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CAUSATIVE AGENTS OF BLOODSTREAM INFECTIONS IN TWO CROATIAN HOSPITALS AND THEIR RESISTANCE MECHANISMS

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ABSTRACT

Blood samples were collected alongside with routine blood cultures (BC) from patients with suspected sepsis, to evaluate the prevalence of different causative agents in patients with bacteraemia. Among 667 blood samples, there were 122 positive BC (18%). Haemoglobin content, platelet number, and systolic blood pressure values were significantly lower in patients with positive BC, whereas serum lactate levels, CRP, creatinine and urea content were significantly higher in patients with positive BC. The rate of multidrug (MDR) or extensively drug resistant (XDR) bacteria was 24% (n=29): *Klebsiella pneumoniae* (9), *Pseudomonas aeruginosa* (9), *Acinetobacter baumannii* (4), *Escherichia coli* (1), vancomycin resistant *Enterococcus* spp *(*VRE*)* (3), and methicillin-resistant *Staphylococcus aureus* MRSA (3). The dominant resistance mechanisms were the production of extended-spectrum β-lactamases, OXA-48 carbapenemase, and colistin resistance in *K. pneumoniae*, VIM metallo-β-lactamases in *P. aeruginosa* and OXA-23-like oxacillinases in *A. baumannii*. The study revealed high rate of MDR strains among positive BCs in Zagreb, Croatia.

Key words: multidrug-resistant bacteria, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, bloodstream infections, extended-spectrum β-lactamases

INTRODUCTION

Bloodstream infections (BSI) are life-threatening conditions. Inappropriate initial antimicrobial therapy leads to higher mortality in patients with septic shock.[1] Infections due to multidrug resistant (MDR) pathogens are prone to treatment failures and inappropriate empiric antimicrobial therapy, but whether drug resistance alone increases mortality in the setting of appropriate therapy is unclear. Bacterial resistance to antibiotics is growing up day by day, in both community and hospital setting, increasing morbidity and mortality. Therapy of invasive infections due to MDR Gram-negative bacteria is challenging, and some of the few active drugs are not available in many countries. Global spread of multiresistant pathogens and the lack of new antibiotics are limiting clinicians in the therapy of septicemia. Particularly important are ESKAPE pathogens: *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species.[2-3] Therapeutic failures are usually associated with methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *Enterococcus faecium* (VRE), extended-spectrum β-lactamase (ESBL) producing Enterobacterales, carbapenem-resistant Enterobacterales (CRE), carbapenem resistant *Pseudomonas aeruginosa* (CRPA), and *Acinetobacter baumannii* (CRAB)[3]. Risk factors for acquiring bloodstream infections with MDR bacteria are: frequent antibiotic treatments, indwelling urinary and intravascular catheters, recent surgical procedures and stay in the hospital ICU. ESBLs hydrolyse expanded-spectrum cephalosporins (ESC) and render them inactive. The spread of plasmids carrying CTX-M type genes in the community beginning mostly in the 2000s is the main driver of ESBL dissemination in infections associated with Enterobacterales, including BSI isolates and replaced other ESBL enzymes (TEM and SHV) that were identified in hospital infections in the past.[4,5] Previous studies revealed very high rates of ESBL producing Enterobacteriaceae associated with BSI in South America, the Far East and Mediterranean

countries.[6-8] The rate of ESBLs in the previous studies ranged from 3% to 15%. CTX-M-15 ESBL was found in *E. coli* causing bloodstream infections in Croatia[9]. The isolates exhibited resistance to expanded-spectrum cephalosporins (ESC), gentamicin and ciprofloxacin. CTX-M- ESBLs are also dominant in South America[6-7], but the SHV and TEM type are still present in some geographic areas such as the Far East[8]. In the previous studies the clinical outcome did not differ between patients with ESBL- positive and ESBL- negative infections. However, the prior usage of antibiotics and inappropriate empirical therapy was more frequent among patients with ESBL- positive organisms.[8] Haematologic malignancies and recent chemotherapy were more frequent in the ESBL- positive group (35% vs 8,3%) and (54% vs 34%) respectively. A high level of resistance to ESC, gentamicin, and ciprofloxacin was noticed. Except for ESBLs, multidrug- resistant AmpC- producing organisms such as *Enterobacter* spp, *Serratia* spp and *Citrobacter* spp can also cause septicaemia and are challenging for treatment[10]. Carbapenems are usually the antibiotics of choice for the treatment of BSI due to ESBL- or AmpC- producing organisms. Recently, carbapenem-resistant isolates, associated with carbapenemases belonging to class A, B or D, were reported to be associated with BSI, particularly in developing countries,[11,12] contributing to the poor outcome. Carbapenem resistance in *P. aeruginosa* was shown to cause increased mortality in BSI.[13]In this study, blood samples were collected alongside with routine blood cultures (BC) from the patients with suspected sepsis, to evaluate the prevalence of different causative agents/positive BCs in patients with suspected bacteraemia, taken in the frames of the European FAPIC project (Fast assay for pathogen identification and characterization, Horizon, 2020).

MATERIAL AND METHODS

Blood samples were collected in two periods: 21st January to 3rd October 2017 and 21st December 2019 to 22nd September 2020 for the purpose of the FAPIC project, aiming to develop new method for pathogen identification and characterization. The protocol of the project required two periods for sample collection. In the first period two hospitals in Zagreb were involved: University Hospital Centre Zagreb (UHCZ) and University Hospital Merkur (UHM), whereas in the second period the samples were taken only UHCZ. UHCZ is a 1724-bed university hospital and the largest hospital in Croatia with all medical specialties including organ and tissue transplantation patients. UHCZ provides services to a part of the Zagreb population and acts as a referral hospital for the entire Croatian population for specific patients/procedure groups, thus covering a population of about 4,000,000 people. UHM is the main transplantation hospital in Croatia.

The hospitals wards included were: surgery ICU (AIK), neurosurgery ICU (AIN), cardiosurgery ICU (AKA), medical ICU and haematology in UHCZ and medical ICU in Merkur. Inclusion criteria were age >18 and suspected bacteremia. In addition to two sets of blood cultures, an additional sample of 9 ml was taken in the first period and 1 ml in the second period. Standard care was provided further on. The study was approved by the Ethical Committee of the University Hospital Centre Zagreb and University Hospital Merkur. All patients signed informed consent.

In this study, sepsis was defined using the definition based on the Systemic Inflammatory Response Syndrome (SIRS)[1]. Sepsis was defined as the presence of causative agents in the blood accompanied by SIRS (elevated or decreased temperature (>38.5ºC or <36 ºC), increased heart rate (>90), respiratory rate (>20) and white blood cell count ($12x10^9$). Septic shock was characterized by sepsis induced hypotension, not responding to the fluid replacement and vasopressors. The hospital BSI was defined as sepsis acquired while in

hospital, occurring 48 h or more after admission and up to 48 h after discharge. BSI developing within first 48 h upon admission was defined as community acquired.

Blood culture (BC) bottles were used for routine cultivation and diagnosis, which included BACTEC FX (bioMerieux, Marcy, l'Etoile, France) and MALDI-TOF. Positive BCs were subjected to Gram staining and subcultured on solid medium (blood, chocolate and Columbia agar) and after 18 to 24 h incubation (overnight) identified by MALDI-TOF (matrix-assisted laser desorption ionization-time of flight mass spectrometry) Biotyper (Bruker, Daltonik GmbH, Bremen, Germany). Susceptibility testing was done on overnight subcultures by disk-diffusion test according to EUCAST standards[14] and by the broth microdilution method (for colistin, meropenem, imipenem, vancomycin). Pathogens were plated for susceptibility testing in the afternoon, while results were available the morning after. The isolates were classified as multidrug-resistant (MDR), extensively-drug resistant (XDR) or pandrug-resistant (PDR) as described previously by Magiorakos *et al*.[15] For resistant isolates, additional laboratory analysis was performed. The BC was considered contaminated if growing a typical skin flora like coagulase- negative- staphylococci or diphteroid bacteria in only one bottle, whereas the other bottle turned out to be negative. The following data were collected from the patient's medical file: demography (age, gender), outcome (death or survival), underlying disease, septic parameters (temperature, serum lactate, heart rate, blood pressure, O_2 saturation, central venous catheter, antibiotic treatment); laboratory data (white blood cells - WBC, C-reactive protein - CRP, haemoglobin, platelet count, creatinine, urea, lactate dehydrogenase - LDH, alanine aminotransferase - ALT, aspartate aminotransferase - AST) and time points (time of blood collection for blood culture, blood culture arrival in laboratory, positive blood culture, identification, antibiotic susceptibility results communicated to the treating physician).

Laboratory analysis of MDR isolates

Antimicrobial susceptibility

Minimum inhibitory concentrations (MICs) of a wide range of antibiotics were determined using the broth microdilution method in Mueller-Hinton broth and 96 well microtiter plates, according to CLSI standards[16] and for colistin according to the EUCAST standards.[14]

The panel of the used antibiotics is shown in Tables 1a to e. The double disk synergy test (DDST) was performed with a 20 mm spacing between the disks as previously described.[17]. The ESBL production was confirmed by the CLSI combined disk test using disks with extended-spectrum cephalosporins (ceftazidime, cefotaxime, ceftriaxone) or ESC alone and with addition of clavulanic acid.[16] For *A. baumannii* and *P. aeruginosa*, the test was done with the addition of cloxacillin in the medium (200 mg/L) to inhibit the chromosomal AmpC β-lactamase, which can antagonize the synergistic effect with clavulanate.[18] Plasmid-mediated AmpC β-lactamases were detected by combined disk test using cephalosporin disks combined with 3-aminophenylboronic acid (PBA).[19] The modified Hodge test (MHT) and CIM test were used to screen for the production of carbapenemases.[20-21] 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.[20,22]

Conjugation

The transferability of cefotaxime or ertapenem resistance in Enterobacterales was determined by conjugation (broth mating method) employing *E. coli* J65 resistant to sodium azide.[23] The transconjugants strains were selected on MacConkey agar supplemented with either cefotaxime (2 mg/L) or ertapenem (1 mg/L) to inhibit the growth of the recipient strain and sodium azide (100 mg/L) to supress the donor strains.

Molecular detection of resistance genes

The nature of ESBL, carbapenemases and fluroquinolone resistance genes was determined by PCR. To amplify the genes conferring resistance to β-lactams including broad-spectrum and extended-spectrum β-lactamases (*bla*$_{SHV}$, *bla*$_{TEM}$, *bla*$_{CTX-M,}$ and *bla*$_{PER-1}$)[24-27], plasmid-mediated AmpC β-lactamases (p-AmpC)[28], and carbapenemases of class A (*bla*$_{KPC}$), class B or metallo-β-lactamases-MBLs (*bla*$_{VIM,}$ *bla*$_{IMP,}$ and *bla*$_{NDM}$) and class D or carbapenem hydrolysing oxacillinases-CHDL (*bla*$_{OXA-48}$)[29] and to fluoroquinolones (*qnr*A, *qnr*B, *qnr*S)[30], we used primers and cycling conditions, previously described. The phylogenetic cluster of CTX-M β-lactamases was detected by multiplex PCR according to Woodford et al.[31] The *mcr* -1 and *mcr*-2 genes were sought by PCR in two colistin resistant *K. pneumoniae* isolates.[32] In *A. baumannii* isolates, genes encoding KPC, MBLs (*bla*$_{VIM,}$ *bla*$_{IMP,}$ *bla*$_{SIM}$ and *bla*$_{NDM}$) and CHDL *(bla*$_{OXA-51-like}$, *bla*$_{OXA-23-like}$, *bla*$_{OXA-24/40-like}$, *bla*$_{OXA-58-like}$ and *bla*$_{OXA-143-like}$) were sought by PCR using protocols and conditions as described previously.[29,33] *P. aeruginosa* isolates demonstrating reduced susceptibility to carbapenems were subjected to PCR amplifying carbapenemases.[29] MBL positive isolates were subjected to PCR with the primers 5'-CS and 3'-CS combined with forward and reverse primers for *bla*$_{VIM}$ in order to determine the location of *bla*$_{VIM}$ gene within class 1 integron.[34] The genetic context of *bla*$_{OXA-51}$, *bla*$_{OXA-23,}$ genes in *A. baumannii* was determined by PCR mapping with primers for IS*Aba1* combined with forward and reverse primers for *bla*$_{OXA-51}$, and *bla*$_{OXA-23}$ according to Turton et al.[35] Flanking regions of *bla*$_{CTX-M}$ genes were determined by PCR mapping using forward primer for IS*Ecp1* and IS*26* combined with the primer MA-3 (reverse for *bla*$_{CTX-M}$ genes).[36]

The linkeage of *bla*$_{OXA-48}$ and IS1999 was determined using forward primer for IS1999 combined with reverse primer for *bla*$_{OXA-48.}$[37]

Whole genome sequencing (WGS)

One MRSA (18242) and two VRE isolates (50 and 326), respectively and one *K. pneumoniae* (5714) and *A. baumannii* (44921), respectively, were subjected to WGS. First, the strains

were cultivated in Tryptic Soy Broth (TSB) and Casein-Peptone Soymeal-Peptone (CASO) Broth (Merck Millipore, MA, USA) at 37°C overnight. Then, the genomic DNA was extracted using the QIAamp UCP Pathogen Mini Kit (Qiagen, Hilden Germany) according to the manufacturer's instructions. The DNA extracts were sent to the Next Generation Sequencing Facility of the Vienna Biocenter for sequencing using Illumina's NextSeq1000 system according to the manufacturer's instructions. The single reads obtained were assembled and analysed using the webservers and services of the Center for Genomic Epidemiology (http://www.genomicepidemiology.org).[38]

Characterization of plasmids

Plasmids were extracted with the Qiagen Mini kit (Inel, Croatia) according to the manufacturer's instructions. PCR-based replicon typing (PBRT) was performed according to the protocol described by Carattoli *et al.*[39-40]PBRT according to Bertini *et al* was applied for *A. baumannii* to type the resistance plasmids carrying carbapenemase genes.[41]

Molecular typing of *A. baumannii* isolates

Sequence groups (SGs 1-3) corresponding to the international clonal lineages (IC I-III) determination was performed according to the procedure described by Turton *et* al.[42]

Statistical methods

Data were analysed using students-t test (continuous) or with non-parametric tests in case of the non-normal data.

RESULTS

Blood samples and bacterial isolates

In total, 667 blood samples were included in the study (257 in the first period and 410 in the second period). UHCZ provided 641 blood samples whereas 26 were obtained from UHM.

The rate of positivity in general, was 19% (125/667) with higher rates in the first period (22%, n=58) compared to the second period (15%, n=64). The rate of positivity was higher in UHM (30%, n= 8) compared to UHCZ (18%, n=117). The rate of contaminated blood cultures was 0,6% (4/667). Nine patients (7.5%) had two organisms in the BC. All patients had nosocomial BSI. Thirteen patients had septic shock leading to multiorgan failure.

The rate of multidrug- (MDR) or extensively drug- resistant (XDR) bacteria was 24% (n=29) with higher rates in the second period (17%, n=9 in the first period vs. 32%, n=20 in the second period). The rate of resistant bacteria was higher in UHM (50%, n=4) compared to UHCZ (22%, n=25). All MRSA, VRE, and ESBL positive Enterobacterales were MDR, whereas one OXA-48 producing *K. pneumoniae* isolate, all *A. baumannii,* and *P. aeruginosa* were XDR. The source of 121 true BSI (excluding contamination) was cryptogenic or no known source in 39% (n=48), IV catheter in 19% (n=23), pneumonia in 16% (n=19), febrile neutropenia in 10% (n=16), abdominal or urinary tract infection in 6% (n=7), endocarditis in 5% (n=6) and gynaecological operation in 0,8% (n=1) patients. The choice of empiric therapy depended on the hospital ward and the underlying infection which acted as a source of BSI. Meropenem was the most frequently used antibiotic in empirical therapy, administered in 129 (19%) of the patients, followed by vancomycin in 105 (16%), piperacillin/tazobactam in 63 (9%), linezolid in 50 (7,5%), ciprofloxacin in 34 (5%), cefuroxime in 28 (4%), colistin in 24 (3,6%), cefepime in 22 (3%), metronidazole in 21 (3,2%) and co-amoxyclav in 12 (1,7%). Aminoglycosides were administered only sporadically with gentamicin used in four patients and amikacin in nine. Moreover, clindamycin and ampicillin/sulbactam were given to only two patients, respectively. The preferred antibiotic combinations in empirical therapy were meropenem +vancomycin applied in 48 patients (7%), followed by meropenem+linezolid in 20 patients (3%) and meropenem+colistin and meropenem+ciprofloxacin administered in five patients (0,7%), respectively. Triple combination including meropenem, fosfomycin and

colistin was applied in three patients who were previously colonized with resistant strain. Ceftazidim/avibactam as last resort antibiotic was administered in two patients. Ampicillin/sulbactam was given to the patients with lower respiratory tract colonization with *A baumannii*. The general surgery ward (AIK) usually applies piperacillin/tazobactam and metronidazole to cover anaerobic bacteria associated with complicated abdominal surgical procedures. On the contrary, in the neurosurgery unit (AIN) ceftriaxone is often administered because it achieves high concentrations in cerebrospinal fluid. In the case when MDR Gram-negative bacteria were identified, meropenem and colistin were applied, whereas in the case of MRSA vancomycin was the drug of choice. Linezolid was used if VRE was confirmed. BSI in the heamatologic wards was mainly associated with febrile neutropenia, and patients received also antiviral and antifungal agents in addition to an antibiotic.

Deescalation (narrowing of antibiotic spectrum or removal of antibiotics in combination therapy) was observed in 73 (11%) (20 in the first and 53 in the second period) of the patients. A class switch was noticed in 28 cases (4%) (16 in the first and12 in the second period). In 141 patients (22%) the therapy was not changed. For ten patients (1,4 %) the data on antibiotic therapy were not available neither in the hospital computer system nor in the paper records in the hospital wards. These changes in antibiotic treatment were triggered either by blood culture positivity, by MALDI-TOF identification results and/or by susceptibility results. Inappropriate empirical antibiotic therapy was observed in 48,2% (n=14) of the patients harbouring MDR/XDR strain in the BC, meaning that the antibiotic was changed when the attending clinician received the antibiotic susceptibility testing result. In the case of sensitive organisms, an antibiotic switch was indicated in 46% (n=43) of the patients and happened when the clinician obtained the result of the identification of the causative organism. The antibiotics most frequently used in patients with positive BC after receiving antibiotic susceptibility results were: meropenem in 43 (34%), colistin in 19 (15%), vancomycin in 18

(14%), metronidazole in 15 (12%), piperacillin/tazobactam in 12 (9,6%), ciprofloxacin in 10 (8 %) and cefepime and teicoplanin in 9 (7,2%) of the patients, respectively. Linezolid was administered in 6 patients (5%). Meropenem was used in combination with vancomycin and colistin, in 9 (7%) patients, respectively. Meropenem combination with linezolid was given to four patients (3%). Only three patients received triple combination (meropenem+colistin+fosfomycin).

Statistical analysis

The haemoglobin content, platelet number, and the systolic blood pressure (SBP) values were significantly lower in patients with positive BC than those with negative BC (p=0.004, 0.02 and, 0.002, respectively). Serum lactate levels, CRP, creatinine, and urea content were significantly higher in patients with positive BC compared to those with negative BC (p=0.007,0.04, 0.003, and 0.0001, respectively). Moreover, the survival rate was significantly lower in the patients with BSI compared to those with sterile BC (p=0.0001). However, the patients with positive BC were significantly older (p=0.007). The length of the ICU stay was significantly longer in the patients with a positive BC (p=0.03). There was no significant difference in oxygen saturation (p=0.21), ALT (p=0.16), AST (p=0.24), LDH (p=0.12), haemoglobin concentration (p=0.051), rate of previous surgical procedures (p=0.11), length of hospital stay (p=0.24) and gender (p=0.48). Moreover, the infection with resistant strain was not associated with longer hospital stays (p=0.41) or increased mortality (p=0.08)

Microbiology analysis

The pathogens isolated from blood cultures in two study periods were: *P. aeruginosa* (n=29, 12+17*), S. epidermidis* (n=27, 11+16), *K. pneumoniae* (n=14, 5+9), *E. coli*, n=8, (6+2), *E. faecalis* (n= 2, 0+2), *E. faecium* (n=7, 6+1), *S. hominis* (n=4, 1+3), *A. baumannii* (n=4, 2+2), MRSA (n=3, 3+0), *S. anginosus* (n=1, 1+0), *S. sanguinis* (1, 0+1), *S. haemolyticus* (2, 0+2*),*

C. acnes (n=2, 0+2), *S. maltophilia (n*=1, 0+1), *S. enteritidis* (n=1+0), *C. albicans* (n=5, 2+3), *C. glabrata* (n=3, 0+3), and *C. parapsilosis* (5, 2+3).

The rate of MDR/XDR pathogens according to the species were: 100 % in *A. baumannii* (4/4) and *S. aureus* (3/3), 64% in *K. pneumoniae* (9/14), 43% in *E. faecium* (3/7), 31% in *P. aeruginosa* (9/29), and 12% in *E. coli* (1/8).

Laboratory analysis of MDR bacteria

Gram-positive bacteria

All three MRSA isolates exhibited susceptibility to vancomycin, teicoplanin and linezolid as shown in Table 1 a. Aminoglycoside resistance genes *aac*(6'), *aph*(2') and *aph*(3'*), norA*-like for fluoroquinolone resistance, and *erm*(A) genes encoding macrolide resistance were found in the strain 18242 by WGS (accession number CABFJA010000000). The *mec*A gene encoding PBP2a was identified as well. The isolate belonged to ST 111.

Three VRE isolates showed susceptibility to linezolid and daptomycin (Table 1b). All isolates demonstrated the Van A resistance phenotype. The VRE strain 38655 was found to possess *ant*(6') and *aph*(2') responsible for aminoglycoside resistance, *erm*B for macrolide resistance, *tet* (M) for tetracycline resistance and *dfr*G for trimethoprim resistance (accession number CABFJB010000000). The strain 327 carried *dfr*G, *tet*A genes and vanA-like gene. The isolates belonged to ST192 and ST 80, respectively.

Gram-negative bacteria

The DDST and combined disk test with clavulanic acid tested positive in all *Enterobacterale*s resistant to expanded-spectrum cephalosporins (ESC) with augmentation of the inhibition zones around cephalosporin disks of 12 to 25 mm in the presence of clavulanic acid. All ESBL- producing Enterobacterales exhibited resistance to amoxycillin alone and combined

with clavulanic acid, cefazoline, cefuroxime, expanded-spectrum cephalosporins or ESC (ceftazidime, cefotaxime, ceftriaxone), cefepime and ciprofloxacin. Gentamicin resistance was proved in 8 isolates (80%). Three isolates demonstrated resistance to ertapenem (30%) and two to colistin (20%). One isolate (10%) demonstrated resistance to imipenem and meropenem (Table 1c). CTX-M-15 was identified in all isolates and the IS*Ecp* insertion sequence upstream of the encoding gene was detected in half of the isolates. The Hodge and CIM tests were positive in three *K. pneumoniae* isolates resistant to ertapenem, one of which was resistant to imipenem and meropenem as well. Ertapenem resistance was transferable from two strains with reduced susceptibility to carbapenems with an efficiency of transfer of $2.2x10^{-3}$ to $5.1x10^{-4}$ transconjugant cells per donor cell, respectively. The transfer of cefotaxime resistance was not successful. Carbapenem MICs of the transconjugant strains were substantially lower, but reflected the data for the respective donor strains. Ertapenem resistant transconjugants did not harbour resistance genes for non β-lactam antibiotics. OXA-48 encoding gene was detected in three *K. pneumoniae* isolates and their respective transconjugants by PCR and was preceded by IS*1999*. All three OXA-48- positive strains coharboured ESBL responsible for resistance to ESC. Except of OXA-48, additional β-lactam resistance determinants were identified by WGS in *K. pneumoniae* 255 (accession number CABFJS010000000): $bla_{\text{SHV-11}}$, $bla_{\text{CTX-M-15}}$ and $bla_{\text{OXA-1}}$. Several aminoglycoside resistance genes were found: *aac*(6') encoding aminoglycoside acetylases, *oqxA* and *oqx*B for aminoglycoside efflux pumps. Tetracycline resistance was mediated by *tet*A gene) whereas the *fos*A gene encoded fosfomycin resistance. The *mcr* genes encoding colistin resistance were not found in colistin resistant strains. The IncL/M plasmid was identified in all OXA-48 producing organisms (Table 2c). The IncFIB plasmid was found in five ESBL- positive organisms, followed by IncFII and IncFIA, present in four isolates and one isolate,

respectively. One of the OXA-48- producing *K. pneumoniae* was found to belong to ST37 as shown in Table 1c.

Four *A. baumannii* isolates showed resistance to ceftazidime, cefepime, piperacillin/tazobactam, gentamicin and ciprofloxacin, and susceptibility to colistin as shown in Table 1d. Three isolates were intermediate susceptible to ampicillin/sulbactam, whereas one exhibited resistance. The Hodge and CIM tests were positive indicating the production of carbapenemases. OXA-23 was the dominant carbapenem resistance determinant, identified in three isolates, while OXA-72, an allelic variant of OXA-24/40-like, was found in one isolate. *bla*$_{\text{OXA-23}}$ and *bla*$_{\text{OXA-66}}$ and genes possessed IS*Aba1* upstream of the genes. Chromosomal *bla*$_{\text{ADH-25}}$ was found as well. Among aminoglycoside resistance determinants: *aph(3'), aac(3), armA, strB,* and *strA* were found by WGS in the isolate 44921 (accession number CABFJO010000000). All isolates belonged to IC II (SG 1).

P. aeruginosa showed uniform resistance to piperacillin/tazobactam, ceftazidime, cefepime, imipenem, meropenem, gentamicin, amikacin, and ciprofloxacin, but susceptibility to colistin. The Hodge, CIM, and inhibitor- based test with EDTA tested positive in eight out of nine isolates indicating the production of an MBL. Eight positive isolates harboured *bla*$_{\text{VIM-2}}$ genes which were located in the class 1 integron (Table 1e).

DISCUSSION

The main finding of the study is the high rate of MDR bacteria among BC isolates in two participating centers. The rates of MRSA, VRE, and ESBL- positive organisms were higher than in previous report from Germany, with 100%, 43% and 14% of resistant organisms in Croatia vs 10%, 17% and 10%, respectively, in the German study.[43]

The types of resistance determinants found in this investigation are in line with previous reports on ESBL and carbapenemases, pertaining to the included hospitals.[44-48] *A. baumannii*

producing OXA-23- like enzymes and belonging to IC (international clonal lineage) II was identified in UHC Zagreb in early 2010.[48] *P. aeruginosa* with VIM-2 MBL was reported in the same hospital wards which provided blood samples for a present study in 2009.[46] This points out to the fact that these isolates may remain unnoticed in hospital equipment and surfaces which serve than as a source of patient's colonization. Genotyping by PFGE or rep--PCR should be done to determine the clonal relatedness of the isolates, but this was beyond this study. A recent paper described *K. pneumoniae* coharbouring OXA-48 and ESBL (CTX-M-15) from patient's saliva in the same surgical ICU which provided the BC growing *K. pneumoniae* isolate, with exactly the same resistance traits.[49] It seems that L/M plasmid mediated the spread of this important carbapenemase within the hospital, which has now outnumbered KPC-2 and VIM-1 in Croatia. Although OXA-48 does not hydrolyse cephalosporins, elevated MICs of ESC were due to the additional ESBL.

IS1*999* located upstream of *bla*$_{\text{OXA-48}}$ is important for the mobilization of the gene[37], similarly as IS*Ecp* preceding *bla*$_{\text{CTX-M}}$ genes[36]. However, cefotaxime resistance was not transferable indicating the possibility of incorporation of *bla*$_{\text{CTX-M}}$ genes into the chromosome, facilitated by the insertion sequence. IS*Aba* 1 identified upstream of *bla*$_{\text{OXA-51-like}}$ and *bla*$_{\text{OXA-23-like}}$ genes increases the expression of the CHDL genes and the level of carbapenem-resistance. OXA-48 does not hydrolyze cephalosporins, but resistance to ESC was due to the additional ESBL. In a Chinese study, KPC-2 and NDM-1 were the most prevalent carbapenemases among BSI *K. pneumoniae* isolates indicating that the carbapenemase types depend on the local epidemiology.[50] ST37 reported in the present study was previously identified in the first KPC producing *K. pneumoniae* in Croatia in 2011[51]. In the Chinese study, BSIs were associated with ST11.[50]

A Korean study found CTX-M and SHV-12 as dominant ESBLs in *Enterobacter cloacae* in bloodstream infections among cancer patients from South Korea.[8] CTX-M ESBLs were also

predominant among *E. coli* bloodstream isolates in Croatia[9], China[52], and South America[7]. CTX-M-14 and CTX-M-15 were dominant allelic variants in Enterobacteriales from BSI, similarly as in our study. P-AmpC β-lactamases were found as important bloodstream pathogens in another study[10], but were not identified in our investigation. The cases of neonatal septicaemia in India were associated with NDM-1 producing *K. pneumoniae*. The isolates coharboured $bla_{\text{CTX-M-15}}$ and fluoroquinolone resistance determinants: *qnr*S1, *qnr*B1, *aac(*6')-Ib, *aac*(6')-Ib-cr.[11] However, in our study MBLs, belonging to VIM family, were only associated with *P. aeruginosa* although in the earlier studies they were reported in *K. pneumoniae*, mostly from rectal swabs.[44] Production of an ESBL was previously found to be associated with inappropriate empirical therapy and need for antibiotic switch in previous investigations.[53] However, in our study the difference in the need for antibiotic switch or escalation, between patients harbouring MDR isolates and those with sensitive isolates, was not very pronounced. Regardless of the susceptibility category (sensitive or resistant), in half of the patients escalation of the antibiotic therapy was indicated. This could be explained by high number of yeasts in the BCs demanding inclusion of antifungal agents. IV catheter was the dominant source of BSI in our study which could be explained by the fact that all patients had infection, contracted in the hospital. This is line with the high rate of MDR/XDR bacteria, including typical hospital pathogens such as MRSA, VRE and CRAB. MRSA isolates with similar properties were detected in the same surgical ICUs for the first time in 2006[54]. UHM participated only in the first period and we expected to have a higher rate of resistant pathogens among the BCs collected in that period because UHM is specialized for transplantations and has predominantly imunocompromised patients. However, the rate of resistance pathogens increased in the second period.

Meropenem and vancomycin is the preferred antibiotic combination in empiric therapy, but switch to colistin and linezolid occurred in the case of CRE or VRE infection. Combinations

with fosfomycin were used in case of carbapenemase producing *P. aeruginosa*. Aminoglycosides were applied very rarely in contrast to other study. Ceftazidime/avibactam was applied only in a few patients because it is licenced in Croatia recently, mostly for severe infections associated with CRE. The implementation of rapid tests for the identification of resistance determinants such as Rapid ESBL NP or NG-test CTX-M is necessary in countries with high rates of resistant bacteria in BSI.[55]

A limitation of the study is the relatively small number of isolates and inclusion of only two centers in the same city. The strength of the study is the detailed molecular analysis of resistance determinants. The spread of resistant bacteria among BSI isolates emphasises the need for continuous surveillance in order to guide empiric antibiotic treatment strategies and prevention programs. The reports from this geographic region are scarce in the bibliography.

CONFLICT OF INTEREST

None to declare

ETHICAL PERMISSION

The project was approved by The Ethical Committee of the University Hospital Centre Zagreb (number: 02/21/JG. All patients included in the study signed informed consent.

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Table 1a. Antibiotic susceptibility of MRSA isolates.

		Antibiotic susceptibility								
Fapic no and protocol number and center	date	PEN	OXA	SXT	RIF	GM	CIP	VAN	TEICO	LZD
440 (18242)	26.01.2017	R	R	S	S	S	R	S	S	S
444	17. 08. 2017.	R	R	S	S	S	R	S	S	S
442	17. 08. 2017.	R	R	S	S	S	R	S	S	S

Abbreviations: PEN-penicillin; OXA-oxacillin; SXT-sulphamethoxazole/trimethoprim; RIF-rifampicin; GM-gentamicin; CIP-ciprofloxacin; VAN-vancomycin; TEICO-teicoplanin; LZD-linezolid

Table 1 b. Antibiotic susceptibility of VRE isolates.

	Antibiotic susceptibility (MIC in parenthesis)					
Fapic no	date	AMP	GM	VAN	TEICO	LZD
326 (38655)	20.06. 2017	R	R	R(128)	R(128)	S
327	11.02.2017	R	R	R(128)	R(128)	S
50 (57252)	21.02. 2020.	R	S	R (128)	R (128)	S

Abbreviations: GM-gentamicin; CIP-ciprofloxacin; VAN-vancomycin; TEICO-teicoplanin; LZD-linezolid;

Table 1 c. Antibiotic susceptibility and β-lactamases production of Enterobacterales isolates.

Fapic no		Phenotypic tests		Antibiotic susceptibility (MIC)														
	date	ESBL	Hodge/ CIM	AMX	AMC	TZP	CZ	CXM	CAZ	CTX	CRO	FEP	IMI	MEM	GM	CIP	COL	BL	Plasmid content and ST
255 (5714) R K.P.	29.05. 2017.	+	+/+	>128	>128	>128	>128	>128	>128	>128	>128	32	1	1	0.5	32	16	IS*1999*-OXA-48+CTX-M-15, SHV-11, OXA-1	*IncL/M, IncFIB IncFII ST 37*
336 M K.P.	05. 07. 2017.	+	NT	>128	32	32	>128	>128	>128	>128	>128	16	0.5	0.25	32	64	0.25	IS*Ecp*-CTX-M-15	*IncFIA*
90 (38088) R K.P.	06.02. 2020.	+	+/+	>128	>128	>128	>128	>128	>128	>128	>128	16	16	32	32	>128	0.5	IS*1999*-OXA-48+CTX-M-15	*IncL/M, IncFIB*
133 49257 K.P.	15.02. 2020.	+	NT	>128	64	16	>128	>128	>128	>128	>128	32	1	0.5	32	16	0.25	IS*Ecp*-CTX-M-15	*IncFII*
318 (129868) K.P.	23.05. 2020.	+	NT	>128	32	64	>128	>128	>128	>128	>128	64	0.25	0.12	2	32	0.25	CTX-M-15	*IncFII*
344 (171502) K.P.	10.07. 2020.	+	NT	>128	32	32	>128	>128	>128	>128	>128	32	0.25	0.12	16	>128	0.5	CTX-M-15	*IncFIB*
374 (183591) K.P.	27.07. 2020.	+	NT	>128	64	64	>128	>128	>128	>128	>128	32	0.12	0.12	1	>128	0.25	IS*Ecp*-CTX-M-15	*IncFIB*
382 (146269) K.P.	12.06. 2020.	+	NT	>128	32	32	>128	>128	>128	>128	>128	64	0.5	0.12	32	64	0.25	IS*Ecp*-CTX-M-15	*IncFIB*
107614 KP	23. 04. 2020.	+	+/+	>128	>128	>128	>128	>128	>128	>128	>128	32	1	0.5	16	32	32	IS*1999*-OXA-48+CTX-M-15,	*IncL/M, IncFIB*
404 E.C.	28.07. 2017.	+	NT	>128	64	16	>128	>128	32	>128	>128	32	0.06	0.06	0.5	64	0.12	IS*Ecp*-CTX-M-15	IncFIA

Abbreviations: AMX-amoxycillin; AMC-co-amoxyclav; TZP-piperacillin/tazobactam; CZ-cefazoline. CXM-cefuroxime, CAZ-ceftazidime, CTX-cefotaxime, CRO-ceftriaxone, FEP-cefepime, IPM-imipenem, MEM-meropenem, GM-gentamicin, CIP-ciprofloxacin, COL-colistin, FOS-fosfomycin; CIM-carbapenem inactivation method, ESBL-combined disk test using cephalosporins alone and combined with clavulanate for detection of ESBLs, CIM-carbapenem-inactivation method; ST – plasmid Inc group and sequence type, K.P: *Klebsiella pneumonia*; E.C: *Escherichia coli*

Table 1d. Antibiotic susceptibility, phenotypic tests and resistance genes content of *A. baumanii* isolates

			Antibiotic susceptibility (MIC)									Resistance genes	
Fapic and protocol number	date	Hodge/CIM	CAZ	FEP	TZP	SAM	IMI	MEM	GM	CIP	COL	BL content	Aminoglycoside resistance genes
120 (44921)	07.03.2017	+/+	>128	>128	>128	16	>128	>128	>128	>128	2	*bla*$_{ADC-25}$ ISA*ba1-* *bla*$_{OXA-23}$ *ISAba1-* *blaOXA-66*	*aph(3')* *aac(3)* *armA* *strB* *strA*
121 (35725)	09.03.2017	+/+	>128	>128	>128	16	>128	>128	>128	>128	2	*blaADC-25* *ISAba1-* *bla*$_{OXA-23}$ *ISAba1-* *bla*$_{OXA-66}$	*aph(3')* *aac(3)* *armA* *strB* *strA*
48 (15564)	17.01. 2020	+/+	>128	>128	>128	8	>128	>128	>128	>128	1	*ISAba1--* *blaOXA-23* *blaOXA-66*	*NT*
139346	03.06. 2020.	+/+	>128	>128	>128	64	>128	>128	>128	>128	1	*blaOXA-72* *blaOXA-66*	*NT*

Abbreviations: CAZ-ceftazidime; FEP-cefepime; IMI-imipenem; MEM-meropenem; SAM-ampiciliin/sulbactam; GM-gentamicin; CIP-ciprofloxacin; COL-colistin; ;; BL-beta-lactamase content; CIM-carbapenem inactivation method; NT-not tested; S-susceptible; all isolates harboured intrinsic *bla*$_{OXA-51}$-like gene, the isolate 125 harboured only *bla*$_{OXA-51}$-like gene without having acquried CHDL; NT-not tested

Table 1e. Antibiotic susceptibility, phenotypic tests, resistance genes content, and clinical data of *P. aeruginosa* isolates.

		Phenotypic tests	Antibiotic susceptibility									
Fapic and protocol number	DATE	Hodge/CIM	EDTA	TZP	CAZ	FEP	IMI	MEM	GM	AMI	CIP	COL	BL
28 (2984)	05.01. 2020.	+/+	+	>128	>128	64	32	32	>128	>128	>128	2	VIM-2
247 (82534)	16. 03. 2020.	+/+	+	>128	>128	32	64	32	64	16	32	2	VIM-2
330 (169585)	09.07. 2020	+/+	+	>128	>128	32	64	32	64	16	32	2	VIM-2
347 (169582)	12.07. 2020.	+/+	+	>128	>128	32	64	32	64	16	32	2	VIM-2
354 (168088)	07.07. 2020	+/+	+	>128	>128	32	64	64	64	16	32	2	VIM-2
360 (167974)	09.07. 2020.	-/-	-	>128	>128	16	64	>128	64	>128	32	2	
349(169584)	10.07. 2020.	+/+	+	>128	>128	32	64	64	64	64	>128	2	VIM-2
372 (176370)	17.07. 2020.	+/+	+	>128	>128	32	64	64	64	64	>128	2	VIM-2
382 (107501)	12. 06. 2020.	+/+	+	>128	>128	64	>128	64	>128	>128	>128	1	VIM-2

Abbreviations: TZP-piperacillin/tazobactam; CAZ-ceftazidime; FEP-cefepime; IMI-imipenem; MEM-meropenem; GM-gentamicin; CIP-ciprofloxacin; COL-colistin; ESBL-inhibitor based test with clavulanic acid for detection of extended-spectrum beta-lactamases; CIM-carbapenem inactivation method; BL-β-lactamase content