Osijek OS 10 (4700)	Urine	Cardiology	05.07.2016.	+	-	+	+	64	64	>128	32	0.25	0.12	2	0.5	>128	1	SHV CTX-M TEM	KIc	UTI	MEM	R
5	Osijek OS 4 (1603)	Blood	ICU	12.04.2016	+	+	+	-	32	>128	>128	>128	64	32	32	0.5	>128	1	SHV OXA-1 CTX-M *Qnr* B	KId	Sepsis	MEM VAN	D
6	Osijek OS 19 (8269)	Catheter urine	Infectology	01.12.2016.	+	-	+	+	32	64	64	16	8	8	32	>128	32	0.06	SHV CTX-M	KIe	UTI	IMI	D
7	Osijek OS 22 (20566)	Abdominal swab	Abdominal surgery	14.12.2016	-	-	+	+	0.25	0.5	0.5	0.06	0.12	0.25	1	0.25	>128	2	TEM SHV	KIe	Peritonitis	TIG COL	D
8	Osijek OS 15 (13744)	Axillar swab	ICU	23.08.2016	-	-	+	+	0.5	0.25	0.25	0.12	0.25	0.5	0.5	4	32	0.5	SHV-1 *Qnr* B	KIe	Colonization	FEP	D
9	Osijek OS 21 (8499)	Urine	Neurosurgery	6.12. 2016	+	-	+	+	32	>128	>128	4	0.06	0.25	1	0.12	>128	64	SHV CTX-M *Qnr A,* B	KIe	UTI	MEM	D
10	Osijek OS2 (1365)	Blood	Gastroenterology	28.03.2016	-	-	+	+	8	1	0.5	0.25	0.25	1	2	0.25	>128	1	SHV-1 OXA-1	KIe	Sepsis	CIP MET	R

11	Osijek 3177	Tracheal aspirate	Internal clinic – ICU	22.02.2016.	-	-	+	+	0.12	0.25	0.5	0.25	0.12	0.12	0.5	0.12	0.25	0.25	SHV-1	KIe	Pneumonia	AMC AMI	D
12	Osijek OS 18 (17068)	Tracheal aspirate	ICU	20. 10. 2016.	+	-	+	+	64	>128	>128	8	0.5	0.5	8	0.25	>128	0.25	SHV CTX.-M *Qnr* B	KIf	Pneumonia	TZP	R
13	Osijek OS 419	Tracheal aspirate	ICU	10.01.2016.	+	-	+	+	>128	>128	>128	>128	16	32	32	0.5	>128	1	SHV TEM CTX-M	KIf	Pneumonia	IMI	D
14	Split UR 5429	urine	Internal clinic	24.03.2017.	+	-	+	+	>128	>128	>128	64	4	16	32	1	>128	0.12	SHV CTX-M	KIg	UTI	MEM AMI	I
15	Split UR 5429	urine	Internal clinic	24.03.2017.	+	-	+	+	>128	>128	>128	64	4	16	32	1	>128	0.12	SHV CTX-M	KIg	UTI	MEM AMI	I
16	Split DG 3759	Wound swab	ICU	11.04.2017.	+	-	+	+	64	>128	>128	>128	4	16	4	0.12	>128	0.12	SHV CTX-M	KIg	Postoperative osteomyelitis	COL VAN MEM	I
17	Split UR7213	urine	Infective clinic	21.04.2017.	+	-	+	+	64	>128	>128	32	2	16	8	0.12	>128	0.12	SHV CTX-M	KIg	Osteomyelitis UTI	FOS	I
18	Split UR 5817	urine	Neurosurgery	30.03.2017.	+	-	+	+	>128	>128	>128	>128	4	16	16	4	>128	0.5	SHV CTX-M *Qnr* B	KIg	UTI	GM	R
19	Pula 322-1	Lanolin cream	Nursing home	09.09.2016.	+	-	+	+	16	>128	64	16	2	4	4	0.5	32	0.5	SHV TEM CTX-M *Qnr* B	KIh	Colonization	None	R
20	Osijek OS 8 (4063)	Catheter urine	Neurosurgery	09.06.2016	+	-	+	+	8	>128	>128	4	2	2	4	1	>128	0.5	SHV-1 OXA-1 CTX-M-15	KIIa	UTI	CIP	R

21	Zagreb VG-8166	urine	Urology	19.02.2015.	+	-	+	-	>128	>128	>128	>128	1	0.5	16	>128	32	0.003	SHV-1 CTX-M-15	KIIa	UTI	MEM	R
22	Osijek OS 11 (11461)	Abdominal swab	Abdominal surgery	12.07.2016	-	-	+	+	0.25	0.5	0.25	0.12	0.25	0.12	2	0.5	>128	1	SHV	KIIb	Peritonitis	AMC MET GEN	D
23	UHC ZGB 149765	Perineal swab	Internal clinic	17.08.2016	-	-	+	+	0.25	0.25	0.25	0.06	4	4	4	0.25	1	0.12	SHV	KIIb	Pleuropneumonia	MEM	R
24	Pula 16780-1	Catheter urine	Neurology	21.07.2016.	+	-	+	+	16	>128	>128	64	32	64	32	0.25	>128	0.5	SHV-1 CTX-M-15 *Qnr* B	KIIc	Colonization	None	R
25	Pula 15636-1	Perineal swab	Infectology	08.04.2017.	+	-	+	+	16	64	32	2	2	8	4	0.25	>128	0.5	SHV TEM CTX-M *Qnr* B	KIIc	Colonization	None	D
26	Slavonski brod 15307	Urine	ICU	15.05.2017.	+	-	+	+	4	32	8	8	1	1	4	32	>128	0.12	SHV-1 CTX-M-15 TEM-1	KIIc	UTI	AMC AZI ERT	D
27	Slavonski brod 16098	Urine	Neurosurgery	22.05.2017.	+	-	+	+	32	>128	>128	>128	1	0.5	2	32	>128	0.25	SHV CTX-M TEM	KIIc	UTI	CZ, LEV ERT, AMI	R
28	Split KK 3602	Rectal swab	Neurosurgery	13.4.2016.	+	-	+	+	64	>128	>128	>128	1	4	8	0.12	>128	0.12	SHV CTX-M	S	Pneumonia	COL MEM AMI	I
	UHC ZGB 190807	Urine	Internal clinic (farmacology)	17.10.2016	-	-	+	+	4	0.5	0.12	0.06	32	32	64	0.12	2	1	SHV-1	KIIIa	Urosepsis	CXM	I
30	Rijeka 012552	External ear swab	Dermatovenerology	07.03.2016.	-	-	+	+	4	1	1	1	4	2	16	0.12	2	1	SHV *Qnr* B	KIIIb	Purulent otitis media	TOB MOX drop	R
31	Osijek 10059	Tracheal aspirate	Surgical ICU	31.07.2015.	-	-	+	+	4	1	1	0.5	2	1	16	0.25	2	0.5	SHV *Qnr* B	KIIIc	Pneumonia	MEM	I
32	Osijek OS 7 (8127)	Pharyngeal swab	ICU	27.05.2016	-	-	+	+	4	1	2	0.12	2	1	8	0.5	0.25	1	SHV *Qnr* B	KIIId	Colonization	No	R

33	Zagreb VG-16/3885	Blood	ICU	13.12.2016.	+	-	+	-	>128	>128	>128	16	0.5	4	8	>128	0.5	1	TEM-1 SHV-11 CTX-M-15	KIIIe	Sepsis	MEM	R
34	Pula 15889	Catheter urine	Neurology	30.06.2017.	+	+	+	-	16	>128	>128	64	32	64	32	0.25	>128	0.5	SHV CTX-M-TEM *Qnr* B	KIVa	Colonization	None	R
35	Slavonski brod 24889	Perineal swab	Surgery	15.03.2017.	+	+	+	+	>128	>128	>128	64	32	32	64	>128	>128	128	SHV CTX-M TEM *Qnr* B	KIVa	Colonization	AMI	R
36	Osijek OS 13 (4988)	Catheter urine	ICU	16.07.2016	+	-	+	-	32	64	>128	32	0.25	0.25	1	0.5	>128	1	SHV CTX-M	S	UTI	MEM	D
37	Split UR 22272	Urine	Haematology	31.12.2016.	+	-	+	+	32	>128	>128	>128	4	8	16	0.25	>128	0.12	SHV-11 TEM-1 CTX-M-15 *Qnr* B	KVa	Pyelonephritis	MEM AMI	I
38	Osijek OS 16 (15299)	Wound swab	Abdominal surgery	19.09.2016.	+	-	+	+	32	>128	>128	16	1	0.5	4	0.25	>128	1	SHV CTX-M *Qnr* B	KVa	Wound infection	AMI MEM	R
39	Osijek OS 9 (10454)	Intraoperative swab	Neurosurgery	24.06.2016.	-	-	+	+	0.5	0.25	0.25	0.25	1	0.5	0.25	0.5	>128	0.25	SHV *Qnr* B	S	Wound infection	FEP	R
40	Osijek OS 25 (9585)	Pharyngeal swab	Neurology	5.6.2017.	+	-	+	+	32	>128	>128	>128	0.5	0.5	0.5	0.06	128	2	TEM SHV CTX-M *Qnr* B	NT	Colonization	CIP IMI	R

Table 1.a Antibiotic susceptibility, resistance genes content, genotypes of *Klebsiella pneumoniae* isolates and clinical data.

Abbreviations: CAZ-ceftazidime; CTX-cefotaxime; CRO-ceftriaxone; FEP-cefepime; IMI-imipenem; MEM-meropenem; ERT-ertapenem; GM-gentamicin; CIP-ciprofloxacin; AMI –amikacin; TOB-tobramycin; AZI- azitromycin; LEV-levofloxacin; CXM-cefuroxime; AMC-amoxicillin-clav.acid; COL-colistin; VAN-vancomycin; TIG-tigecycline; FOS-fosfomycine; CZ-cephazoline; ESBL-inhibitor based test with clavulanic acid for detection of extended-spectrum

beta-lactamases; Amp-C-inhibitor based test with phenyloboronic acid for detection of AmpC beta-lactamases; BL-beta-lactamase content; CIM-carbapenem inactivation method; PFGE-pulsed field gel electrophoresis NT-not tested; S-singleton; ICU-intensive care unit; RES-resistance genes; R- released from the hospital; I-improved; D-died.

No	CENTRE AND ISOLATE NUMBER	SPECIMEN	DEPARTMENT	DATE	ESBL	AMPC	Hodge	CIM	CAZ	CTX	CRO	FEP	IPM	MEM	ETP	GM	CIP	COL	Other β-lactamases and *qnr* genes	PFGE cluster	Type of infection	Antibiotic treatment	Clinical outcome
Enterobacter cloacae																							
1	Osijek OS 14(5824)	Catheter urine	Internal clinic	23.08.2016.	+	+	+	+	>128	64	>128	64	2	4	8	32	8	1	CTX-M *Qnr* B	EIa	UTI	COL, RIF	D
2	Osijek 7766	Catheter urine	Neurology	17.11.2015.	+	+	+	+	32	>128	>128	32	2	1	16	32	8	0.5	CTX-M	EIb	NA	NA	NA
3	Pula 17504	Urine	Oncology	23.08.2017.	+	+	+	+	>128	>128	>128	64	4	4	16	>128	>128	2	OXA-1 CTM-15 TEM-1 ACT-7	EIc	Col	NA	D
4	Slavonski Brod 30676	Urine	ICU		+	+	+	+	32	>128	>128	32	0.25	0.5	1	>128	2		TEM-1 b CTX-M-15 OXA-1 ACT-7	EIc	UTI	AMC	R
5	Split HK 1076	Blood culture	Neurosurgery	25.01.2016.	+	-	+	+	>128	>128	>128	>128	4	8	16	4	>128	0.12	CTX-M	EId	Pneumonia	MEM, VAN	I
E.coli																							
1	Pula 18464	urine	Urology	28.08.2017.	-	-	+	+	1	0.5	0.25	0.12	16	2	4	32	>128	2	TEM-1 *Qnr* B		Colonization	No	R
2	Osijek OS 24 (9322)	Wound swab	Traumatology	30.05.2017.	-	-	+	+	0.12	0.5	0.5	0.12	0.5	0.5	0.5	0.06	128	2			Wound infection	AMC MET MEM VAN	R
Citrobacter freundii																							
1	Osijek 20 (19409)	Wound swab	Abdominal surgery	28.11.2016.	-	+	+	+	1	0.5	0.5	0.06	0.06	0.06	1	0.25	0.06	1	CMY		**NA**	NA	D

Table 1.b Antibiotic susceptibility, resistance genes content, genotypes of *Enterobacter cloacae, Eshcerichia coli* and *Citrobacter freundii* isolates and clinical data.

Abbreviations: CAZ-ceftazidime; CTX-cefotaxime; CRO-ceftriaxone; FEP-cefepime; IMI-imipenem; MEM-meropenem; ERT-ertapenem; GM-gentamicin; CIP-ciprofloxacin; AMI –amikacin; TOB-tobramycin; AZI- azitromycin; LEV-levofloxacin; CXM-cefuroxime; AMC-amoxicillin-clav.acid; COL-colistin; VAN-vancomycin; TIG-tigecycline; FOS-fosfomycine; CZ-cephazoline; ESBL-inhibitor based test with clavulanic acid for detection of extended-spectrum beta-lactamases; Amp-C-inhibitor based test with phenyloboronic acid for detection of AmpC beta-lactamases; BL-beta-lactamase content; CIM-carbapenem inactivation method; R- released from the hospital; I-improved; D-died, ICU-intensive care unit; NA-not applicable

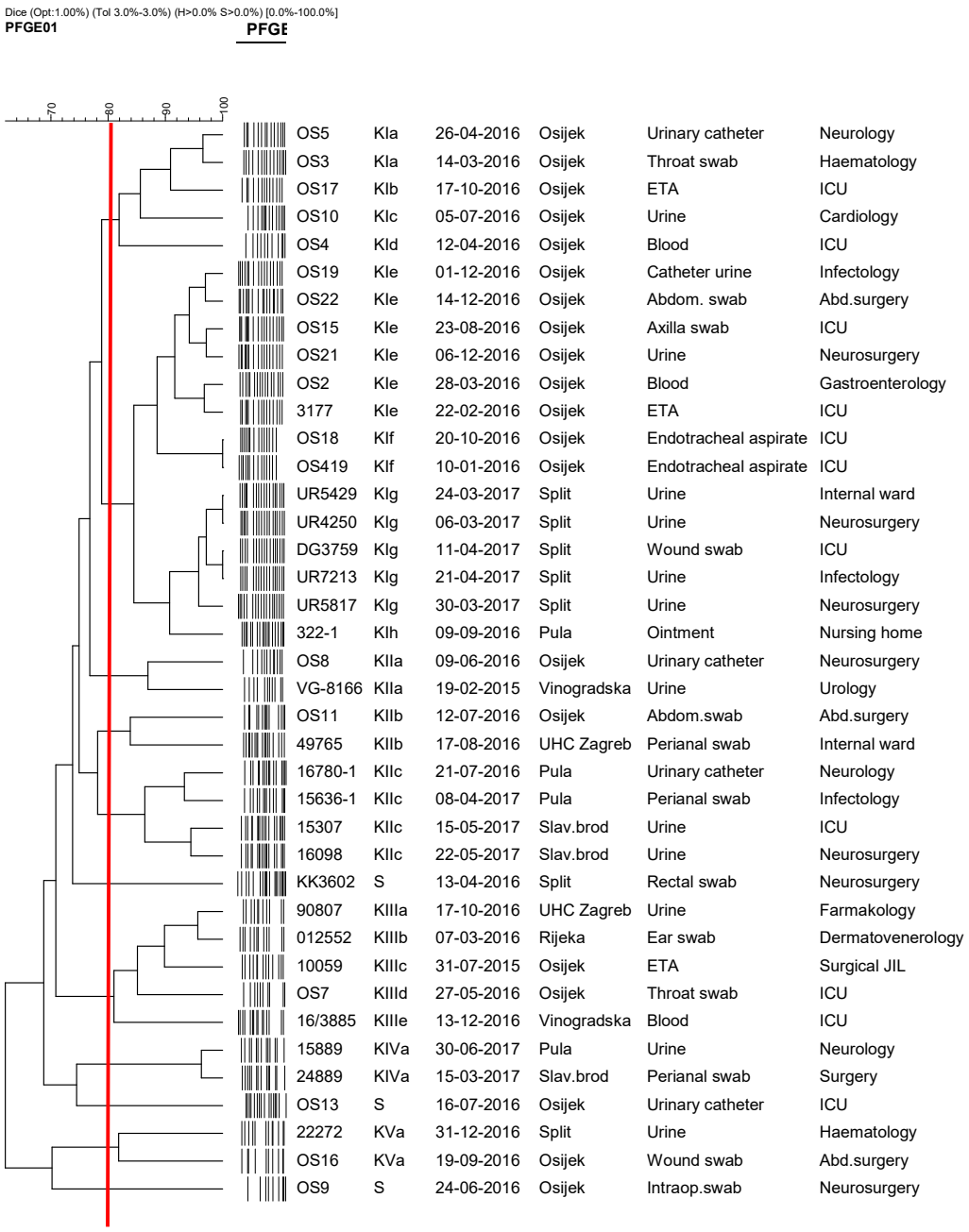

Fig S 2a. (supplementary material) PFGE dendrogram of OXA-48-producing *K. pneumoniae* isolates. The strain number, protocol number, PFGE cluster, date of isolation, hospital ward and specimen are shown. The cut of value of 80% was used to define a clone. The subclones differed in one or two bands. Abbreviations: NT-not tested, S- singleton

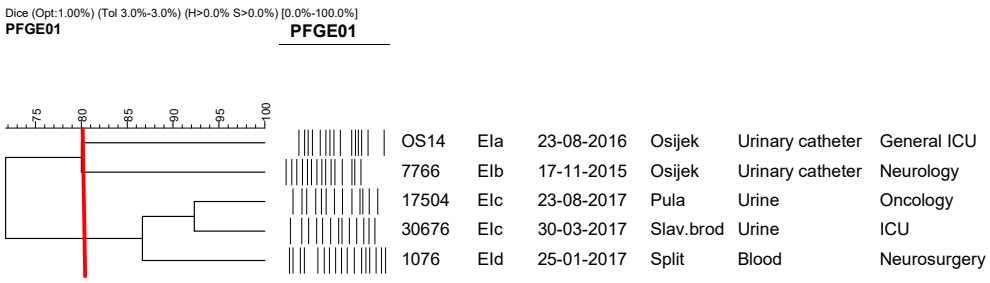

Fig. S 2b . (supplementary material). PFGE dendrogram of OXA-48 producing *E. cloacae* isolates. The strain number, protocol number, PFGE cluster, date of isolation, hospital ward and specimen are shown. The cut of value of 80% was used to define a clone. The subclones differed in one or two bands. Abbreviations: NT-not tested, S- singleton