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Research Paper

Epidemiology of colistin-resistant, carbapenemase-producing Enterobacteriaceae and *Acinetobacter baumannii* in Croatia

Valentino D'Onofrio[a,b,c], Rick Conzemius[d], Dijana Varda-Brkić[e], Maja Bogdan[f,g], Andrea Grisold[h], Inge C. Gyssens[a,c], Branka Bedenić[e,i], Ivan Barišić[d,*]

[a] Faculty of Medicine and Life Sciences, Hasselt University, 3500 Hasselt, Belgium.
[b] Department of Infectious Diseases and Immunity, Jessa Hospital, 3500 Hasselt, Belgium
[c] Department of Internal Medicine and Center for Infectious Diseases, Radboud University Medical Center, 6663 Nijmegen, The Netherlands.
[d] AIT, Austrian Institute for Technology, 1210 Vienna, Austria
[e] University Hospital Center Zagreb, 10000 Zagreb, Croatia
[f] Public Health Institute of Osijek-Baranja County, 31000 Osijek, Croatia
[g] School of Medicine, University of Osijek, 31000 Osijek, Croatia
[h] Institute for Hygiene, Microbiology and Environmental Medicine, Medical University of Graz, Graz, Austria
[i] School of Medicine, University of Zagreb, 10000 Zagreb, Croatia

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ABSTRACT

Colistin is a last-resort antibiotic for the treatment of infections caused by multidrug and carbapenem-resistant Gram-negative bacteria. Colistin resistance has been emerging and multiple outbreaks have been reported in Europe and elsewhere. It has been most frequently reported in carbapenem-resistant *K. pneumoniae.* In this study, 24 multidrug and colistin-resistant clinical isolates (14 *K. pneumoniae*, one *E. aerogenes*, one *E. cloacae,* and eight *A. baumannii)* were collected from four hospitals in Croatia from 2013 to 2018, in order to analyse the molecular epidemiology and mechanisms of antibiotic resistance. β-lactamase and carbapenemase genes were detected by PCR. Genotyping was done on selected isolates by rep-PCR. Whole genome sequencing (WGS) was performed to discover possible molecular mechanisms for the observed colistin resistance. All isolates, except two *K. pneumoniae* isolates, were extensively drug resistant. Ten out of 16 (63%) *K. pneumoniae* isolates possessed $bla_{\text{OXA-48}}$, which is the most common carbapenem resistance gene in Croatia and in other parts of Europe. All *A. baumannii* isolates possessed the OXA-23-like carbapenem hydrolysing oxacillinase and five turned out to be pandrug-resistant. Colistin resistance was most likely chromosomally mediated. After sequence analysis, none of the isolates were found to possess any of the *mcr* gene variants. Several previously reported mutations were found in PmrB, PhoP, PhoQ, and MgrB, which are associated with colistin resistance. In the global phylogenetic analysis, DNA mutations causing mutations in the MgrB protein were present mostly in lineages comprising colistin resistant isolates, and the second most prevalent mutation (K3X) was also encountered in our isolates. In addition, based on genotyping by rep-PCR, the spread of colistin resistance is most likely to be clonal. Most importantly, the presence of colistin resistance together with carbapenemase genes in extensively drug resistant isolates poses real threats in the use of carbapenems and colistin to fight infections.

1. Introduction

Colistin, which was discovered more than 50 years ago (Li et al., 2006), is a polypeptide antibiotic with a narrow spectrum of activity (Poirel et al., 2017). It is active against common Gram-negative bacteria, including *Klebsiella* and *Enterobacter* species, *Pseudomonas aeruginosa*, and *Acinetobacter baumannii* (Falagas and Kasiakou, 2005). These pathogens are reported as the "ESKAPE" pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species) to emphasize their growing resistance and ability to escape the effects of antibacterial drugs (Boucher et al., 2009; Ripabelli et al., 2018). In the 1970's, the use of colistin was terminated due to nephro- and neurotoxicity, and the emergence of novel aminoglycosides, expanded-spectrum cephalosporins (ESC), and carbapenems, which were less toxic (Poirel et al., 2017; Falagas and Kasiakou, 2005). However, recent

* Corresponding author.
E-mail address: Ivan.Barisic@ait.ac.at (I. Barišić).

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reports show that the risk of colistin's side effects has been overestimated, and studies confirm its safety and efficacy of intravenous use to battle Gram-negative infections (Li et al., 2005). Due to this risk reassessment and the rise of multidrug resistant (MDR) Gram-negative bacteria, polymyxins, and more specifically colistin, were reintroduced in clinical practice (Poirel et al., 2017). Higher dosages of colistin are associated with better microbiological success and with lower mortality rates, and combinations with amikacin, meropenem, or fosfomycin are recommended (Vicari et al., 2013).

Colistin is currently one of the last-resort antibiotics, effective against MDR bacteria, especially carbapenemase-producing bacteria (Poirel et al., 2017; Di Tella et al., 2019). Unfortunately, colistin resistance has been emerging and colistin monotherapy is associated with the development of heteroresistance and therapeutic failure, even with high doses (Poirel et al., 2017; Bedenic et al., 2018a; Pragasam et al., 2016; Falagas et al., 2010; Jayol et al., 2014; Liu et al., 2016; Wang et al., 2018). The clinical breakpoint for Enterobacteriaceae and *A. baumannii*, as determined by the European Committee on Antimicrobial Susceptibility Testing (EUCAST)(The European Committee on Antimicrobial Susceptibility Testing, 2018) is a MIC of 2 mg/L colistin. Both chromosomally mediated and plasmid mediated resistances to colistin have been described (Poirel et al., 2017; Pragasam et al., 2016; Falagas et al., 2010; Jayol et al., 2014; Liu et al., 2016; Poirel et al., 2015; Bialvaei and Samadi Kafil, 2015; Nicolet et al., 2016; Wright et al., 2015; Adams et al., 2009). The two-component regulatory systems PmrA/PmrB and PhoP/PhoQ regulate the modification of lipopolysaccharide (LPS) (Poirel et al., 2017; Pragasam et al., 2016; Falagas et al., 2010; Jayol et al., 2014; Bialvaei and Samadi Kafil, 2015; Wright et al., 2015; Adams et al., 2009). The plasmid-located Mobilized Colistin Resistance (*mcr*) -1 gene modifies lipid A. Both mechanisms result in the loss of the negative charge, necessary to bind colistin, and making it less susceptible (Poirel et al., 2017; Wang et al., 2018; Nicolet et al., 2016).

Although the overall prevalence is low, colistin resistance is an upcoming threat. Colistin resistance in *K. pneumoniae* represents the biggest concern, since *K. pneumoniae* is one of the main pathogens causing nosocomial infections. Colistin resistance in *K. pneumoniae* has been reported mostly together with carbapenem resistance (Poirel et al., 2017). The increasing prevalence of carbapenem resistant *K. pneumoniae* has already been thoroughly described in the last years (Bedenic et al., 2018b). A recent report shows that the production of OXA-48 carbapenemases are now the most common mechanism for carbapenem resistance in Croatia (Bedenic et al., 2018b).

The emergence of colistin resistance has been reported in Croatia and other countries (Poirel et al., 2017; Pragasam et al., 2016; Falagas et al., 2010; Bialvaei and Samadi Kafil, 2015). The SENTRY antimicrobial surveillance program reported a worldwide increase in colistin resistance rates from 1.2% in 2006 to 1.8% in 2009 among Gram-negative bacteria (Gales et al., 2011). Resistance rates of 2.4% for *K. pneumoniae* and 2.0% for *A. baumannii* were reported in Europe in 2016 (Ears-Net) (European Centre for Disease Prevention and Control, 2017).

Multiple colistin resistance outbreaks with carbapenemase-producing (KPC) *K. pneumoniae* attributed to the endemic clone type ST258 have been reported in Europe, including the Netherlands, Hungary, Greece, and Italy (Poirel et al., 2017). In an ICU in Rome, a colistin resistance rate of 36.1% was observed, with ST258 as the major causative organism, and resulting in a worse outcome (Capone et al., 2013).

The emergence and rising prevalence of colistin resistance, especially in MDR and carbapenemase-producing bacteria (CRE), will be a major health concern in the future. In spite of the increasing number of reports on colistin-resistant *K. pneumoniae* from different countries, such isolates are still rare in Croatia. The first report of colistin resistance in Croatia were published in 2018 in *E. aerogenes* and *K. pneumoniae* but the mechanism of colistin resistance was not clarified (Bedenic et al., 2018a; Bedenic et al., 2018b). In the meantime, pandrug-resistant isolates of *A. baumannii* with combined carbapenem, tigecycline and colistin resistance emerged in the University Hospital Osijek in Croatia, leaving no therapeutic options. This prompted us to analyse the mechanisms of colistin resistance and the molecular epidemiology of colistin-resistant enterobacterial and *A. baumannii* clinical isolates. We also used the colistin-resistance associated genes *mgrB* and *lpxC* to investigate the global phylogenetic diversity of *K. pneumoniae* and *A. baumannii* isolates, respectively, and included in the calculations whole genome sequencing (WGS) from this and our previous studies (Wright et al., 2015; Petrovic et al., 2018)

2. Materials and methods

2.1. Bacterial isolates

In total, 16 *Enterobacteriaceae* resistant to colistin were collected in the University Hospital Zagreb, University Hospital Osijek, General Hospital Pula, and General Hospital Slavonski brod from 2013 to 2018. Eight *A. baumannii* isolates resistant to colistin were collected in the University Hospital Osijek and General Hospital Pula from 2017 to 2018. In the period before 2017, no colistin-resistant *A. baumannii* strains were observed in these hospitals. Demographic and clinical details of patients were collected from electronic medical files. The University Hospital Center Zagreb is a 1724-bed university hospital and the largest hospital in Croatia with all medical specialties including organ and tissue transplantation patients. It serves a part of the Zagreb population and acts as a referral hospital for the whole Croatian population for specific patients/procedure groups, thus covering a population of about 4.000,000 people. The Clinical Department for Clinical and Molecular Microbiology provides conventional bacteriological and mycological diagnostic, molecular identification of microorganisms, and serology testing. This was a retrospective study, and isolates were collected during routine diagnostic procedures, which does not need Ethical Permission in Croatia.

2.2. Antimicrobial susceptibility testing

Antibiotic susceptibility of all isolates was tested by a broth microdilution test in Mueller-Hinton broth and 96 well microtiter plates, according to CLSI recommendations (The European Committee on Antimicrobial Susceptibility Testing, 2018; CLSI, 2018). Enterobacteriaceae were tested for susceptibility to amoxicillin alone and combined with clavulanic acid, expanded-spectrum cephalosporins (ceftazidime, cefotaxime, ceftriaxone) (ESC), cefepime, imipenem, meropenem, piperacillin/tazobactam, gentamicin, ciprofloxacin, and colistin. *A. baumannii* isolates were tested for susceptibility to ceftazidime, cefepime, cefotaxime, ceftriaxone, imipenem, meropenem, piperacillin/tazobactam, ampicillin/sulbactam, gentamicin, ciprofloxacin, tigecycline, and colistin. Susceptibility testing was done according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI)(CLSI, 2018), and the European Committee on Antimicrobial Susceptibility Testing (EUCAST clinical breakpoints v8.1, 2018) (The European Committee on Antimicrobial Susceptibility Testing, 2018). The strains were classified as MDR (multidrug-resistant), XDR (extensively-drug-resistant), or PDR (pan-drug resistant) (Magiorakos et al. (2012)) according to definitions by Magiorakos et al. (2012), MDR pathogens are non-susceptible to at least one agent in three or more antimicrobial categories. XDR pathogens are non-susceptible to at least one agent in all but two or fewer antimicrobial categories (i.e. bacterial isolates remain susceptible to only one or two categories).

2.3. Phenotypic tests for detection of ESBLs, plasmid-mediated AmpC β-lactamases and carbapenemases

The double disk synergy test (Jarlier et al., 1988) and CLSI

combined disk test with addition of clavulanic acid were performed to detect ESBLs (CLSI, 2018). In *A. baumannii*, the testing was done with the addition of cloxacillin (100 mg/L) to inhibit AmpC β-lactamases which could antagonize the synergistic effect with clavulanic acid. Plasmid-mediated AmpC β-lactamases in Enterobacteriaceae were detected by a combined disk test using cephalosporin disks with and without 3-aminophenylboronic acid (PBA) (Coudron, 2005). A modified Hodge test (MHT) (Lee et al., 2003) and CIM test (van der Zwaluw et al., 2015) were used to screen for the production of carbapenemases in both Enterobacteriaceae and *A. baumannii*. Additionally, all 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 (Lee et al., 2003; Pasteran et al., 2009).

2.4. Conjugation assay

The transferability of colistin, cefotaxime, and meropenem resistance in Enterobacteriaceae was determined by the broth mating method at 35°C using *E. coli* J65 resistant to sodium azide as described previously (Elwell and Falkow, 1986). In brief, the sodium azide resistant *E. coli* J65 was used as the recipient. Donor and recipient strains were mixed at a ratio of 1:2 in Brain–heart infusion broth. The transconjugants were selected on MacConkey agar containing either colistin (1 mg/L), cefotaxime (2 mg/L), or meropenem (1 mg/L) and sodium azide (100 mg/L). The frequency of conjugation was determined relatively to the number of donor cells. The co-transfer of resistance determinants to sulphonamides, trimethoprim, aminoglycosides and chloramphenicol was tested as well.

2.5. Molecular detection of resistance genes

The genes conferring resistance to β-lactams including broad spectrum and extended-spectrum β-lactamases (bla_{SHV}, bla_{TEM}, bla_{CTX-M}, and bla_{PER-1}) (Nuesch-Inderbinen et al., 1996; Arlet et al., 1995; Woodford et al., 2006; Woodford et al., 2004; Saladin et al., 2002), plasmid-mediated AmpC β-lactamases (Perez-Perez and Hanson, 2002), class A carbapenemases (bla_{KPC}, bla_{SME}, bla_{IMI}, and bla_{NMC},) (Yigit et al., 2001; Naas et al., 1994), class B carbapenemases (bla_{VIM}, bla_{IMP}, and bla_{NDM}) (Giakkoupi et al., 2003), carbapenem hydrolyzing oxacillinases (bla_{OXA-48}) (Gulmez et al., 2008), and to fluoroquinolones (*qnrA*, *qnrB*, *qnrS*) were determined by PCR using protocols and conditions as described previously (Robicsek et al., 2006; Poirel et al., 2011). *A. baumannii* isolates were tested for bla_{GES} genes, and for blaOXA-23-like, blaOXa-24-like, blaOXA-58-like, blaOXA-51-like and blaOXA-143-like genes. The DNA was extracted by the boiling method. Reference strains producing SHV-1, SHV-2, TEM-1, CTX-M-3, CTX-M-15, IMP-1, VIM-1, KPC-2, and OXA-48 were used as positive control. The amplification products were column-purified with the Qiagen DNA purification kit (Hilden, Germany) and sequenced by Eurofins sequencing services (Graz, Austria).

2.6. Characterization of plasmids

Plasmids were extracted with the Qiagen Mini kit according to the manufacturer's instructions. PCR-based replicon typing (PBRT) (Carattoli et al., 2005) was applied to type the resistance plasmids carrying carbapenemase genes according to Carattoli *et al* for Enterobacteriaceae (Carattoli et al., 2005) and according to Bertini *et al* for *A. baumannii* (Bertini et al., 2010).

2.7. Genotyping by rep-PCR

Five *A. baumannii* isolates and five *K. pneumoniae* isolates were subjected to molecular typing by repetitive element sequence based-PCR (rep-PCR) as described previously (Overdevest et al., 2011). DNA was isolated by the Ultra-Clean microbial DNA isolation kit (Mo Bio Laboratories, Carlsbad, CA, USA), as recommended by the manufacturer. The DNA concentration was measured and set between 25 and 30 ng/L. Subsequently, the DNA was amplified using the Bacterial fingerprinting kit (Bacterial barcodes, bioMerieux, Athens, GA, USA), according to the manufacturer's instructions. PCR was performed using the following parameters: initial denaturation (94 °C) for 2 min, and then 35 cycles of 30 s of denaturation (94 °C), 30 s of annealing (60 °C), and 90 s of extension (70 °C), followed by 3 min of final extension (70 °C) and ending at 4 °C. The amplification products were separated with the Agilent B2100 bioanalyzer. Five microliters of DNA standard markers (used for the normalization of sample runs) and 1 μl of the DNA product were used. All data were entered in the DiversiLab software system. A cut-off value of 97% was used to define a clone (Overdevest et al., 2011). The same strains were also genotyped by MLST according to Diancourt (Diancourt et al., 2005).

2.8. Whole genome sequencing and sequence analysis

Seven Enterobacteriaceae and three *A. baumannii* were subjected to WGS: *K. pneumoniae* 24899, *K. pneumoniae* 609815, *K. pneumoniae* 8449, *K. pneumoniae* 22774, *K. pneumoniae* 196662, *K. pneumoniae* 208158, *E. aerogenes* 12264, *A. baumannii* 1966/18, *A. baumannii* 1306/18, *A. baumannii* 1475/18. The genomic DNA was extracted with the QiAmp DNA mini kit (Qiagen). WGS was performed using the Ion Torrent PGM platform using 400bp read chemistry. Sequencing was performed according to the protocol recommended by Life Technologies. 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 analyzed 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). Raw reads were assembled *de novo* using Assembler SPAdes software (Nurk et al., 2013). The genome was annotated using the RAST (Rapid Annotations using Subsystems Technology) database (Aziz et al., 2008; Overbeek et al., 2014). Multilocus sequence typing and plasmid replicon typing were done using the tools from the Center for Genomic Epidemiology website (Larsen et al., 2012; Carattoli et al., 2014). Antibiotic resistance genes were found using the ResFinder database (Zankari et al., 2012) and PRIMEval (Conzemius et al., 2019). WGS data is deposited in the European Nucleotide Archive (ENA) with the following accession numbers: CABFJU010000000 (*K. pneumoniae* 24899), CABFJT010000000 (*K. pneumoniae* 609815), CABFJS010000000 (*K. pneumoniae* 22774), CABFIZ010000000 (*K. pneumoniae* 196662), CABFJV010000000 (*K. pneumoniae* 208158), CABEFN010000000 (*E. aerogenes* 12264), CABFJK010000000 (*A. baumannii* 1966/18), CABFJP010000000 (*A. baumannii* 1306/18), CABFJF010000000 (*A. baumannii* 1475/18).

Reference genomes from *K. pneumoniae* (HS11286; NCBI accession number NC_016845.1), *E. aerogenes* (KCTC2190; NCBI accession number NC_015663.1), and *A. baumannii* (AB030; NCBI accession number NZ_CP009257) were screened for reference amino acid sequences of PmrA, PmrB, PhoP, PhoQ, MgrB, CrrB, LpxA, LpxC. All isolates were screened for known (Poirel et al., 2017) and new SNPs and indels in the amino acid sequence using the BLAST tool. Additionally, reference genes for all *mcr* variants were searched; *mcr*-1.1, *mcr*-2, *mcr*-3.5, *mcr*-3.11, *mcr*-4, *mcr*-5.1, *mcr*-5.2, *mcr*-6, *mcr*-7, *mcr*-8.1. Isolates were screened for the presence of any of these genes using the BLAST tool.

Table 1
Minimum inhibitory concentrations (MIC), resistance gene content of colistin-resistant *K. pneumoniae*, *E. cloacae* and *E. aerogenes* and clinical data.

Isolate	Centre	Department	Isolation date	Specimen	AMX	AMC	TZP	CAZ	CTX	CRO	FEP
142 456	UHC Zagreb	Medical ICU	20.08.2015	Blood	> 128	32	32	> 128	> 128	> 128	32
175 223	UHC Zagreb	Medical ICU	08.10.2015	Urine	> 128	> 128	> 128	> 128	> 128	> 128	> 128
219 749	UHC Zagreb	Medical ICU	10.12.2015	Perianal swab	> 128	64	32	> 128	> 128	> 128	16
23267	UHC Zagreb	Outpatient	31.12.2015	Urine	> 128	> 128	> 128	> 128	> 128	> 128	> 128
16000	UHC Zagreb	Medical ICU	27.01.2016	Blood	> 128	64	16	> 128	> 128	> 128	16
69677	UHC Zagreb	Outpatient	14.04.2016	Sputum	> 128	64	> 128	> 128	> 128	> 128	32
8499	UHC Osijek	Neursurgery	06.12.2016	Urine	> 128	> 128	> 128	32	> 128	> 128	4
20566	UHC Osijek	Abdominal Surgery	14.12.2016	Abdominal swab	> 128	> 128	> 128	0.25	0.5	0.5	0.06
24889	Slavonski brod	Surgery	15.03.2017	Perineal swab	> 128	> 128	> 128	> 128	> 128	> 128	> 128
22774	UHC Zagreb	Surgery ICU	29.05.2017	Blood	> 128	> 128	> 128	16	> 128	> 128	16
609 815	UHC Zagreb	Haematology	28.05.2018	Wound swab	> 128	> 128	> 128	> 128	> 128	> 128	> 128
196 662	UHC Zagreb	Medical ICU	05.09.2018	Rectal swab	> 128	> 128	> 128	1	0,5	0,5	0,12
208 158	UHC Zagreb	Medical ICU	19.09.2018	Pharyngeal swab	> 128	> 128	> 128	> 128	> 128	> 128	> 128
270 776	UHC Zagreb	Medical ICU	27.11.2018	Urine	> 128	> 128	> 128	32	> 128	> 128	32
143 666	UHC Zagreb	Haematology	25.08.2013	Blood	> 128	> 128	> 128	> 128	> 128	> 128	> 128
12264	General Hospital Pula	Neurosurgery ICU	06.07.2016	Tracheal aspirate	> 128	> 128	64	> 128	> 128	> 128	8

Isolate	IPM	MEM	GEN	CIP	COL	ESBL	ampC	Hodge	CIM	β-lactamases	Antibiotic treatment	Clinical outcome
142 456	0.5	0.5	1	32	16	+	-	-	-	SHV CTX-M-15 TEM-1	None	D
175 223	0.12	0.12	64	> 128	16	+	-	+	+	SHV CTX-M-15 OXA-48	COL+ LZD	R
219 749	1	8	0.5	64	32	+	-	-	+	SHV CTX-M TEM	None	R
23267	16	32	32	64	32	+	-	+	+	SHV TEM KPC-2	MEM	R
16000	0,5	4	16	64	32	+	-	-	-	SHV CTX-M TEM	MEM	D
69677	1	8	64	> 128	4	+	-	+	+	SHV CTX-M TEM OXA-48	None	R
8499	0,06	0,25	0,12	> 128	64	+	-	+	+	TEM-1 CTX-M-15 OXA-48	MEM+ TGC+ COL	D
20566	0.12	0.25	0.5	> 128	32	-	-	+	+	SHV-1 OXA-48	MEM+ TGC+ COL	D
24889	32	32	> 128	> 128	> 128	+	+	+	+	OXA-48 CTX-M-15 TEM-1	None	R

(*continued on next page*)

Table 1 (*continued*)

Isolate	IPM	MEM	GEN	CIP	COL	ESBL	ampC	Hodge	CIM	β-lactamases	Antibiotic treatment	Clinical outcome
22774	0,12	0,25	0,5	> 128	16	+	-	+	+	SHV CTX-M OXA-48	None	R
609 815	128	128	> 128	> 128	8	+	-	+	+	SHV CTX-M OXA-48	COL	D
196 662	128	128	0,5	> 128	32	-	-	+	+	SHV OXA-48	COL	D
208 158	32	64	> 128	> 128	16	+	-	+	+	SHV CTX-M TEM OXA-48	COL	D
270 776	32	64	> 128	> 128	32	+	-	+	+	TEM-1 CTX-M-15 OXA-48	MEM + VAN + AMK	R
143 666	8	16	16	32	32	-	-	+	+	SHV-1 VIM-1	MEM	R
12264	0,5	0,25	0,5	32	16	+	-	-	-	CTX-M-15 TEM-1	COL	R

AMX, Amoxicillin; AMC, Amoxicillin/Clavulanic acid; AMK, Amikacin; TZP, Piperacillin/Tazobactam; CAZ, Ceftazidime; CTX, Cefotaxime; CRO, Ceftriaxone; FEP, Cefepime; IPM, Imipenem; MEM, Meropenem; GEN, Gentamicin; CIP, Ciprofloxacin; LZD, Linezolid; VAN, Vancomycin; COL, Colistin; ESBL, inhibitor-based test with clavulanic acid for detection of extended-spectrum beta-lactamases; AmpC, inhibitor-based test with phenyloboronic acid for detection of AmpC beta-lactamases; CIM, carbapenem inactivation method; ICU, intensive care unit; R, patient recovery; D, patient death (all-cause).

2.9. Phylogenetic analysis

All publicly available sequence databases at the NCBI FTP server were downloaded on a local server. Subsequently, all *mgrB* and *lpxC* sequences obtained from our isolates that were collected in this and previous studies were compared with all publicly available sequences using in-house software and stand-alone BLAST + . A sequence database comprising 80% sequence similarity to the input sequences was created. Then, alignments for each gene were calculated using MAFFT (Katoh and Standley, 2013). Genbank files including the alignment data were imported in the phylogenetic software analysis package ARB (Ludwig et al., 2004). Partial sequences and those with sequencing errors and poor quality ("Ns" instead of nucleotide code) were removed. The DNA alignment was manually curated and translated into the amino acid code. Phylogenetic trees using the Neighbour-Joining ARB implementation (1000 bootstrap replicates) were calculated. DNA- and amino acid-based phylogenetic trees were compared.

3. Results

3.1. Patient Characteristics and bacterial isolates

Between 2013 and 2018, 24 colistin-resistant isolates were collected from four hospitals in Croatia. Enterobacteriaceae originate from the University Hospital Center Zagreb (12 colistin-resistant isolates out of 4854 isolates; 0.25%), General Hospital Pula (one colistin-resistant isolates out of 8151 isolates; 0,012%), General Hospital Slavonski brod (one colistin-resistant isolates out of 13,750 isolates; 0,007%), and University Hospital Center Osijek (two colistin-resistant isolates out of 10,474 isolates; 0,019%). Seven colistin-resistant *A. baumannii* isolates (out of 611 isolates in 2018; 1,15%) were collected from the University Hospital Center Osijek and one (out of 192 isolates in 2017; 0,52%) from the General Hospital Pula. The characteristics of the isolates are shown in Table 1 for Enterobacteriaceae and Table 2 for *A. baumannii*.

Among 24 patients harbouring colistin-resistant isolates, 12 were males and 12 females. The age ranged from 6 to 84 years (median 63.5 years). All patients were hospitalized. Twelve patients were treated with colistin during hospitalization and prior to the isolation of the colistin-resistant isolate. The predominant specimen were blood cultures (9 patients), followed by urine samples (5 patients). Other specimen were tracheal aspirates, sputum, or bronchoalveolar lavage fluid (BAL) (4 patients), wound swab (1 patient), or pus (1 patient). Four isolates were collected from surveillance cultures (pharyngeal swab, perianal swab, rectal swab). In total, seven (44%) isolates were obtained from colonized patients. Eleven patients died (all-cause mortality). Attributable mortality due to septic shock was recorded in two patients. Thirteen patients recovered.

3.2. Antimicrobial susceptibility

The MIC-values for colistin ranged from 4 to 128 mg/L for Enterobacteriaceae and from 4 to 64 mg/L for *A. baumannii* and are shown in Table 1 and Table 2 respectively, together with patient characteristics. All enterobacterial isolates were resistant to amoxycillin alone and combined with clavulanic acid, piperacillin/tazobactam, and ciprofloxacin (16/16). Fourteen (14/16) isolates (96%) were resistant to cefotaxime, ceftriaxone, ceftazidime, and cefepime. Nine isolates (57%) were resistant to meropenem and gentamicin, and seven (46%) to imipenem. The MIC_{90} of all antibiotics except of colistin, exceeded 128 mg/L. Thirteen Enterobacteriaceae isolates (81%) were phenotypically positive for ESBL by the double disk synergy test (DDST) and combined disk test with clavulanic acid (10 mg/ml). The Hodge test and Carbapenem Inactivation Method (CIM) test demonstrated carbapenemase activity in 12 (69%) and 13 (81%) Enterobacteriaceae isolates, respectively.

All *A. baumannii* isolates were resistant to all tested antibiotics. Only

Table 2
Minimum inhibitory concentrations (MIC), resistance gene content of colistin-resistant *A. baumannii* and clinical data.

Isolate	Centre	Department	Isolation date	Specimen	SAM	TZP	CAZ	CTX	CRO	FEP	IPM
A. baumannii											
4802-1	Pula	Surgery ICU	09.03.2017	BAL	> 128	> 128	> 128	> 128	> 128	64	64
1966/18	Osijek	Surgery ICU	13.03.2018	Catheter Urine	16	> 128	> 128	> 128	> 128	> 128	16
1306/18	Osijek	Cardiosurg-ery ICU	16.03.2018	Blood	32	> 128	> 128	> 128	> 128	> 128	16
5813/18	Osijek	Surgery ICU	18.03.2018	Drain aspirate	64	> 128	> 128	> 128	> 128	> 128	16
1475/18	Osijek	Cardiosurg-ery ICU	18.03.2018	Blood	32	> 128	> 128	> 128	> 128	> 128	16
17318/18	Osijek	surgery ICU	01.10.2018	CVC	32	64	> 128	> 128	> 128	32	32
17376/18	Osijek	Surgery ICU	02.10.2018	Endotracheal aspirate	32	> 128	> 128	> 128	> 128	64	64
5330/18	Osijek	Surgery ICU	12.11.2018	Blood	32	64	> 128	> 128	> 128	32	32

Isolate	MEM	GEN	CIP	TGC	COL	Hodge	CIM	PCR	EDTA	Antibiotic treatment	Clinical outcome
A. baumannii											
4802-1	> 128	> 128	> 128	?	16	+	+	OXA-23	+	None	D
1966/18	16	> 128	> 128	3	64	+	+	OXA-23	+	AMC + COL + Metronidazole	D
1306/18	32	> 128	> 128	3	4	+	+	OXA-23	+	CIP + COL	R
5813/18	32	> 128	> 128	1	16	+	+	OXA-23	+	MEM	R
1475/18	32	> 128	> 128	3	4	+	+	OXA-23	+	SAM + VAN + COL	D
17318/18	64	> 128	> 128	4	16	+	+	OXA-23	+	MEM + VAN + COL	R
17376/18	> 128	> 128	> 128	2	16	+	+	OXA-23	+	FEP + GEN	R
5330/18	> 128	> 128	> 128	3	16 (64)	+	+	OXA-23	+	LZD + IPM + COL	D

SAM, Ampicillin-sulbactam; TZP, Piperacillin/Tazobactam; CAZ, Ceftazidime; CTX, Cefotaxime; CRO, Ceftriaxone; FEP, Cefepime; IPM, Imipenem; MEM, Meropenem; GEN, Gentamicin; CIP, Ciprofloxacin; TGC, Tigecycline; COL, Colistin; VAN, Vancomycin; LZD, Linezolid; CIM, carbapenem inactivation method; PCR, resistance genes detected with PCR; EDTA, Inhibitor based test with EDTA; ICU, intensive care unit; CVC, Central Venous Catheter; R, patient recovery; D, patient death (all-cause).

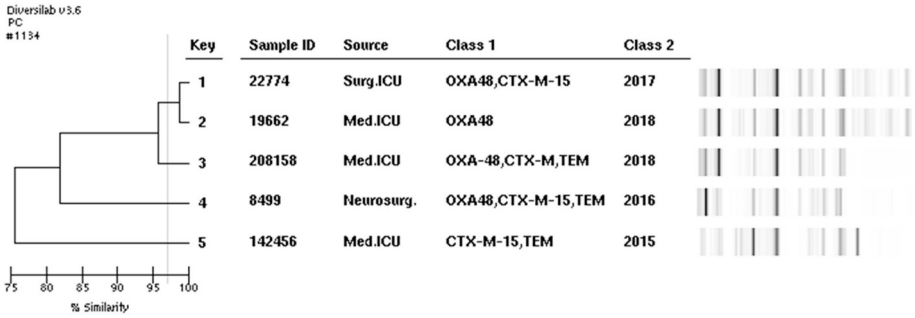

Fig. 1. *K. pneumoniae* dendrogram after genotyping by rep-PCR

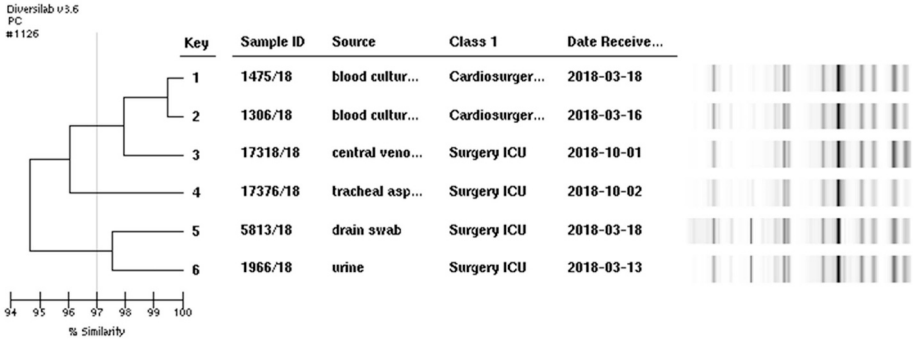

Fig. 2. *A. baumannii* dendrogram after genotyping by rep-PCR

one isolate was intermediate susceptible to ampicillin/sulbactam and two were intermediate susceptible to piperacillin/tazobactam. Imipenem and colistin were the most efficient antibiotics with MIC_{90} of 16 mg/L. All *A. baumannii* isolates were positive in the Hodge and CIM test indicating carbapenemase activity. The inhibitor based test with EDTA was positive suggesting the production of metallo-β-lactamases (MBL).

In our study, all but two Enterobacteriaceae (*K. pneumonia*e 20566 and *K. pneumonia*e 196662), can be classified as extensively drug resistant (XDR). *K. pneumoniae* 20566 and *K. pneumonia*e 196662 are MDR as they are susceptible to several agents in more than two antimicrobial categories (aminoglycosides, cephalosporins, and carbapenems). Five *A. baumannii* isolates were classified as pandrug–resistant (PDR) since they are resistant to all tested antibiotics, including tigecycline. Three *A. baumannii* isolates were XDR; *A. baumannii* 1966/18 was susceptible to Ampicillin-Sulbactam, *A. baumannii* 4802-1 and *A. baumannii* 5813/18 were susceptible to tigecycline.

3.3. Conjugation assay

Colistin resistance was not transferable to the *E. coli* recipient strain. Six ESBL positive isolates transferred cefotaxime resistance to the *E. coli* recipient strain with the frequency of 10^{-5}. Tetracycline resistance was co-transferred alongside with cefotaxime resistance from four isolates, gentamicin resistance from three isolates, and chloramphenicol resistance from two isolates. The reduced susceptibility to meropenem was not transferable to the *E. coli* recipient strain.

3.4. Molecular detection of resistance genes

Enterobacteriaceae showed diverse resistance mechanisms to antibiotics. Twelve isolates were positive for ESBLs of group 1 of the $bla_{CTX\text{-}M}$ family. In three isolates, it was the sole β-lactam resistance mechanism. Twelve isolates (12/16; 75%) that were phenotypically positive for carbapenemases (Hodge and CIM test), were shown to possess $bla_{OXA\text{-}48}$ (10/12 isolates; 83%), $bla_{VIM\text{-}1}$ (1/12 isolate; 8,3%) and $bla_{KPC\text{-}2}$ (1/12 isolate; 8,3%). All except two OXA-48 producing strains possessed additional CTX-M β-lactamases conferring resistance to expanded-spectrum cephalosporins (ESC). In all *A. baumannii* isolates, resistance was associated with the presence of a OXA-23-like carbapenem hydrolysing oxacillinase (CHDL). The PCR for common MBLs of *A. baumannii* was negative.

3.5. Characterization of plasmids

The L plasmid was found in all OXA-48 positive Enterobacteriaceae. IncA/C was found in the VIM-1 positive *E. cloacae* (143666), whereas the KPC-2 positive *K. pneumoniae* (23267) was negative for any plasmids reported in Enterobacteriaceae so far. *A. baumannii* isolates were positive for group 2 plasmids.

3.6. Genotyping by rep-PCR

K. pneumoniae showed distinct rep-PCR patterns with only two isolates being related (22774 and 196662) originating from different hospital wards and different time periods. The dendrogram for *K. pneumoniae* is shown in Fig. 1.

Fig. 2 represents the dendrogram for *A. baumannii*. Rep-PCR demonstrated the existence of two clusters with > 97% similarity in *A. baumannii*: one comprising two isolates from the neurosurgery ICU (1306 and 1475) and one from the surgery ICU (17318). The other cluster contained the isolates 1966 and 5813, both from the surgery ICU, collected in the same week. One isolate was a singleton (17376).

3.7. Whole genome sequencing

The antimicrobial resistance genes that were identified from 10 isolates (*K. pneumoniae n = 6, E. aerogenes n = 1, A. baumannii n = 3*) are shown in Table 3. WGS revealed $bla_{CTX\text{-}M\text{-}15}$ genes in all Enterobacteriaceae isolates. Furthermore, all *K. pneumoniae* isolates were found to possess $bla_{OXA\text{-}48}$ genes, and all *A. baumannii* isolates possessed $bla_{OXA\text{-}23}$ genes. The *E. aerogenes* isolate was found to possess the $bla_{OXA\text{-}}$

Table 3
Antimicrobial resistance genes identified in colistin-resistant *K. pneumoniae*, *E. aerogenes*, and *A. baumannii* with WGS

Isolate	AG	β-lactam	FQ	FOF	CHL	SUL	TET	TMP
K. pneumoniae 24889	*aac(6')-Ib-cr* *aac(3)-IIa*	*bla*$_{SHV-182}$ *bla*$_{TEM-1B}$ *bla*$_{CTX-M-15}$ *bla*$_{OXA-1}$ *bla*$_{OXA-48}$	*aac(6')-Ib-cr* *qnrS1* *oqxA* *oqxB*	*fosA*	*catB4* *catA1*	*sul1*	*tet(A)*	*dfrA1*
K. pneumoniae 609815	*aac(6')-Ib-cr* *aac(3)-IIa*	*bla*$_{SHV-81}$ *bla*$_{CTX-M-15}$ *bla*$_{OXA-1}$ *bla*$_{OXA-48}$	*aac(6')-Ib-cr* *oqxA* *oqxB*	*fosA*	*catB4*	-	*tet(A)*	-
K. pneumoniae 8449	*aac(6')-Ib-cr* *aph(3")-Ib* *aph(6)-Id*	*bla*$_{SHV-28}$ *bla*$_{TEM-1B}$ *bla*$_{CTX-M-15}$ *bla*$_{OXA-1}$ *bla*$_{OXA-48}$	*aac(6')-Ib-cr* *qnrB1* *oqxA* *oqxB*	*fosA*	*catB4*	*sul2*	*tet(A)*	*dfrA14*
K. pneumoniae 22774	*aac(6')-Ib-cr*	*bla*$_{SHV-81}$ *bla*$_{CTX-M-15}$ *bla*$_{OXA-1}$ *bla*$_{OXA-48}$	*aac(6')-Ib-cr* *oqxA* *oqxB*	*fosA*	*catB4*	-	*tet(A)*	-
K. pneumoniae 196662	*aac(6')-Ib-cr* *aac(3)-IIa*	*bla*$_{SHV-81}$ *bla*$_{CTX-M-15}$ *bla*$_{OXA-1}$ *bla*$_{OXA-48}$	*aac(6')-Ib-cr* *oqxA* *oqxB*	*fosA*	*catB4*	-	*tet(A)*	-
K. pneumoniae 208158	*aac(6')-Ib-cr* *aac(3)-IIa*	*bla*$_{SHV-81}$ *bla*$_{CTX-M-15}$ *bla*$_{OXA-1}$ *bla*$_{OXA-48}$	*aac(6')-Ib-cr* *oqxA* *oqxB*	*fosA*	*catB4*	-	*tet(A)*	-
E. aerogenes 12264	*aac(3)-IIa* *aac(6')-Ib-cr* *aph(6)-Id* *aph(3")-Ib*	*bla*$_{TEM-1B}$ *bla*$_{CTX-M-15}$ *bla*$_{OXA-1}$	*aac(6')-Ib-cr* *qnrB1*	-	*catB4*	*sul2*	-	*dfrA14*
A. baumannii 1966/18	*aac(3)-Ia* *aph(3')-VIa* *aadA1* *aph(3")-Ib* *aph(6)-Id* *armA*	*bla*$_{ADC-25}$ *bla*$_{OXA-23}$ *bla*$_{OXA-66}$	-	-	*catA1*	*sul1*	*tet(B)*	-
A. baumannii 1306/18	*aac(3)-Ia* *aph(3')-VIa* *aadA1* *aph(3")-Ib* *aph(6)-Id* *armA*	*bla*$_{ADC-25}$ *bla*$_{OXA-23}$ *bla*$_{OXA-66}$	-	-	-	*sul1*	*tet(B)*	-
A. baumannii 1475/18	*aac(3)-Ia* *aadA1* *aph(3")-Ib* *aph(6)-Id* *armA*	*bla*$_{ADC-25}$ *bla*$_{OXA-23}$ *bla*$_{OXA-66}$	-	-	-	*sul1*	*tet(B)*	-

AG, aminoglycosides; FQ, fluoroquinolones; FOF, fosfomycin; CHL, chloramphenicol; SUL, sulphonamides; TET, tetracycline; TMP, trimethoprim; MLS, macrolides, lincosamides, and streptogramin B

Table 4
Sequence type and plasmids identified in colistin-resistant *K. pneumoniae*, *E. aerogenes*, and *A. baumannii* with WGS

Isolate	MLST	Plasmids
K. pneumoniae 24889	395	lncL/M(pOXA-48); lncR; Col440II
K. pneumoniae 609815	37	lncFII; lncL/M(pOXA-48)
K. pneumoniae 8449	15	lncFII; lncFIB(K); ColpVC
K. pneumoniae 22774	37	lncFII; lncL/M(pOXA-48)
K. pneumoniae 196662	37	lncFII; lncL/M(pOXA-48); ColRNAI
K. pneumoniae 208158	37	lncFII; lncL/M(pOXA-48); ColRNAI
E. aerogenes 12264	Unknown[a]	lncFII(pBK30863); lncL/M(pMU407)
A. baumannii 1966/18	1421	-
A. baumannii 1306/18	195	-
A. baumannii 1475/18	1816	-

[a] The ST is not trusted since there was a gap in the rpoB gene. The nearest MLST were 59 and 16. MLST, multilocus sequence type

$_1$ gene. Resistance genes for aminoglycosides were found in all isolates. Resistance genes for fluoroquinolones were observed for all Enterobacteriaceae.

The multilocus sequence type (MLST) and plasmids that were detected are shown in Table 4. Most common detected plasmids were lncL/M(pOXA-48) and lncFII plasmids in Enterobacteriaceae. Plasmids such as lncL and lncFIB, which were detected here, were previously shown to carry *mcr*-genes. No plasmids were found in *A. baumannii* isolates.

No *mcr* variants were detected in any isolates after sequence analysis, although compatible plasmid backbones have been detected. For all but one *K. pneumoniae* isolate, the amino acid substitutions R256G in PmrB, L26Q in PhoP, and D150G in PhoQ were found. Three *K. pneumoniae* strains had variations in the MgrB protein sequence. The *E. aerogenes* strain had variations in PmrB and PhoQ. All *A. baumannii* isolates had the amino acid substitution A138T in PmrB. All variations are shown in Table 5.

Table 5
Amino acid changes identified after sequence analysis in proteins associated with colistin resistance

Isolate	PmrB	PhoP	PhoQ	MgrB	LpxC
K. pneumoniae 24889	R256G	L26Q	D150G L482Q[a] S475G[a]	-	
K. pneumoniae 609815	R256G P167T[a] V177R[a] Q356A[a]	L26Q	D150G	-	
K. pneumoniae 8449	V177R[a] A246T[a]	L26Q	D150G L482Q[a] S475G[a]	-	-
K. pneumoniae 22774	R256G	L26Q	D150G	Disrupted by IS26 transposon at G7	
K. pneumoniae 196662	R256G	L26Q	D150G	K3X	-
K. pneumoniae 208158	R256G	L26Q	D150G	K3X	-
E. aerogenes 12264	T167P[a] V200A[a] G210S[a] A228K[a] N275K[a]	-	A178T[a]	-	-
A. baumannii 1966/18	S14L A138T[a]	-	-	-	
A. baumannii 1306/18	A138T[a] S183F[a] T269P[a]	-	-	-	
A. baumannii 1475/18	A138T[a] S183F[a] T269P[a]	-	-	-	

[a] These amino acid changes were, to our knowledge, not previously described.

3.8. Phylogenetic analysis

Phylogenetic calculations were performed to obtain more information regarding our clinical isolates and their global context. All publicly available gene sequences of *mgrB* for *K. pneumoniae* and *lpxC* for *A. baumannii* were used to assign isolates collected within this and our previous studies to global lineages. Only lineages that were observed at two (*mgrB*) or three (*lpxC*) different sites at least were included in the phylogenetic analysis. All our *K. pneumoniae* isolates except the three mutated *mgrB* variants (196662, 208158, 22774) belonged to the dominant wildtype sequence (100% sequence similarity). In the global analysis, DNA mutations causing mutations in the MgrB protein were present mostly in lineages comprising colistin resistant isolates (Fig. 3). The most frequent mutation observed at 12 different sites is a point mutation introducing a stop codon at amino acids position 30. The second most prevalent mutation (K3X) also encountered in our isolates was previously reported at seven different sites. Other mutations were significantly less prevalent. The lineages comprising only silent DNA mutations did not include any colistin resistant isolates but have been globally reported at many different sites.

The diversity within LpxC was significantly lower in comparison to MgrB (Fig. 4). Only three amino acid sequences deviating from the wildtype have been reported so far. The point mutation D287N was reported at four different sites and is the only mutated lineage comprising colistin resistant isolates. All *A. baumannii* isolates from this study belonged to the dominant wildtype sequence. Interestingly, five *A. baumannii* isolates originating from Bosnia and Hercegovina that were analysed by our group in a former study belong to the globally second most prevalent group reported at 37 different sites.

4. Discussion

In this study, 24 colistin-resistant isolates collected from Croatian hospitals were analysed. All but two isolates were XDR, and five were even PDR. Twelve out of 16 (75%) enterobacterial isolates were found to possess ESBL genes. Also, 12 enterobacterial isolates (75%) were phenotypically positive for carbapenemases, and ten isolates (63%) were found to possess the most common carbapenemase gene; bla_{OXA-48}. All *A. baumannii* isolates were phenotypically positive for carbapenemases, which was found to be associated with the presence of a OXA-23-like CHDL. Colistin resistance most likely was due to chromosomal mutations leading to amino acid changes in key regulator proteins, which are responsible for the modification of the principle target of colistin, the LPS. In addition to previously reported mutations, we observed also new mutations in these key regulator proteins. None of the isolates possessed any of the *mcr* gene variants.

Enterobacteriaceae showed diverse resistance mechanisms to other antibiotics. CTX-M ESBLs conferred high level of resistance to ESC (ceftazidime, cefotaxime, and ceftriaxone) and were also associated with fluoroquinolone resistance shown by WGS to be due to aac(6')-Ib-cr or qnrA or qnrB genes. Plasmids encoding CTX-M β-lactamases usually harbour resistance genes to sulphonamides, trimethoprim, tetracyclines, chloramphenicol, aminoglycosides, and fluoroquinolones resulting in the MDR phenotype. *Enterobacteriaceae* harbouring VIM and KPC exhibited high levels of resistance to carbapenems (MICs for imipenem and meropenem respectively were: 16 and 32 for KPC-positive isolate, and 8 and 16 for VIM-positive isolates) due to a high hydrolysis activity of carbapenems by these carbapenemases. On the other hand, OXA-48 producing organisms demonstrated variable levels of carbapenem susceptibility/resistance which is typical for oxacillinases and the reason why this type of carbapenemase often get missed in routine laboratory testing (Tomic Paradzik et al., 2019). Four OXA-48 producing organisms had meropenem and imipenem MICs below 0.5 mg/L. Unlike MBL and KPC, OXA-48 does not hydrolyse ESC, which was the reason for the bimodal distribution of ESC MICs observed in this study. *Enterobacteriaceae* positive for carbapenemases of class A, B, and D are known to often co-harbour ESBLs or plasmid-mediated AmpC beta-lactamases very often (Wilson and Torok, 2018).

The L/M plasmid was found in all OXA-48 producing organisms and, although not demonstrated by Southern blots, is most likely responsible for the horizontal spread of this important resistant determinant. Previous studies found polyclonal dissemination of OXA-48 producing *Enterobacteriaceae* in Croatia (Bedenic et al., 2018b) which is in concordance with the present study

In *A. baumannii*, colistin resistance was combined with OXA-23-like CHDL. OXA-23-like CHDL are now the most prevalent type of CHDL all over the world and replaced OXA-58 due to a better hydrolysis of carbapenems providing a selection advantage in an antibiotic rich environment (Towner et al., 2008). In Croatia, OXA-24-like CHDL was always the dominant type of CHDL in hospitals, nursing homes and environment. The four *A. baumannii* isolates were PDR and were resistant not only to all β-lactam antibiotics and colistin, but also to trimethoprim/sulphamethoxazole (data not shown), leaving no therapeutic option left. Furthermore, all *A. baumannii* isolates tested positive in the inhibitor-based test with EDTA. However, PCRs for common MBLs in *A. baumannii* were negative. Similar observation of false-positive MBL tests in strains positive for CHDL have been previously reported (Bedenic et al., 2019). A possible explanation is that oxacillinases change to a less active state in the presence of EDTA, leading to a drastic reduction in the MIC or augmentation of the inhibition zone (Villalon et al., 2013).

Most *K. pneumoniae* showed distinct rep-PCR patterns, and mutations that could be responsible for colistin resistance probably arouse *de novo* in each isolate. However, a significant proportion of *Enterobacteriaceae* were obtained from colonized patients (7/16, 44%), indicating the existence of a reservoir of colistin resistance among colonized patients. This could be the source for the emergence of hospital outbreaks and the dissemination of colistin resistance determinants to the community and the environment. *A. baumannii* isolates showed

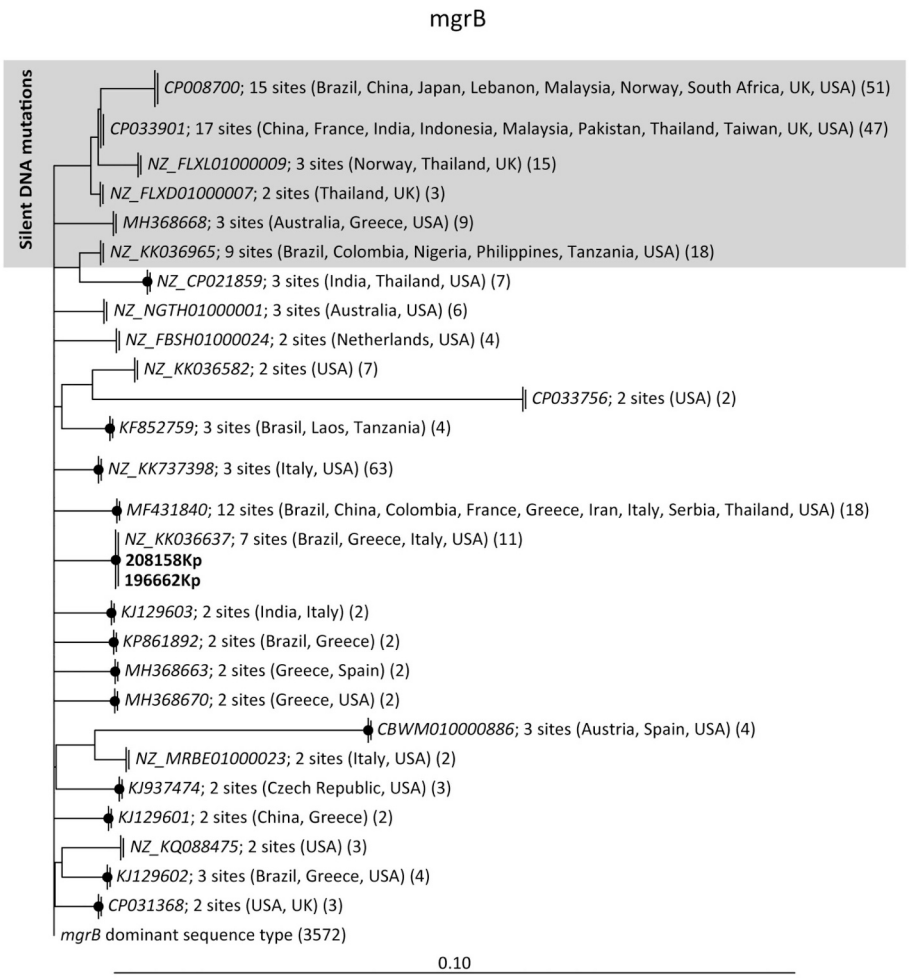

Fig. 3. Neighbour-joining tree based on all publicly available *mgrB* gene sequences from *K. pneumoniae.* Only lineages observed at more than two sites were included. Black dots indicate lineages with colistin resistant isolates. The number in brackets indicates the total number of publicly available sequences with 100% sequence similarity to the selected reference.

similar rep-PCR patterns, demonstrating the existence of two clusters with > 97% similarity, which points out to the clonal spread of colistin resistance determinants. Furthermore, all *A. baumannii* isolates originated from the surgical wards in the University Hospital Center Osijek indicating that surgical procedures or lapses in the hospital hygiene measures could be a risk factors for acquiring colistin resistant isolates (Falagas and Kopterides, 2006).

In published reports, more than 40% of colistin resistance in *K. pneumoniae* worldwide is mediated by mutations in *MgrB* (Olaitan et al., 2014; Cannatelli et al., 2014). This inactivation can be mediated by insertions, nonsense, or missense mutations (Olaitan et al., 2014). The inactivation of MgrB is reported as the most common mechanism for colistin resistance (Poirel et al., 2015; Cannatelli et al., 2014). Three *K. pneumoniae* isolates in this study were found to have a non-functional *mgrB* gene. In the strains 208158 and 196662, a point mutation introduced a stop codon at the amino acid position 3. This mutation was previously reported in isolates from Brazil, Greece, Italy and the USA. In strain 22774, an insertion of an IS26 transposase element at amino acid position 7 in the *mgrB* gene was observed creating a nonsense protein sequence. This mutation was not previously reported. Both the mutation and the insertion could lead to gene inactivation, and thus the phenotypic colistin resistance. However, these resistance mechanisms were not confirmed by additional *in vitro* experiments. A set of variations were found in the proteins PmrB, PhoP, and PhoQ in all *K. pneumoniae* isolates. Three previously reported amino acid changes have been observed in all but one *K. pneumoniae* isolate; R256G in PmrB (Cheng et al., 2015), D150G in PhoQ (Cheng et al., 2015), and L26Q in PhoP (Olaitan et al., 2014). These amino acid changes are reported to be deleterious mutations, although no increased MIC was reported with these changes alone, and secondary factors are probably needed to acquire colistin resistance (Cheng et al., 2015).

Multiple variations were found in all *A. baumannii* isolates. One *A. baumannii* isolate (1966/18) had the previously reported S14L amino acid change in PmrB (Beceiro et al., 2011). This change was associated with a MIC value of 128 μg/ml in previous reports (Beceiro et al., 2011), while *A. baumannii* 1966/18 had the highest MIC value (64 μg/ml) compared to other *A. baumannii* isolates in this study. However, mutations in other colistin resistance associated genes were not detected. The low number of point mutations in the lipid A biosynthesis gene, *lpxC*, that was globally observed might be an indicator that mutations in these genes result in a significantly reduced fitness, and that they are only selected under a high, antibiotics-driven evolutionary pressure.

Although several *mcr* compatible (Poirel et al., 2017) plasmid backbones were detected in the *K. pneumoniae* and *E. aerogenes* isolates, but not in *A. baumannii*, no *mcr* genes have been found in any of our isolates. The FII plasmid was previously found in Enterobacteriaceae harbouring CTX-M-15(Literacka et al., 2009), and the lncL plasmid was found in *K. pneumoniae* harbouring OXA-48 in Croatia (Bedenic et al., 2018b). Colistin resistance in these isolates was most likely of chromosomal origin. Although, only some previous described variations have been detected *in situ,* the lack of *mcr* genes, and the negative

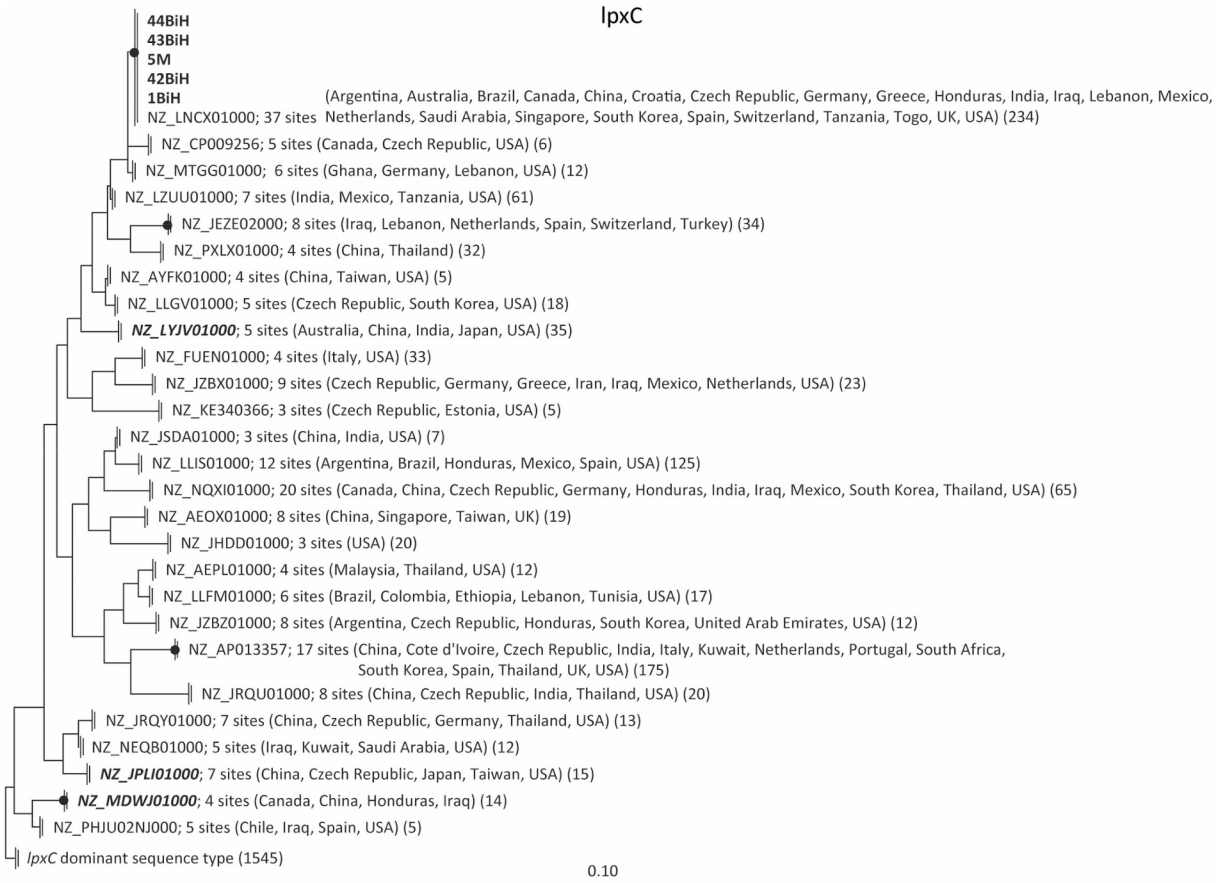

Fig. 4. Neighbour-joining tree based on all publicly available *lpxC* gene sequences from *A. baumanni*. Only three lineages have mutations in their protein sequence (bold italic). Isolates sequenced in our previous studies belong to the second most prevalent *A. baumannii* lineage (bold). Only lineages observed at more than three sites were included. Black dots indicate lineages with colistin resistant isolates. The number in brackets indicates the total number of publicly available sequences with 100% sequence similarity to the selected reference.

results from the conjugation experiments point towards a chromosomal origin.

Croatia has a national surveillance system and specific guidelines for the detection of carbapenemase producing Enterobacteriaceae and the obligation to report these to health authorities. However, such protocols have not yet been established for colistin-resistant isolates. The detection of colistin resistance can be a problem for the majority of routine laboratories since the disk-diffusion test does not provide reliable results due to poor diffusion of colistin in agar medium. Furthermore, the E-test was shown to yield lower MICs, resulting in a very major error (resistant strains being identified as susceptible) (The European Committee on Antimicrobial Susceptibility Testing, 2018; CLSI, 2018). Thus, the broth microdilution method is recommended by EUCAST as the most reliable method for susceptibility testing. However, the method is complicated and time consuming (The European Committee on Antimicrobial Susceptibility Testing, 2018; CLSI, 2018). Furthermore, this study showed a good correlation between phenotypic tests for the detection of ESBLs (DDST, combined disk test) and carbapenemases (Modified Hodge test, CIM test) and the molecular detection of resistance genes. This indicates that results of the phenotypic tests, which are cheap and easy to perform, are reliable for the detection of carbapenemases and ESBLs, although they are not as precise as molecular tests which are able to detect the causative genetic mechanisms. The surveillance of colistin-resistant organisms is very important, since spread to other countries can be expected. Based on MLST analysis, we found that the most common sequence type of *K. pneumoniae* is 37. This is different from the ST258 which was responsible for previous colistin-resistance outbreaks in Europe, as mentioned earlier. Furthermore, colistin resistance in *K. pneumoniae* with ST307 and S7306 has been described in Serbia (Novovic et al., 2017). However, several cases of KPC-producing *K. pneumoniae* with ST37 have been described in Italy (Richter et al., 2012), and in Croatia (Bedenic et al., 2012).

In conclusion, the colistin resistance in these MDR and XDR isolates, along with the mechanisms for antibiotic resistance, shows that for these patients, colistin is not a last resort option anymore. Other antibiotic options, such as tigecycline and sulbactam, are available to treat infections with MDR and XDR pathogens but with less clinical and microbiological cure rates than colistin, except as triple therapy, which potentially prevents the emergence of colistin-resistant *A. baumannii* strains as is suggested by *in vitro* and *in vivo* studies (Kengkla et al., 2018). Since some of the isolates exhibited high level resistance to carbapenems combination therapy without colistin and carbapenems, including gentamicin, tigecycline and fosfomycin should be recommended which was previously proved to be efficient in the prospective cohort studies (Machuca et al., 2017). In addition, the combination of colistin with vancomycin or teicoplanin was shown to be effective in the treatment of severe infections caused by colistin-resistant *A. baumannii* (Vidaillac et al., 2012). Last, ceftazidim/avibactam and ceftolozan/tazobactam seem to have promising effects on CRE (except MBL positive) and carbapenem-resistant non-fermentative bacteria, regardless of colistin non-susceptibility, which is particularly important since the majority of our isolates possessed either OXA-48. However, both inhibitors do not inhibit MBLs. New therapeutic options are urgently needed for colistin-resistant MDR, and especially carbapenemase-producing bacteria. Novel approaches, for example

antimicrobial peptides (AMP) and ceragenins (small non-peptide-based molecules) could play a role, since observations of resistance to AMPs are limited. Colistin-resistant isolates remain susceptible to AMPs and ceragenins. *In vivo* studies show an effective elimination of bacterial isolates by ceragenins (Hashemi et al., 2017). Overall, the presence of colistin resistance in MDR/XDR and carbapenemase-producing isolates demonstrates the emerging threat to healthcare.

Declaration of Competing Interests

All authors: no conflict of interest.

Contributions

VD did experimental work, collection and analysis of the data, and wrote the article. RC did experimental work and collected and analysed the data. DV-B did laboratory work. MB did laboratory work. ICG did critical analysis of the manuscript. BB designed the study, did experimental work, and did critical analysis of the manuscript. IB designed the study and did critical analysis of the manuscript.

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