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ARRIVAL OF CARBAPENEM- HYDROLYZING- OXACILLINASES *IN ACINETOBACTER BAUMANNII* IN BOSNIA AND HERZEGOVINA

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ABSTRACT

Multidrug-resistant *Acinetobacter baumannii* (MDR-AB) is an important threat for critically ill patients. It can infect the respiratory tract, blood, soft tissues, urinary tract and central nervous system. Recently, carbapenem-resistance was observed in *A. baumannii* clinical isolates from Bosnia and Herzegovina. This prompted us to analyze these isolates with regards to genotypic diversity, antibiotic susceptibility and occurrence of acquired carbapenem resistance genes. Twelve carbapenem-resistant isolates were collected at a University hospital during two different periods of 2011 and 2015-2016: four isolates in 2011 and eight isolates 2015-2016 and compared to determine the dynamic changes in carbapenemase resistance mechanisms and population structure. All twelve isolates were positive for intrinsic *bla*$_{\text{OXA-51-like}}$, nine for *bla*$_{\text{OXA-40-like}}$ and one for the *bla*$_{\text{OXA-23-like}}$ gene. IS*Aba1* was found upstream of *bla*$_{\text{OXA-51}}$ in all and upstream of *bla*$_{\text{OXA-23-like}}$ gene in one isolate. Sequencing of the selected PCR products revealed the presence of OXA-72 β-lactamase (strain 1) and OXA-23 β-lactamase (strain 41). WGS of the selected isolate (AB 5) revealed the presence of *bla*$_{\text{OXA-72}}$, chromosomal genes *bla*$_{\text{OXA-69}}$ and *bla*$_{\text{ADC}}$. Moreover, the *aac (3)*-1a and aadA1 genes encoding aminoglycoside resistance, and *sul1*encoding sulphonamide resistance were identified. PFGE and rep-PCR revealed two clones containing highly similar isolates positive for OXA-40-like; one from 2011 and the other from 2015-2016.

Implementation of hospital hygiene measures, screening of the patients on admission for carriage of MDR-AB, and the early and accurate detection, with restriction of antibiotic use should be recommended to control the spread of these important hospital pathogenes. To our knowledge, this is the first report of *A. baumannii* isolates producing carbapenem-hydrolyzing oxacillinases (CHDL) from Bosnia and Herzegovina.

1.Introduction

Multidrug- resistant *Acinetobacter baumannii* (MDR-AB) is an important threat for critically ill patients (Coelho et al., 2004). It can infect the respiratory tract, blood, soft tissues, urinary tract and central nervous system. This organism is frequently resistant to multiple antimicrobial agents and there are recent reports on isolates that are pandrug-resistant bacteria (Livermore and Woodford, 2006; Durante-Mangoni et al, 2014). Hospital outbreaks are usuall associated with clusters of highly similar strains which are designated as International clonal lineages I, II and III or worldwide clonal lineages (Nemec et al., 2008). Carbapenem resistance is usually associated with ICL II. Resistance to carabapenems can be due to impaired permeability or alternation in penicillin-binding proteins (Poirel and Nordmann, 2006). However, β-lactamase-mediated resistance is the most common mechanism of carbapenem resistance in this species. Carbapenemases found in *Acinetobacter* spp. belong to molecular class A (KPC) (Robledo et al., 2010), class B (metallo-β-lactamases of IMP, VIM, SIM or NDM family) (Lee et al., 2005; Cornaglia et al., 1999; Hrabak et al., 2012; Perilli et al., 2011) or class D (OXA enzymes) recently known as CHDL (carbapenem-hydrolyzing class D oxacillinases) (Brown and Amyes, 2006). The OXA enzymes of *Acinetobacter* are classified into five phylogenetic groups comprising the in *A. baumannii* intrinsic OXA-51-like and the acquired enzymes: OXA-23-like, OXA-24/40-like, OXA-58- like, OXA-143-like and OXA-235-like (Brown and Amyes, 2006; Higgins et al., 2009). Enzymes belonging to OXA-51 group are naturally occuring β-lactamases of *A. baumannii* and are normally expressed at low levels but can be overexpressed as a consequence of the IS*Aba1* location upstream of the genes (Turton et al., 2006). Recently carbapenem-resistance was observed in *A. baumannii* from Bosnia and Herzegovina. This geographic region is characterised by a great cultural diversity and population mixing. Thus, the molecular characterization and strain typing of such epidemic isolates is important for the identification of the sources and the mode of spread, in order to develop the targetted infection control strategies. This prompted us to

analyze these isolates with regards to genetic diversity, antibiotic susceptibility and occurrence of acquired carbapenem resistance genes. The previous studies from 2009 found that reduced susceptibility in *A. baumannii* from Bosnia was solely atributed to overexpression of intrinsic OXA-51 β-lactamase with IS*Aba1* upstream of the gene (Ibrahimagić et al, 2017). The new isolates were collected during two periods: in 2011 and 2015 and compared to determine the dynamic changes in carbapenemase resistance mechanisms and the population structure.

2. Material and methods

2.1Bacterial isolates

Twelve MDR-AB were collected at the University Hospital Mostar in Bosnia and Herzegovina. The University Hospital Mostar is a tertiary hospital with 900 beds, covering a population of 400 000 inhabitants. Four non-duplicate (one per patient), consecutive isolates were collected from July 2011 to November 2011. The remaining eight consecutive non-duplicate isolates were collected from November 2015 till January 2016. Two periods were selected in order to study the dynamic changes in the epidemiology of carbapenem-resistance in a major opportunistic pathogen such as *A. baumannii.* The total number of *A. baumannii* isolates in the first study period (July to November 2011) was 131 with four showing carbapenem-resistance (3%). In the second study period (November 2015 to January 2016) the rate of carbapenem resistant *A. baumannii* was 9 % (7/73). The isolates originated from nephrology, surgical ICU and urology hospital ward with two isolates being obtained from outpatients previously hospitalized in the same hospital. Eight isolates were obtained from patients with signs and symptoms of infection and four from colonized patients according to standard infection definitions (Horan et al., 2008).

2.2Susceptibility tests and β-lactamase detection

The antimicrobial susceptibility to ceftazidime, cefotaxime, ceftriaxone, cefepime, imipenem, meropenem, gentamicin, ciprofloxacin, tigecycline and colistin was determined by disk-diffusion and broth microdilution method in 96 well microtiter plates and interpreted according to CLSI breakpoints (Clinical Laboratory Standards Institute, 2014). Minimum inhibitory concentrations (MICs) of imipenem and meropenem were determined also by agar dilution with addition of cloxacillin (200 mg/L) and sodium chloride in order to determine the effect of chromosomal AmpC β-lactamases on the susceptibility to carbapenems and to detect enzymes of OXA-58 group which are susceptible to inhibition with sodium chloride, respectively (Pournaras et al., 2006). The isolates were classified as multidrug-resistant, extensively drug resistant or pan drug resistant bacteria (Magiorakis et al., 2012).

Extended-spectrum β-lactamases (ESBLs) were detected by combined disk test with cephalosporins and clavulanic acid according to CLSI with addition of cloxacillin in the medium (200 mg/L) (Clinical Laboratory Standards Institute, 2014) to inhibit the chromosomal AmpC β-lactamase which can antagonize the synergistic effect with clavulanate. Augmentation of the inhibition zones around ceftazidime, and cefepime disks of at least five mm in the presence of clavulanic acid was considered indicative of ESBL production.

A modified Hodge test (MHT) was used to screen for the production of carbapenemases (Lee et al., 2003). Additionally the isolates were tested by combined disk tests with imipenem and meropenem alone and combined with 3-aminophenylboronic acid test (PBA), 0.1 M EDTA or both to screen for KPC, MBLs, or simultaneous production of KPC and MBL, respectively (Pasteran et al 2009).

2.3. Molecular detection of resistance genes

The genes conferring resistance to β-lactams including broad spectrum and extended-spectrum β-lactamases (*bla*$_{SHV}$, *bla*$_{TEM}$, *bla*$_{CTX-}$ *bla*$_{GES}$ and *bla*$_{PER-1}$) (Nuesch-Inderbinen et al., 1996; Arlet et al., 1995; Woodford et al., 2004; Pagani et al., 2004; Bonnin et al, 2011.,), class

A carbapenemases (*bla*$_{KPC}$,) (Robledo, et al., 2010) , class B (*bla*$_{VIM,}$ *bla*$_{IMP}$ and *bla*$_{NDM}$) (Poirel et al., 2011), and CHDL (*bla*$_{OXA-51-like}$, *bla*$_{OXA-23-like}$, *bla*$_{OXA-40-like}$, *bla*$_{OXA-58-like}$ and *bla*$_{OXA-143-like}$) (Woodford et al., 2006) were determined by PCR as previously described. Reference strains producing SHV-1, SHV-2, TEM-1, CTX-M-15, IMP-1, VIM-1, KPC-2, OXA-23, OXA-40, OXA-58 and OXA-143 were used as positive control strains. The PCR product were purified by using the QIA DNA minikit (Inel, Zagreb, Croatia) and the selected amplicons (strain 1- *bla*$_{OXA-40}$ and strain 41-*bla*$_{OXA-23}$) were sequenced by the Eurofins sequencing services (Eurofins Genomics GmbH, Ebersberg, Germany). The genetic context of *bla*$_{OXA-51}$ and *bl*a$_{OXA-23}$ genes was further investigated by PCR mapping with primers for IS*Aba1* combined with forward and reverse primers for *bla*$_{OXA-51}$ and *bl*a$_{OXA-23}$ (Turton et al., 2006).

Isolate 5 was randomly selected for further whole genome sequencing (WGS). The bacterial genomes were sequenced using the IonTorrent PGM platform (Life Technologies, Carlsbad, USA) according to the manufacturer's instructions. The Ion Xpress Plus Fragment Library Kit was used to enzymatically shear 100 ng of the genomic DNA. The target fragment size was 400 bp. Subsequently, the fragmented DNA was processed using the Ion DNA Barcoding kit (Life Technologies) and its size selected using the E-Gel SizeSelect 2% Agarose kit (Life Technologies). The size distribution of the DNA fragments was analysed using the High Sensitivity Kit (Agilent, Santa Clara, USA). Further sample processing was performed using the Ion OneTouch Kit (Life Technologies). Finally, the amplified DNA was sequenced using the 318 chip (Life Technologies). The single reads obtained were *de novo* assembled using MIRA 3.9.9, which is part of the Assembler plugin on the Ion Torrent server. Subsequently, the contigs were aligned to a reference genome of the corresponding species using Mauve and submitted to the RAST analysis platform (Gan et al., 2015). RAST is an automated annotation platform for bacterial genomes. The annotated genes were screened in RAST for antibiotic

resistance genes and their genetical context. Additionally, the contigs were analyzed using the ResFinder web-service (Zankari et al., 2012).

2.4. Characterization of plasmids

The transferability of meropenem resistance was determined by conjugation (broth mating method) employing *E. coli* J65 resistant to sodium azide (Elwell and Falkow, 1986). Plasmids were extracted with Qiagen Mini kit according to the manufacturer's instructions and subjected to transformation experiments using *A. baumannii* ATCC 19606 as recipient (Choi et al., 2006). PCR-based replicon typing (PBRT) was applied to type the resistance plasmids carrying carbapenemase genes according to Bertini for A. *baumannii* (Bertini et al., 2010).

2.5. Genotyping

Sequence groups of *A. baumannii* (SGs 1-3) corresponding to international clonal lineages (ICL I-III) were determined according to the procedure described by Turton et al. (Turton et al., 2007). PFGE genotyping of *Apa* I-digested genomic DNA was performed on eleven out of twelve isolates (1, 2, 4, 5, 11, 17, 41, 42, 43, 44 and 45) with a CHEF-DRIII system (Bio-Rad); the images were processed using Gel-Compar software. The dendrogram was computed after band intensity correlation using global alignment with 1.5 % optimization and tolerance and UPGMA (unweighted pair-group method using arithmetical averages) clustering. The strains were considered to be clonally related if they showed more than 80 % similarity of their PFGE patterns (Kaufman, 1998; Tenover et al., 1995).

Ten (1, 2, 4, 5 and 17, 41, 42, 43, 44, and 45) were subjected to molecular typing by rep-PCR as described previously (Overdest et al, 2011). DNA was isolated by 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 ng/L 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. All

data were entered in the DiversiLab software system. Cut-off value of 97% was used to define a clone (Healy et al., 2005).

Seven isolates (1, 2, 41, 42, 43, 44 and 45) were also genotyped by MLST according to protocols on Pasteur website.

3. Results

3.1. *Bacterial isolates*

The isolates were identified by conventional biochemical tests and MALDI-TOF, and confirmed as *A. baumannii* by the presence of the intrinsic *bla*$_{\text{OXA-51}}$ gene. The patients did not have a history of a travel abroad within three months before the sample collection.

3.2. Susceptibility tests and β-lactamase detection

All isolates except AB 11, and AB 45, were uniformly resistant to third generation cephalosporins, cefepime, piperacillin/tazobactam, imipenem, meropenem, gentamicin and ciprofloxacin and susceptible to colistin. All but one isolate were susceptible to tigecycline as shown in Table 1. Apart of the isolates 11, and 45, all were classified as extensively drug resistant according to Magiorakos et al. The addition of NaCl and cloxacillin did not lower the MICs of carbapenems indicating the absence of OXA-58 and lack of hyperproduction of chromosomal AmpC β-lactamase. All isolates tested negative in combined disk test with clavulanic acid indicating the lack of an ESBL. The production of class B carbapenemase was suspected based on positive inhibitor based test with EDTA.

3.3. Molecular detection of resistance genes

All isolates were positive for *bla*$_{\text{OXA-51-like}}$, nine for *bla*$_{\text{OXA-40-like}}$ genes and one for *bla*$_{\text{OXA-23-like}}$ gene. Reduced susceptibility to carbapenems in two isolates (11 and 45) was associated with IS*Aba1* upstream of *bla*$_{\text{OXA-51}}$. PCR was negative for ESBLs reported in *A. baumannii* so far. Sequencing of the selected PCR products revealed OXA-72 β-lactamase (strain 1 and OXA-23 β-lactamase (strain 41). MBLs were not found among the isolates. WGS of the

strain 5 revealed the presence of *bla*$_{\text{OXA-72}}$, chromosomal genes *bla*$_{\text{OXA-69}}$ and *bla*$_{\text{ADC}}$ in the isolate 5. Moreover, the genes *aac(3)*-Ia and *aadA1* encoding aminoglycoside resistance and *sul1* encoding sulphonamide resistance were identified. Furthermore, virulence genes such as htpB encoding heat shock protein, man B for phosphomanomutasel, pilF for type IV pilus assembly, IspE for general secretion pathway protein E, clpP for ATP-dependent Clp protease, tviB for polysaccharide biosynthesis protein, endopeptidase Cip ATP protein chain, lpxC for UDP-3-O-3-hydroxymyristail acetylglucosamin, gspE-general secretion pathway protein, fepA for ferrienterobactin outer membrane transportation system, capD for capsular polysaccharide synthesis enzyme and many other virulence factors were detected by WGS. The sequence is deposited in the Gene bank with the accession number PRJEB23482.

3.4. Characterization of plasmids

Repeated attempts failed to transfer imipenem resistance from each of eleven isolates to *E. coli* recipient strain by conjugation or *to A. baumannii* ATCC 19606 by transfomation. PBRT was negative for any of the plasmid inocompatibility groups reported in *A. baumannii* so far, except of the strain 41 with OXA23 which-belonged to Inc group 6 encoding aci6-replicase gene originally found on plasmid pACICU2.

3.5. Genotyping

Rep-PCR and PFGE identified two distinct clusters with OXA-40 positive isolates: one containing four isolates from 2011 (1, 2, 4 and 5) and the other comprising three isolates from 2015 (42, 43 and 44) whereas the isolate 17 from 2015 showed distinct rep-PCR profile (Figure 1a, Figure 2). A certain degree of diversification was observed among the isolates belonging to the same cluster. (Figure 2). Four isolates were singletons by PFGE (11, 17, 41 and 45). All isolates belonged to IC I (SG 2). Since *bla*$_{\text{OXA-72}}$ has been associated with isolates belonging to IC I we compared the rep-PCR profiles obtained in this study with OXA-72 positive isolates from an outbreak in a nursing home in Zagreb which also belonged to IC I and the isolates from 2011 matched with 97% similarity with those from Zagreb

(Figure 1b). Two isolates from 2011 (1 and 2) were found to belong to ST 642 whereas five from 2015 (41, 42, 43, 44 and 45) were allocated to ST636.

4. Discussion

Carbapenem-resistant *A. baumannii* are a source of deep concerns due to its multidrug resistance properties and ability to persist in the environment and cause outbreaks in the hospital setting. Three different carbapenem resistance mechanisms were involved: OXA-72 β-lactamase belonging to OXA-40- like group, OXA-23 β-lactamase and upregulation of OXA-51 β-lactamase by IS*Aba1*. The study performed in 2009 found that reduced susceptibility to carbapenems was associated only with upregulation of *bla*$_{\text{OXA-51}}$ gene by IS*Aba1* (Ibrahimagić et al., 2017). In this study a shift to CHDL was observed

Sequencing of *bla*$_{\text{OXA-40}}$ gene revealed the OXA-72 allelic variant associated with high level of resistance to carbapenems. This type of CHDL with similar properties was previously reported in two hospital centers in the neighbouring Croatia and also in the nursing home in Zagreb (Goić-Barišić et al., 2011; Franolić-Kukina et al., 2011; Bedenić et al., 2015). The OXA-72 β-lactamase with similar properties was previously reported in France (Barnaud et al., 2010), South Korea (Lee et al., 2009), Taiwan (Lu et al., 2009), China (Wang et al., 2007), Brazil (Werneck et al., 2010) and Serbia (Dortet et al., 2015). Similarily as in our study, they proved the chromosomal location of *bla*$_{\text{OXA-72}}$ gene.

OXA-23 was found in only one isolates. This type of CHDL is widespread and reported all over the world (Towner et al, 2007, Coelho et al, 2004, Zong et al, 2008). It was reported previously from two hospital centers in Zagreb (Vranić-Ladavac et al., 2014) in the multicenter study conducted in 2009-2010 and it was also previously found in a nursing home in Zagreb, Croatia (Bedenić et al., 2015) which is a neighbouring country to Bosnia. The isolate carrying *bla*$_{\text{OXA-23}}$ gene was found to be less resistant to carbapenems compared to highly resistant

isolates harbouring *bla*$_{\text{OXA-40}}$-like genes. The majority of studies proved plasmid location of OXA-23 gene, but in our study transformation experiments failed. However, the OXA-23 positive isolate was found to posses the plasmid belonging to Inc 6 group by PBRT. Unlike the isolates from Croatia which were associated with outbreaks (Vranić-Ladavac et al, 2013) the isolate from Bosnia was single.

PFGE and rep-PCR revealed two distinct clones harbouring OXA-40-like positive isolates one from 2011 and the other from 2015-2016, both containing highly similar isolates. High genetic similarity of the isolates could be due to the fact that the isolates were obtained from the same hospital over a short time period, which might indicate cross infection. However, the isolates originating from different time periods demonstrated diverse banding patterns generated by PFGE and rep-PCR, thus showing a dynamic changes in the population structure and pointing out to the introduction of a new clone.. The isolates from Bosnia positive for OXA-72 from 2011 clustered together with those from a nursing home in Zagreb collected in 2012, also positive for OXA-72, indicating the ability of isolates for crossing borders. The origin of this clone remains unclear, as it is the reason for its epidemic success. It is very likely that the spread occurred via patient transfer, but there is no direct epidemiologic proof of this view, and it remains possible that the isolates at different location have arisen by independent events. PFGE is still considered to be a gold standard in genotyping of *A. baumannii*. High correspondence between pattern differentiations produced by rep-PCR and PFGE was observed with PFGE being more discriminative. Two isolates 41 and 45 were classified as pair by rep-PCR and as singletons by PFGE which could be explained by better discriminatory power of PFGE. A link between the carbapenemase gene, PFGE and rep-PCR typing results was observed, suggesting that the both methods could effectively be applied for epidemiologic typing of *A. baumannii.* The main drawback of PFGE is the lack of portability of typing results. Interestingly, all imipenem-susceptible *A. baumannii* isolates belonged to IC I. Carbapenem-resistance was strongly associated with IC 1, in contrast to other studies in

which it was linked to IC 2 (Healy et al., 2005; Nemec et al, 2008). The ST 642 associated with two OXA-40 positive isolates from 2011, is rare and was previously reported in Mongolia (Kao et al., 2014). All isolates from 2015 belonged to ST 636, confirming the introduction of a new clone in 2015. This ST was previously reported in Colombia (Correa et al, 2017). Interestingly, all isolates from 2015 including those with OXA-40-like and OXA-23-like genes belonged to ST636 pointing out to the absence of link between population structure and resistance gene content. Different genotyping methods were applied because PFGE is more suitable for outbreak analysis whereas MLST and rep-PCR, as portable methods, enable international and interlaboratory comparison of the isolates. The dissemination of MDR-AB in various clinical wards within the hospital was observed including surgery, neurology, medical ICU and urology. The fact that also outpatients were also colonized points out to the dissemination of MDR-AB into the community setting.

All imipenem-resistant isolates were phenotypically suspected for MBLs, but PCR for common MBLs was negative. False positive results in inhibitor based tests can occur because in the presence of EDTA oxacillinase is converted to a less active state leading to the augmentation of the inhibition zone around the carbapenem disk (Vilalon et al., 2011). Many virulence factors found in WGS contribute to the severity of diseases associated with MDR-AB.The drawback of the study is relatively small number of isolate and the fact that outbreak isolates were compared with non-outbreak. Unfortunately, the isolates were not collected in the period between 2011 and 2015 to analyze the evolution of carbapenem-resistance and MDR-AB genotypes in the meantime.

Implementation of hospital hygiene measures, and the early and accurate detection, with restriction of antibiotic use should be recommended to control the spread of these important hospital pathogens. Moreover, surveillance programs to monitor antibiotic resistance in *A. baumannii* should be implemented. Bosnia and Herzegovina is a epidemiologically interesting

region with a great diversity and population mixing and representing a possible reservoir for multiresistant pathogens.

FIGURE LEGEND

Figure 1a. Rep-PCR dendrogram of ten representative isolates. Specimen, date of colection, and hospital unit are shown. Cut off value of 97% similarity was applied to define a clone. Existence of two clusters is visible.

Figure 1b. Comparison of Rep-PCR profiles of isolates from Mostar with those from Zagreb.

Figure 2. PFGE dendrogram of 11 *A. baumannii* isolates from two periods. Cut off value of 80% similarity was applied to define a clone. Specimen, hospital unit, and date of isolation are shown. Existence of two clusters is visible.

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