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Emergence of carbapenem hydrolyzing oxacillinases in *Acinetobacter baumannii* in children from Croatia

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Short Title: Carbapenem hydrolyzing oxacillinases in *Acinetobacter baumannii* in children

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Abstract

Introduction: Carbapenem resistance in *Acinetobacter baumannii* can be mediated by carbapenemases of class A, class B metallo-β-lactamases (MBLs) and class D carbapenem-hydrolyzing oxacillinases (CHDL). The aim of the study was to investigate the antimicrobial susceptibility and β-lactamase production of carbapenem-resistant *A.baumannii* isolates (CRAB) from Children's Hospital Zagreb, Croatia.

Methods: A total of 12 *A. baumannii* isolates collected between August 2016 and March 2018 were analyzed. Antibiotic susceptibility was determined by broth microdilution method. The presence of MBLs was explored by combined disk test with EDTA. The presence of carbapenemases of class A, B and D was explored by PCR. The occurrence of the IS*Aba1* upstream of the $bla_{\text{OXA-51-like}}$ or $bla_{\text{OXA-23-like}}$ was determined by PCR mapping. Epidemiological typing was performed by determination of sequence groups (SG). Genotyping was performed by sequence group determination, rep-PCR and MLST.

Results: All CRAB were resistant to piperacillin/tazobactam, ceftazidime, cefotaxime, ceftriaxone, cefepime, imipenem, meropenem, gentamicin and ciprofloxacin. Moderate resistance rates were observed for ampicillin/sulbactam (67%) and tigecycline (42%). The isolates were uniformly susceptible to colistin. PCR revealed the presence of genes encoding OXA-24-like CHDL in nine and OXA-23-like CHDL in three isolates. $bla_{\text{OXA-51}}$ genes were preceeded by IS*Aba1*. PCR for the common MBLs in Acinetobacter was negative. All isolates belonged to SG 1 corresponding to ICL II. Rep-PCR identified four major clones.

Conclusions: The study found OXA-24-like beta-lactamase to be the dominant CHDL among children's CRAB. The predominant spread of OXA-24-like is in contrast with recent global dissemination of OXA-23 reported all over the world. In contrast to the previous studies in

which emergency of OXA-24-like positive isolates was monoclonal we found considerable genetic diversity of the isolates.

Introduction

The emergence of carbapenem resistance in *Acinetobacter* spp is a significant public health concern leaving few therapeutic options remaining [1]. Carbapenem hydrolyzing oxacillinases belonging to molecular Ambler class D (CHDL) [2] have emerged globally as the main mechanism responsible for this resistance, although metallo-β-lactamases are prevalent in some area, particularly Far East [3-5]. Class A carbapenemases (KPC) are rare in *Acinetobacter* spp [6]. Enzymes belonging to OXA-51 group are naturally occuring β-lactamases of *A. baumannii* and are normally expressed at low levels but can be overexpresseed as a consequence of the IS*Aba1* located upstream of the $bla_{\mathrm{OXA-51}}$ genes [7]. However, *Acinetobacter* may develop resistance to carbapenems through various combined mechanisms including decreased permeability, altered penicillin binding proteins (PBPs) and, rarely, efflux pump overexpression [7]. Our previous studies demonstrated that carbapenem resistance was rare in Croatia until 2002. The first carbapenem-resistant isolates in Croatia were found in the University Hospital Split in 2002 [8], followed by carbapenem-resistant *Acinetobacter* isolates found in muliple centers in Croatia [9-16]. The dominant type of CHDL in Split and Zagreb University hospitals was OXA-72 which is widespread in Balcan geographic region [10-11] with sporadic occurrence of OXA-23 and OXA-58.

In spite of the high number of publications concerning carbapenem-resistant *A. baumannii* in Croatia [9-16], there are no bibliographical references on the carbapenemases in *A. baumannii* in paediatric population. The aim of the present study was to analyze the resistance patterns and mechanisms of paediatric carbapenem-resistant *A. baumannii* (CRAB) isolates.

Material and methods

Bacteria

Between August 2016 and March 2018, 28 (72%) out of 39 *A.baumannii* isolates were resistant to carbapenems. A total of 12 CRAB isolates (single isolate from single patient) were analyzed. The isolates were collected in intensive care unit, burn unit, surgery, paediatric and orthopedic ward of the Childen's hospital in Zagreb, Croatia from various clinical specimens. Ten isolates were obtained from infected patients and two from colonized patients. The isolates were identified by conventional biochemical testing and MALDI-TOF. The identification was confirmed by PCR for *bla*$_{OXA-51}$ gene. The Ethics committee of Children's hospital Zagreb approved the study protocol and decided that patient consent was not required.

Susceptibility testing

The antimicrobial susceptibility to a wide range of antibiotics was determined by broth microdilution in Mueller-Hinton broth and 96 well microtiter plates according to CLSI guidelines [17]. Minimum inhibitory concentrations (MICs) were determined for imipenem and meropenem by agar dilution in the presence of sodium chloride (200 mM) for detection of OXA-58 and cloxacillin (200 mg/L) for detection of AmpC hyperproduction [18]. Combined disk test with EDTA was used for detection of metallo-β-lactamases [19]. To assess the possible role of upregulated efflux in resistance, MIC of meropenem was determined in the presence of 12.5µM of an efflux pump inhibitor, carbonylcyanide-m-chlorophenylhydrazon CCCP [20].

The *A. baumannii* isolates were classified as multidrug-resistant (MDR), exstensively drug-resistant (XDR) or pandrug-resistant (PDR) according to Magiorakos [21].

Detection of resistance genes

PCR was used to detect the presence of the genes encoding KPC, MBLs of IMP, VIM and SIM series [22], *bla*$_{OXA}$ (*bla*$_{OXA-51}$, *bla*$_{OXA-23}$, *bla*$_{OXA-24/40}$, *bla*$_{OXA-58}$, *bla*$_{OXA-143}$) [23] and *bla*$_{ESBL}$ (*bla*$_{SHV}$, *bla*$_{TEM}$, *bla*$_{CTX-M}$, *bla*$_{GES}$ and *bla*$_{PER-1}$) genes as previously described [24-28]. The amplicons of the selected representative isolates (2, 3, 7 and 8) were column purified (QIAquick PCR purification kit, Inel Medicinska tehnika, Zagreb) and subjected to sequencing in the Eurofin sequencing service with the same primers used for PCR in order to determine the identity of the enzyme. Sequence alignment analysis was done online by utilizing the BLAST Program. The genetic context of *bla*$_{OXA-51}$ and *bl*a$_{OXA-23}$ genes was determined by PCR mapping with the primers for IS*Aba1* combined with forward and reverse primers for *bla*$_{OXA-51}$ [29].

Characterization of plasmids

Conjugation experiments were performed using *Esherichia coli* J 53 resistant to sodium azide [30]. Since conjugation experiments did not work out the plasmid DNA was extracted and transferred to $CaCl_2$ treated *Acinetobacter baumannii* ATCC 17978 by transformation. Transformants were selected on MacConkey agar containing 10µg/ml imipenem [30]. Plasmid incompatibility groups were determined by PCR-based replicon typing (PBRT) according to Bertini et al [31].

Molecular typing of isolates

Sequence groups (1-3) corresponding to ICL (International Clonal Lineage) I-III were determined by multiplex PCR as described previously [32]. Nine isolates (1, 2, 3, 4, 5, 6, 9, 10 and 11) were subjected to molecular typing by rep-PCR as described previously. All data were entered in the DiversiLab software system. Cut-off value of 97% was used to define a clone [33].

Four isolates (1 ,3, 6 and 9) were also genotyped by MLST according to protocols of the

Oxford scheme (*gltA, gyrB, gdhB, recA, cpn60, gpi* and *rpoD*). Details of the MLST sheme including amplification and sequencing are available at pubMLST web site (https://pubmlst.org/abaumannii/).

Results

Antimicrobial susceptibility testing and phenotypic detection of beta-lactamases

All isolates were resistant to piperacillin/tazobactam, ceftazidime, cefotaxime, ceftriaxone, cefepime, imipenem, meropenem, gentamicin and ciprofloxacin as shown in Table 1. Moderate resistance rates were observed for ampicillin/sulbactam (67%) and tigecycline (42%). The isolates were uniformly susceptible to colistin. They were all classified as XDR according to Magiorakos, as they were susceptible only to colistin and tigecyline (Table 1). Colistin was used to treat the infections caused by resistant *A. baumannii* with successful outcome in all patients.

The addition of either sodium chloride or cloxacillin did not lower the MICs of carbapenems indicating the lack of OXA-58 and no hyperproduction of chromosomal AmpC β-lactamase. CCCP did not decrease the carbapenem MICs either, excluding the effect of hyperexpression of efflux pumps on carbapenem MICs. The production of carbapenemase was identified by CIM test in all isolates. All isolates demonstrated enlargement of the inhibition zone around imipenem and meropenem disks supplemented with EDTA, ranging from 10 to 21 mm, indicating the production of an MBL.

Molecular detection of resistance genes

PCR revealed OXA-24-like CHDL in nine and OXA-23-like CHDL in three isolates (Table 1). Sequencing of the selected *bla*$_{\text{OXA-24}}$ amplicons (2, 3, 7 and 8) revealed OXA-72 allelic variant, *bla*$_{\text{OXA-51}}$ genes and *bla*$_{\text{OXA-23}}$ genes were preceeded by IS*Aba1*. PCR for the common MBLs and ESBLs found in *A. baumannii* was negative.

Characterization of plasmids

Attempts to transfer imipenem resistance by conjugation or transformation were unsuccessful indicating chromosomal location of *bla*$_{OXA-23}$ gene. The plasmids extracted from isolates positive for OXA-23 belonged to Inc group 2 encoding Aci2 replicasa gene.

Molecular typing of isolates

All isolates belonged to SG 1 corresponding to ICL II. Rep-PCR performed on nine isolates identified one large cluster with six isolates which contained subtypes with higly related isolates (Fig. 1). Three isolates were singletons. Two isolates (3 and 9) positive for OXA-24-like were found to belong to ST492 (Table 1) whereas two isolates (1 and 6) were assigned to ST1926, a new multilocus sequence type with the pattern (1/3/3/3/2/96/3).

Discussion

The study found OXA-24-like β-lactamase to be the dominant CHDL among children's *A. baumannii* isolates which is in concordance with the previous studies in Croatia [10-13]. OXA-24-like CHDL is widespread in Iberian Peninsula [34], USA [35], Czech Republic [36] and Bulgaria [37] and was recenlty reported in our neighbouring countires Bosnia and Herzegovina [38] and Serbia [39]. Representative isolates were positive for OXA-72 which is the only allelic variant of OXA-24-like, reported in Croatia so far.

OXA-23 was found in three isolates. This type of CHDL is widespread and reported all over the world [40-42]. It was reported previously from two hospital centers in Zagreb [12] in the multicenter study conducted in 2009-2010 and was previously also found in a nursing home in Zagreb, Croatia [13] and in environment [15]. The isolates carrying *bla*$_{OXA-23}$ gene were found to exhibit similar resistance patterns as those harbouring *bla*$_{OXA-24/40}$-like genes. The majority of studies proved plasmid location of OXA-23 gene, but in our study conjugation experiments failed. However, the OXA-23 positive isolate was found to posses the plasmid belonging to Inc 6 group by PBRT.

The difusion of OXA-24-like producing isolates among children is in contrast with recent global dissemination of OXA-23 reported all over the world. In contrast to the previous studies in which emergency of OXA-24-like positive isolates was monoclonal, in this study we found considerable genetic diversity of the isolates. False positive MBL testing in CHDL positive isolates can be due to the fact that oxacillinase can be converted to a less active state in the presence of EDTA leading to the augmentation of the inhibition zone [43]. The predominace of OXA-23 and OXA-24 could be atributable to better hydrolysis of carbapenem substrates compared to OXA-58 which provides selective advantage in antibiotic rich environment. Two isolates positive for OXA-24-like were found to belong to ST492 which was never reported in Croatia before. Previous studies in Croatia found the predominance of ST487, ST637 and ST195 [13, 44]. A new strain type is also reported in this study. The ST1926 is closly related to ST195 (only differing in recA). ST195 is been known to be present in different (east) asien countries (45).

None of the sulbactam/ampicillin-resistant isolates carried the $bla_{\mathrm{TEM-1}}$ gene, suggesting that in *A. baumannii* resistance to sulbactam may rely on additional mechanisms, besides TEM-1 overexpression although this was the most important mechanism as reported previously [46]..

The strength of the present study is the detailed molecular analysis of resistance mechanism while the few number of the strains and their relatedness are a weekness. This investigation will give rise to future multicenter studies to obtain insight on molecular epidemiology of paediatric *A.baumannii* isolates from different geographic regions in Croatia.

This is the first report of carbapenem resistant *A. baumannii* in children population in Croatia.

Disclosure Statement

The authors have no conflicts of interest to declare.

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Author contributions

Isolate collecting: ALG, MK

Laboratory work: ALG,MK,MŽ,JL,GZ,BB

Critical review: AG

Writting the manuscript: ALG, MK, BB

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Table 1. Antibiotic susceptibility, genotypes and beta-lactamase content of *A. baumannii* isolates from children

Fig.1. Molecular typing of isolates

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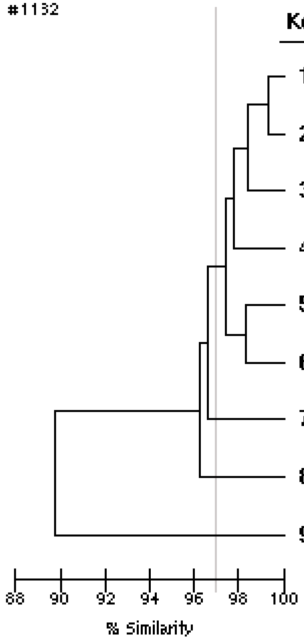
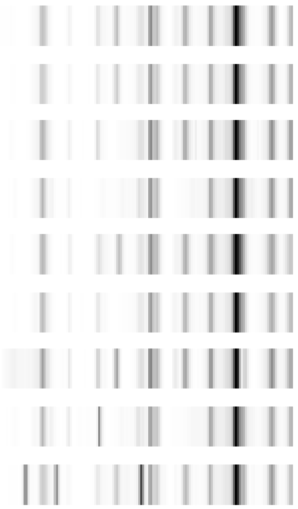


Key	Sample ID	Source	Class 1	Date Received
1	K10a	wound swab	OXA-24 like	2017-10-05
2	K3	bum swab	OXA-24 like	2017-09-14
3	K5	blood culture	OXA-24 like	2017-04-21
4	K11	rectal swab	OXA-23 like	2018-03-14
5	K9a	blood culture	OXA-24 like	2016-08-08
6	K2	wound swab	OXA-24 like	2017-11-22
7	K4	stomach fluid	OXA-24 like	2017-07-05
8	K1	bum swab	OXA-23 like	2017-11-22
9	K6	wound swab	OXA-24 like	2017-06-01



Ordinal numbrer	Date of isolation (month/year)	Specimen	Protocol number	Hospital ward	TZP	SAM	CAZ	CTX	CRO	FEP	IMI	MEM	GM	CIP	TIG	COL	SG/Rep-PCR cluster/ST	BL
1	11/2017	Burn swab	22742	ICU	>128	16	>128	>128	>128	32	64	64	>128	>128	2	1	SG 1/S ST492	Isaba 1/OXA-23
2	11/2017	Wound swab	22698	ortopedics	>128	32	>128	>128	>128	64	64	>128	>128	>128	1	1	SG1/A4 ST-1926	OXA-24
3	09/2017	Burn swab	17180	Burn unit	>128	16	>128	>128	>128	32	64	>128	>128	>128	2	1	SG1/A1 ST492	OXA-24
4.	07/2017	Stomach fluid	18255	paediatrics	>128	64	>128	>128	>128	64	64	>128	>128	>128	4	1	SG1/S	OXA-24
5.	04/2017	Blood culture	8100	ICU	>128	32	>128	>128	>128	128	32	>128	>128	>128	2	0.5	SG1/A2	OXA-24
6.	06/2017	Wound swab	12241	ICU	>128	16	>128	>128	>128	128	64	>128	>128	>128	2	1	SG1/S 1926	OXA-24
7.	08/2016	IV catheter tip	18285	ICU	>128	16	>128	>128	>128	128	64	>128	>128	>128	4	1	SG1	OXA-24
8.	12/2016	Burn swab	27097	ICU	>128	64	>128	>128	>128	>128	64	>128	>128	>128	2	0.5	SG1	OXA-24
9	08/2016	Blood culture	17476	ICU	>128	64	>128	>128	>128	32	64	>128	>128	>128	4	1	SG1/A4, 1926	OXA-24
10	10/2017	Wound swab	18440	surgery	>128	32	>128	>128	>128	64	64	>128	>128	>128	2	0.5	SG1/A1	OXA-24
11	03/2018	Rectum swab	5712	ICU	>128	64	>128	>128	>128	>128	64	>128	>128	>128	4	0.5	SG1/A3	ISAba 1/OXA-23
12	07/2017	Burn swab	14900	ICU	>128	64	>128	>128	>128	>128	64	>128	>128	>128	4	0.5	SG1	ISAba 1/OXA-23

Table 1. Antibiotic susceptibility, β-lactamase production and genotypes of *A. baumannii* isolates from children.

Abbreviations; TZP-piperacillin/tazobactam; SAM; sulbactam/ampicillin; CAZ-ceftazidime; CRO-ceftriaxone; FEP-cefepime;IMI-imipenem; MEM-meropenem; GM-gentamicin; CIP-ciprofloxacin; TIG-tigecycline; COL-colistin; ST-sequence type SG-sequence group; BL: beta-lactamase content: ICU-intensive care unit