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Epidemic spread of OXA-48 beta-lactamase in Croatia

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Abstract

Purpose. A dramatic increase in OXA-48 β-lactamase was observed recently not only in large hospital centres, but also in smaller suburban hospital centres in geographic areas bordering 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 and 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 from seven hospital centres in Croatia from May 2016 to May 2017, were analysed (40 *Klebsiella pneumoniae*, 5 *Enterobacter cloacae*, 2 *Escherichia coli* and one *Citrobacter freundii*). Thirty-three isolates were ESBL positive and harboured group 1 CTX-M 1 β-lactamases. In addition to the β-lactam resistance genes detected by PCR (bla_{SHV-1}, bla_{OXA-48} and bla_{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 found in this study showed wide variability in antibiotic susceptibility, β-lactamase content and PFGE banding patterns. This study revealed a switch from the predominance of VIM-1 in 2012–2013 to that of OXA-48 in the 2015 to 2017.

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

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The gene sequences are submitted to the Gene bank under accession numbers ERS2513354–ERS2513358.

Two supplementary tables are available with the online version of this article.

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 class D [1, 2]. OXA-48 β-lactamase was reported for the first time in Turkey in 2004 [3]. In the last decade a marked increase in 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 the Far East [11]. The rapid dissemination of 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 also been reported [13].

The first carbapenem-resistant enterobacterial strain detected in Croatia was NDM-1-producing *K. pneumoniae*, isolated at the University Hospital Centre Zagreb in 2008 [14]. In 2012 the first KPC-positive *K. pneumoniae* was reported [15]. A marked increase in the number of carbapenem-resistant isolates was observed in 2012. This observation gave rise to a multi-centre study on carbapenem-resistance in *Enterobacteriaceae* from Croatia, conducted in 2012,which revealed the predominance of the VIM-1 metallo-β-lactamase in two large hospital centres [16]. There were 36 VIM-1-positive isolates (90 %) among 40 carbapenemase-producing isolates. Three of the remaining isolates were positive for NDM-1 and one for KPC-2. The proportion of VIM-1-producing isolates compared to the total number of carbapenem- non-suceptible isolates was 63 % (36/57). Two years later a clonal outbreak of VIM-1-positive *Enterobacter cloacae* and *Citrobacter freundii* was observed in the largest hospital centre in Croatia. In the same study, the emergence of OXA-48 β-lactamase in *Enterobacteriaceae* was reported for the first time in two hospital centres in Croatia [17]. In total, 34 *E. cloacae* isolates were found to possess VIM-1 and were allocated to eight clusters with one large clone comprising 18 identical isolates. Seventeen *C. freundii* isolates were identified as VIM-1 producers, and these belonged to two clusters with one containing 11 identical isolates. In total, 65 patients were either infected or colonized with VIM-1-producing organisms (14 with *K. pneumoniae*, 34 with *E. cloacae* and 17 with *C. freundii*). In that study, the co-existance of VIM-1, NDM-1 and OXA-48 was reported for the first time in Croatia, similar to that 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, a monoclonal outbreak associated with VIM-1 *E. cloacae* (6 isolates) was reported from University Hospital Split in 2012 [19]. VIM-1 was the sole carbapenemase in that hospital centre.

The first OXA-48-producing organisms in Croatia, in 2011/2012 from the northwestern area of Croatia, were reported in a multi-centre study performed from 2010 to 2012 but published only recently [20].

Following sporadic cases between 2010 and 2013 [17, 20], between 2015 and 2017 an increase in OXA-48 β-lactamase was observed by clinical microbiologists in participating centres, not only in large hospital centres but also in the smaller hospitals in the peripheral, outlying areas of Croatia. Since all carbapenem-resistant Enterobacteriaceae are sent to the reference laboratory for the identification of carbapenemase type, clinical microbiologists are notified about the results of carbapenemase identification. All OXA-48-positive organisms were sent to the University Hospital Centre Zagreb for analysis of the epidemiology, the mechanisms of antibiotic resistance and routes of dissemination of OXA-48 carbapenemase in Croatia.

METHODS

Bacterial isolates

In total, 48 enterobacterial isolates were collected from May 2015 to May 2017, from 10 centres in Croatia which participated in the study: University Hospital Centre 'Sestre Milosrdnice', University Hospital Centre Zagreb, University Hospital Centre Split, University Hospital Centre 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 (available in the online version of this article). The isolates were identified to the species level by conventional biochemical testing using Vitek 2 or MALDI-TOF, depending on the routine laboratory where they were isolated. Only those isolates confirmed as $bla_{\text{OXA-48}}$-positive in the reference centre were included in the study. All enterobacterial isolates with reduced susceptibility to at least one carbapenem by disk diffusion test, in all routine diagnostic laboratories in Croatia, are obliged to be sent to the Reference Centre for Antibiotic Resistance Surveillance at 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_{VIM}, bla_{NDM}, $bla_{\text{OXA-48}}$ and bla_{KPC} genes was conducted for those isolates positive in phenotypic tests. Clinical microbiologists in the routine laboratories are notified by the reference laboratory about the type of carbapenemase, and isolates confirmed as possessing OXA-48 were sent to the Clinical Department for Clinical and Molecular Microbiology of the University Hospital Centre Zagreb for further analysis.

All other analyses of isolates, including antimicrobial susceptibility testing, phenotypic detection of β-lactamases, molecular detection of other β-lactamases, *qnr* genes, and insertion sequence, plasmid characterization and genotyping, were carried out at the Clinical Department for Clinical and Molecular Microbiology, University Hospital Centre Zagreb. Whole-genome sequencing was performed at the Austrian Institute for Technology.

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

Antimicrobial susceptibility to amoxicillin alone and in combination 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). Susceptibility to fosfomycin was determined by agar dilution test. *E. coli* ATCC 25922 and *K. pneumoniae* 700603 were used as quality control strains for minimum inhibitory concentration (MIC) determination. Susceptibility to sulphametoxazole/trimethoprim, tetracycline and chloramphenicol was determined by disk-diffusion test. Isolates were classified as multi-drug-resistant (MDR), extensively drug-resistant (XDR) or pan-drug-resistant (PDR) as described previously by Magiorakos *et al.* [22].

The double-disk synergy test (DDST) [23] and CLSI combined disk test with addition of clavulanic acid were performed to detect extended-spectrum beta-lactamases (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].

Molecular detection of resistance genes

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} and bla_{NMC}), class B carbapenemases (bla_{VIM}, bla_{IMP} and bla_{NDM}), carbapenem-hydrolysing oxacillinases ($bla_{OXA-48-like}$) and fluoroquinolone resistance genes (*qnr*A, *qnr*B and *qnr*S) were determined by PCR using protocols and conditions as described previously [29–36]. Grouping of CTX-M β-lactamases was detected by multiplex PCR [33]. Inactivation of *mgrB* genes and plasmid-encoded colistin resistance genes *mcr-1* were analysed 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 centre (*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 variance of the TEM, SHV and CTX-M β-lactamases. The positive control strains producing TEM-1, TEM-2, SHV-1 and SHV-2 were kindly provided by Professor 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 determined by gel electrophoresis after staining with ethidium bromide. The amplification products from selected strains (*E. cloacae* 17504, *K. pneumoniae* 7213, *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}. The genetic context of bla_{CTX-M} genes was determined by PCR mapping with forward primer for IS*Ecp1* and IS*26* combined with primer MA-2 (reverse for bla_{CTX-M} genes) [39].

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 ($0.5\,mg\,l^{-1}$) or cefotaxime ($2\,mg\,l^{-1}$) and sodium azide ($100\,mg\,l^{-1}$). The frequency of conjugation was determined relative to the number of donor cells. Co-transfer of resistance to gentamicin, tetracycline, sulfamethoxazole/trimethoprim, chloramphenicol and ciprofloxacin was determined. Those isolates which did not yield transconjugants were subjected to a 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 MacConkey medium containing $1\,mg\,l^{-1}$ of meropenem.

Whole-genome sequencing (WGS)

WGS was carried out at the Austrian Institute for Techology. Five representative OXA-48-producing isolates of different species and from various hospital centres 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. 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 analysed using the RAST analysis platform and the ResFinder web-service to screen for antibiotic resistance genes and their genetical context [42].

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, DNA was visualized 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 and to PBRT to determine the plasmid ncompatibility groups. Positive control strains for PBRT were kindly provided by Dr A. Carattoli (Insituto Superiore di Sanita, Rome, Italy).

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 Gel-Compar software. The dendrogram was computed after band intensity correlation using global alignment with 1.5 % optimization and 1 % tolerance and the unweighted pair-group method using arithmetical averages (UPGMA) clustering. PFGE cluster analysis was carried out with Gel Compare II (Applied Maths, Belgium) using the Dice similarity coefficient and clustering by UPGMA [45]. Band patterns were visually compared to define indistinguishable and closely related subtypes 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. *K. pneumoniae* clusters were designated as K, and *E. cloacae* as E.

RESULTS

Patients data and bacterial isolates

Thirty-six patients had an infection with an isolate including OXA-48, whereas 12 patients were only colonized. The type of infection, antibiotic treatment and outcome are shown in Tables 1 and 2. Urinary tract infection was the predominant type of infection (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 data were not available.

In total, 48 isolates were analysed in the study: 25 from University Hospital Centre Osijek, eight from University Hospital Centre Split, six from General Hospital Pula, four from General Hospital Slavonski Brod, two each from University Hospital Centre Zagreb and University Hospital Centre 'Sestre Milosrdnice' and one from University Hospital Centre Rijek. 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. The rate of carbapenem-resistant Enterobacteriaceae varied from 0.03 % in Dubrovnik to 4 % at the University Hospital Centre 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 Centre Osijek, −0.9 % (29/3181) in General Hospital Slavonski Brod, −1 % (93/8958) in University Hospital Centre 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 resistance rate and hospital size was observed. Large hospitals such as University Hospital Centre 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: 1.25 % (1/8) in University Hospital Centre Rijeka, 13 % (47/355) in University Hospital Centre Zagreb, 14 % (4/29) General Hospital Slavonski Brod, 15 % (14/93) in University Hospital Centre Split, 50 % (24/48) in University Hospital Centre Osijek and 100 % (6/6) in General Hospital Pula. Fifteen patients died and the other 21 recovered from their infection. Various antibiotic combinations, including meropenem, colistin and amikacin, were used for treatment (Tables 1 and 2). Meropenem was the most frequently prescribed antibiotic, administered in 15 cases.

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 1. Eleven out of 40 isolates (27 %) were resistant to fosfomycin. Twenty-four isolates (60 %) were resistant to

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

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

Table 1. cont.

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

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.

Table 2. Antibiotic susceptibility, resistance genes content, genotypes of *Enterobacter cloacae, Eshcerichia coli* and *Citrobacter freundii* isolates and clinical data

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

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.

sulfamethoxazole/trimethoprim and eight (20 %) to tetracycline. *E. cloacae* isolates were uniformly resistant to amoxicillin alone and in combination with clavulanate, piperacillin/tazobactam, cefazoline, expanded-spectrum cephalosporins and cefepime (Table 2). 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 2. They exhibited variable MICs for carbapenems. One isolate was resistant to sulfamethoxazole/trimethoprim and tetracycline. One *C. freundii* isolate was susceptible to all tested antibiotics except amoxicillin alone and in combination with clavulanate and piperacillin (Table 2). In summary, 31 isolates were MDR and two were XDR (OS21 and 24889), since they were resistant also to colistin (MIC values of 64 and 128 mg l^{-1}, 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 (Tables 1 and 2). Inhibitor-based testing with PBA for the detection of AmpC- β-lactamases was positive in four *E. cloacae*, one *C. freundii* and four *K. pneumoniae* isolates. Hodge's 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 1). Two (VG 8166 and OS5) of the six CIM negative isolates were resistant only to ertapenem, with MIC values of 16 and 4 mg l^{-1}, respectively. One isolate (VG 16/3885) was susceptible only to imipenem, with a MIC value of 0.5 mg l^{-1}, whereas the other two (OS4, 158889) were resistant to all three carbapenems with MIC values ranging between 32 and 64 mg l^{-1}. One isolate (OS 13) was fully susceptible to all three carbapenems, with MIC values equal or below 1 mg l^{-1} as shown in Table 1.

Molecular detection of resistance genes

All 28 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 $bla_{CTX\text{-}M\text{-}15}$ (Table 1). $bla_{CTX\text{-}M\text{-}15}$ genes were preceded by an IS*Ecp* insertion sequence. Twelve CTX-M-producing isolates harboured TEM-1 addition to OXA-48 and three OXA-1 (Table 1). 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 2). The *E. coli* isolates possessed only OXA-48 combined with the broad-spectrum TEM-1 β-lactamase in one isolate (Table 2). One *C. freundii* isolate

possessed chromosomal CMY in addition to OXA-48 (Table 2). Genes *Qnr*A and *Qnr*B, contributing to fluoroquinolone resistance, were found in two and 20 isolates, respectively. *K. pneumoniae* was the dominant species carrying *qnr* genes (20 out of 22). The *mcr*-1 gene, contributing to colistin resistance, was not found in colistin-resistant *K. pneumoniae* isolates. Other species did not show colistin resistance.

PCR analysis detected wild-type *mgr*B genes. PCR mapping revealed augmentation of the PCR product obtained with forward primer for IS*1999* and reverse for $bla_{\text{OXA-48}}$ 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 $bla_{\text{OXA-48}}$ gene and *lys*R, IS*1999* and *tnp*A downstream of the $bla_{\text{OXA-48}}$ gene.

Conjugation and transformation

Reduced susceptibility to meropenem of 31 isolates was transferred to the *E. coli* recipient strain with a frequency ranging from 1.2 to 8.4×10^{-6}. Sixteen out of 33 ESC-resistant isolates transferred cefotaxime resistance to the *E. coli* recipient with a frequency ranging from 8×10^{-8} to 4×10^{-4}. The transconjugants obtained with cefotaxime as selective agent showed similar resistance patterns to ESC as their respective donors. Resistance to sulphametoxazole was co-transferred alongside with cefotaxime resistance in six, and to tetracycline in two tested isolates that transferred cefotaxime resistance. The transconjugants obtained with meropenem as selective agent did not exhibit resistance to sulphonamides, tetracyclines, chloramphenicol or gentamicin. The 16 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.

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 which extends its drug targets to include also 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 in addition to blaCTX-M-15. *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 $bla_{\text{TEM-1b}}$ genes.

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 the $bla_{\text{OXA-48}}$ gene flanked with IS*1999* 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.

Plasmid characterization

A plasmid of 60–70 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 [44]. The PCR for L/M plasmid was negative. The transconjugants obtained with meropenem as selective agent harboured L plasmid as their respective donors.

Pulsed-field gel electrophoresis (PFGE)

Genotyping revealed the existence of five clusters among 39 *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. S2a). Isolates originating from different hospital wards in the same centre and with different β-lactamase content, including ESBL-positive and -negative, clustered together. The second cluster contained eight isolates, arranged in three subclusters with isolates obtained from five hospital centres (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 centres (University Hospital 'Sestre Milosrdnice', Rijeka, University hospital Centre Zagreb, Osijek and Rijeka). Clusters IV and V each comprised only two isolates. Three isolates (OS 9, OS 13 and KK3602) had unique banding patterns and were designated as singletons (Fig. S2a). The isolates belonging to one cluster in one hospital were usually from the same period but included different hospital wards, indicating cross-infection.

five *E. cloacae* isolates from different centres (Pula, Slavonski Brod, Osijek and Split) were allocated to two clusters E I and EII. The isolaes within one cluster showed >85 % similarity, but some diversification was observed within the clones and thus four subclusters were identified (Fig. S2b).

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

DISCUSSION

After the first report of OXA-48 in a large hospital centre in Croatia in 2012, clinical microbiologists in smaller hospitals in peripheral, outlying geographic areas of Croatia have noticed a dramatic increase in OXA-48 among carbapenem-resistant Enterobacteriacae according to data from the Reference Centre for Antibiotic Resistance Surveillance at the University Hospital for Infectious Diseases in Zagreb, from 0 % up to 2014 to almost 100 % in the 2015 to 2017, depending on the centre.

Croatia has a national surveillance system and specific guidelines for the management of carbapenemase-producing Enterobacteriaceae, and an obligation to report these to the health authorities, but in spite of these measures there is an increase in OXA-48-producing organisms, similar to 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 hydrolyses penicillins and, similar to other OXA β-lactamases, is inhibited by neither clavulanic acid nor tazobactam. The production of additional OXA-1 β-lactamase in some isolates may have contributed to the resistance to β-lactam combinations with inhibitors.

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

Colistin resistance observed in two *K. pneumoniae* isolates was not associated with inactivation of *mgrB* gene or acquisition of *mcr- 1*, and thus is probably caused by 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 to date for *K. pneumoniae* [48, 49]. Mutations in the two-component signalling transduction system *phoP/phoQ* or *pmrA/pmrB,* regulating genes necessary for lipopolysaccharide modification, may play a role in colistin resistance [50], but investigation of outer membrane lipopolysaccharides is beyond the scope of the present study. Our results are in disagreement with 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 BLAST analyses of the WGS contigs suggests the presence of $bla_{OXA\text{-}48}$ on plasmids. Moreover, $bla_{\text{OXA-48}}$-carrying transconjugants obtained in our study did not harbour any additional resistance genes, as reported in one previous study [20]. An 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 gene expression. Analysis of the flanking regions of $bla_{\text{OXA-48}}$ gene revealed a structure similar to that previously reported by Gianni *et al.* with an IS*1R* element between IS*1999* and the OXA-48-encoding gene [12].

PFGE showed the existence of five different *K. pneumoniae* clusters with isolates from different centres and with different β-lactamase content belonging to the same clusters indicating transfer of the related strains by patient or staff transfer. Isolates from the same centre but different clinical wards showed high similarity in PFGE banding patterns, pointing to cross-infection. PFGE patterns did not correlate with resistance gene content, and 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 67 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 a previous study conducted in 2013/2014 in four hospital centres 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 susceptibility, 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 Centre Zagreb and as a sole carbapenemase in one *K. pneumoniae* isolate from Split [17]. Similar to a recently published nationwide study on early OXA-48-positive *K. pneumoniae* collected in 2011/2012 in Croatia [20], the dissemination of OXA-48-positive isolates was polyclonal. The results points to 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 an earlier study in which all OXA-48 strains co-produced CTX-M-15 [20], in our investigation only some 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.

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

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

The study demonstrated dynamic changes in the carbapenem resistance mechanisms of major hospital pathogens like *K. pneumoniae* or *E. cloacae*, with VIM and NDM as the dominant carbapenem-resistance mechanism in 2012–2014 and OXA-48 becoming predominant in the period 2015–2017. We have also shown the complex epidemiology of OXA-48-producing Enterobacteriaceae including cross-infection, transmission 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 among the community. The large differences in the prevalence of OXA-48 β-lactamase among the participating centres may suggest that Croatia is not yet at an advanced stage of dissemination of OXA-48, or may reflect variations in the success of infection control at particular sites.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

Ethical statement
The experiments were not done on human or animal subjects.

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