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ARTICLE

Genetic diversity of OXA-51-like genes among multidrug-resistant *Acinetobacter baumannii* in Riyadh, Saudi Arabia

M. Aly · H. T. Tayeb · S. M. Al Johani · E. J. Alyamani · F. Aldughaishem · I. Alabdulkarim · H. H. Balkhy

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Abstract We explore the genetic diversity of class D oxacillinases, including OXA-23, -24 (-40), -58 and, particularly, the intrinsic OXA-51-like genes, among multidrug-resistant (MDR) *Acinetobacter baumannii* strains from inpatients at a tertiary care hospital in Riyadh, Saudi Arabia. Sequence-based typing (SBT) of the OXA-51-like gene was carried out on 253 isolates. Selected isolates (n=66) were subjected to multilocus sequence typing (MLST). The polymerase chain reaction (PCR) typing results showed that all isolates (n=253) contained the OXA-51-like and OXA-23 genes. However, the OXA-58 gene was detected in five isolates. Further, none of the isolates had the OXA-40 (identical to the OXA-24) gene. SBT revealed a high OXA-51-like genotypic diversity and showed that all isolates were clustered into four main groups: OXA-66 (62.3 %), followed by OXA-69 (19.1 %), OXA-132 (7.6 %) and other OXA-51-like genes (10.3 %), including OXA-79, -82, -92, -131 and -197. MLST revealed four main sequence types (STs), 2, 19, 20 and 25, among the isolates, in addition to six isolates with newly designated ST194–ST197 singletons. Further, a high prevalence (81.4 %) of OXA-66 and OXA-69-like genes in *A. baumannii* was identified. More studies are essential in order to explore the molecular mechanisms that confer carbapenem-resistant phenotypes for *A. baumannii* isolates and to investigate the genetic diversity of other OXA-D genes.

M. Aly · I. Alabdulkarim · H. H. Balkhy
King Abdullah International Medical Research Centre, Riyadh, Saudi Arabia

H. T. Tayeb
King Faisal Specialist Hospital & Research Centre, Riyadh, Saudi Arabia

S. M. Al Johani · H. H. Balkhy (✉)
King Saud bin Abdulaziz University for Health Sciences, WHO CC and GCC Center for Infection Control, P.O. Box 22490, Riyadh 11426, Saudi Arabia
e-mail: balkhyh@hotmail.com

S. M. Al Johani · F. Aldughaishem
Department of Pathology, King Abdulaziz Medical City, Riyadh, Saudi Arabia

E. J. Alyamani
King Abdulaziz City for Science and Technology, Riyadh, Saudi Arabia

H. H. Balkhy
Department of Infection Prevention and Control, King Abdulaziz Medical City, Riyadh, Saudi Arabia

Introduction

Healthcare-associated infections (HAIs) due to *Acinetobacter baumannii* are an increasing problem globally, including countries of the Gulf Cooperation Council (GCC) [1, 2]. *A. baumannii* is widespread in hospitals and the healthcare environment, and plays an important role as a causative agent for HAIs [3]. OXA-51-like genes are genetically diverse and have been identified worldwide, including Europe, North and South Americas, and the Far East [4]. Recent publications from neighbouring countries have identified the prevalence of *A. baumannii* in HAIs and have further identified the presence of OXA-51 and OXA-23 among their isolates [5, 6]. However, the prevalence and genetic diversity of OXA-51-like oxacillinases in the Middle East in general and Saudi Arabia in particular have not been explicitly described [7]. The purpose of the current study was to identify the genetic diversity of OXA-51-like genes using sequence-based typing (SBT)

and multilocus sequence typing (MLST) among multidrug-resistant (MDR) *A. baumannii* in Saudi Arabia [8].

Methods

Bacterial identification and susceptibility testing

The clinical isolates used in this study were collected at the microbiology laboratory of King Abdulaziz Medical City (KAMC) hospital in Riyadh between 2006 and 2008. Isolates that exhibited aerobic growth, and were catalase-positive and oxidase-negative, were further identified as *Acinetobacter* by commercial biochemical system MicroScan® WalkAway (Siemens Healthcare). Isolates were initially identified at the species level using MicroScan and were then confirmed by the RNA polymerase β-subunit (rpoB) gene polymerase chain reaction (PCR) and sequencing [2]. Isolates were tested for their minimum inhibitory concentrations (MICs) using the Clinical and Laboratory Standards Institute (CLSI) guidelines [9]. All results that exhibited resistance were confirmed by the Etest® (bioMérieux) to detect the exact MIC value. Only one isolate per patient per year was included in the current analysis. Quality control was performed by testing these same antimicrobials against *Escherichia coli* ATCC 25922, *E. coli* ATCC 35218 and *Pseudomonas aeruginosa* ATCC 27853. The isolates were defined as MDR if they were found to be resistant to three or more classes of antibiotics, including: β-lactam/β-lactamase inhibitors, combinations of extended-spectrum cephalosporins, aminoglycosides, fluoroquinolones and carbapenems [10].

DNA extraction and detection of resistance genes

Genomic DNA was extracted from 100 μL of fresh tryptic soy broth subcultured samples using the DNA extraction kit MagNA Pure Compact (Roche), according to the manufacturer's instructions. Isolate species were confirmed to be *A. baumannii* using rpoB PCR primers Ac696F (5′-TAYC GYAAAGAYTTGAAAGAAG-3′) and Ac1093R (5′-CMACACCYTTGTTMCCRTGA-3′) [2]. Samples were stored at −20 °C until further analysis. Eleven pairs of primers were used for *A. baumannii* resistance genotyping (Eurofins, Germany), including OXA A, B, C and 58, as previously explained by Hujer et al. [11]. Some modifications to the annealing temperature, number of cycles etc. were made to optimise the amplification for certain genes. The PCR products were analysed by electrophoresis on a 1.5 % (wt/vol) agarose gel containing 0.125 μg/mL ethidium bromide.

SBT and sequencing

The PCR amplicons were purified by using the QIAquick PCR extraction kit (Qiagen). Both strands of the amplicons were sequenced by the same gene amplification primers using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems™, Austin, TX, USA). Similarity searches were done using the BLAST program of PubMed National Centre for Biotechnology Information.

MLST and lineage tree

We performed MLST of 66 randomly selected isolates representing all four clusters from the SBT phylogenies. MLST was carried out using seven housekeeping genes as described by Diancourt et al. [12] and the PCR conditions were as previously described elsewhere [12, 13]. Data were collected and analysed by Excel, SPSS and START2 software. A lineage tree was generated using the unweighted pair group method with arithmetic mean.

Results

MDR phenotypes and OXA genotyping

A total of 253 isolates were collected from the clinical microbiology laboratory between 2006 and 2008. The sources of the clinical isolates were as follows: respiratory 117/253 (46.24 %), wound and burn 61/253 (24.11 %), blood 34/253 (13.43 %), urine 8/253 (3.16 %) and others 33/253 (13.04 %). Susceptibility testing showed that all isolates were resistant to four or more antimicrobial groups. Antibiotic susceptibility and MIC values are presented in Table 1. Carbapenem resistance, where the MIC was≥8 μg/ml for meropenem and imipenem, was found in 96.44 % (244/253) and 88.53 % (224/253) of the isolates, respectively. OXA-D PCR typing showed that all 253 isolates carried OXA-51-like and OXA-23 genes. The OXA-58 gene was detected in only five isolates, while none of the isolates carried the OXA-40 (identical to the OXA-24) gene.

SBT

The SBT OXA data showed *A. baumannii* isolates carrying a wide range of OXA-51-like genes, including OXA-66, -69, -79, -82, -98, -94, -95, -131, -132 and -197. These were clustered into four main groups, including OXA-66 (62.3 %), OXA-69 (19.1 %), OXA-132 (7.6 %) and OXA-51-like genes (11 %), such as OXA-79, -82, -98, -94, -95, -131 and -197. Moreover, OXA-66 gene sequencing showed the partial insertion sequence of IS*Aba1* in the upstream position,

Table 1 Antibiotic susceptibility of MDR *A. baumannii* isolates represented as the minimum inhibitory concentration (MIC) μg/ml, then the number of isolates (*n*), followed by the percentage

Antibiotic	Resistant			Intermediate			Susceptible		
	MIC[a]	*n*	%	MIC[a]	*n*	%	MIC[a]	*n*	%
Amikacin	>32	147	58.1	32	33	13.0	≤16	73	28.9
Cefepime	>16	250	98.8	16	0	0.0	≤8	3	1.2
Cefotaxime	>32	251	99.2	16	1	0.4	8	1	0.4
Ceftazidime	>16	251	99.2	16	0	0.0	≤8	2	0.8
Ceftriaxone	>32	250	98.8	32	0	0.0	≤8	3	1.2
Ciprofloxacin	>2	252	99.6	2	1	0.4	≤1	0	0.0
Colistin	>4	3	1.19	4	0	0.0	≤2	250	98.8
Gentamicin	>8	244	96.4	8	1	0.4	≤4	8	3.2
Imipenem	>8	200	79.1	8	30	11.9	≤4	23	9.1
Meropenem	>8	233	92.1	8	16	6.3	≤4	4	1.6
Tobramycin	>8	105	41.5	8	14	5.5	≤4	134	53.0
Trimethoprim/sulphamethoxazole	>2/38	249	98.4	2	0	0.0	≤1	4	1.6

[a] MIC: interpretive CLSI 2011 standards

while the insertion sequence IS*Aba3*-like sequence transposase (tnpA) was identified with the carbapenem-hydrolysing oxacillinase OXA-58 (blaOXA-58)

STs of *A. baumannii* isolates and identification of regional clones

The sequence types (STs) of 66 isolates typed by MLST were grouped into four clusters. The most common ST was ST2 (*n*=26), which is part of clonal complex 2 (CC2) and represents the European clone 2 (EU2) or worldwide lineage 2 (WW2). The next most common type was ST20 (CC1) (*n*= 15), followed by other STs (ST19, ST49, ST141, ST154). Eleven isolates were not designated with STs due to missing one or more MLST allele. Nevertheless, they were identified with mixed OXA-51-like genotypes, such as OXA-66, -131 and -197. MLST revealed four new isolates (194–197) with novel allelic profiles (Table 2). The MLST allelic profiles of

Table 2 Multilocus sequence typing (MLST) alleles and OXA-D genotyping among MDR *A. baumannii* isolates from Riyadh

Number of isolates	ST	Clonal complex	MLST allele scheme							OXA-D genotype
			cpn60	fusA	gltA	pyrG	recA	rplB	rpoB	
1	1	1	1	1	1	1	5	1	1	69
26	2	2	2	2	2	2	2	2	2	66
1	19	2	1	2	1	1	5	1	1	58
16	20	1	3	1	1	1	5	1	1	69
2	25	2	3	3	2	4	7	2	4	na
1	49	1	3	3	6	2	3	1	5	98
1	141	1	26	2	2	1	43	4	5	197
1	154	1	3	41	6	1	3	4	5	197
2	**194**	**Singleton**	**3**	**1**	**15**	**1**	**5**	**1**	**1**	**58**
1	**195**	**Singleton**	**2**	**2**	**2**	**2**	**2**	**1**	**2**	**197**
1	**196**	**Singleton**	**2**	**2**	**13**	**2**	**2**	**1**	**2**	**58**
2	**197**	**Singleton**	**3**	**3**	**3**	**4**	**39**	**4**	**4**	**132**
11	UNK	–	Missing alleles							*

Bold type = new sequence types, UNK = unknown due to being untypeable

*=mixed genotypes

the four novel ST isolates were entered into the Institut Pasteur MLST database (http://www.pasteur.fr/mlst) on September 2012 with the following Pasteur website IDs: 602, 612, 617, 620, 633 and 640.

Association of OXA-51 diversity and MLST typing

A lineage tree was generated from the MLST allelic profile and SBT OXA using the unweighted pair group method with arithmetic mean (Fig. 1). MDR *A. baumannii* isolates were grouped into four clusters, where the first cluster consisted of isolates that carried the OXA-66 gene and belonged to ST2 (CC2). On the contrary, isolates typed as ST20 (CC1) and possessed mostly the OXA-69 gene were the main contributors to the second cluster. However, the third and fourth clusters were more intermixed, where the OXA-51-like genes and MLST included: 65 (ST197), 69 (ST1), 98 (ST49), 132 (ST25) and 197 (ST154, ST196).

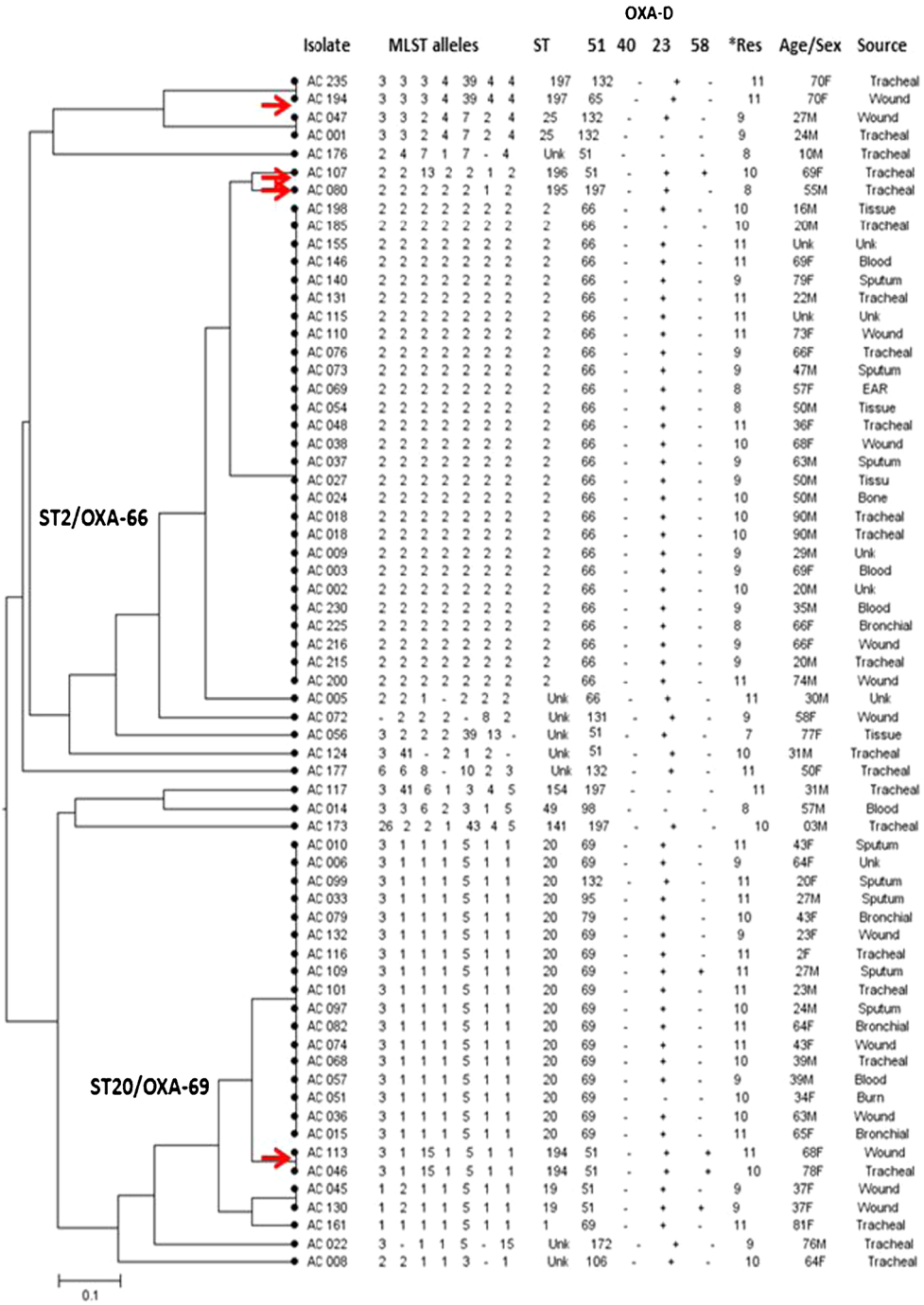

Fig. 1 Lineage tree generated from the multilocus sequence typing (MLST) allelic profile showing the MLST alleles, sequence type, corresponding OXA-D genotypes, number of resistant antibiotics and patient demographic data (age, gender, isolate source) of MDR *A. baumannii* isolates form Saudi Arabia. The new sequence type isolates are shown by the *arrows*

Discussion

We identified OXA-66 as the major OXA-51-like gene, which is consistent with several other reports from Europe, the United States, South Americas, Turkey and China [14–16]. The sequences showed that our isolates carried the OXA-66 gene, along with the upstream partial insertion sequence of IS*Aba1*, which was previously described as an OXA-66 promotor associated with carbapenem resistance among *A. baumannii* isolates [15, 17]. Another gene identified, OXA-69, known to confer carbapenem resistance, was found to a lesser extent among our clinical isolates, 19.1 % [18]. Both OXA-66 and OXA-69 were associated to a particular epidemic lineage of *A. baumannii*, which is consistent with the findings of other research groups [18, 19]. The OXA-132 gene was present in 7.6 % of our isolates, a gene which has been previously reported among clinical isolates from Saudi Arabia and Portugal [7, 20]. Five of our isolates showed that OXA-58 coexisted with the partial insertion sequence IS*Aba3* and *tnpA* gene, which may contribute to the resistance phenotype by integrating the insertion sequences into host genomes to confer drug resistance [21–24]. Three OXA-58 isolates contained new MLST alleles which could not be categorised into the major worldwide clonal lineage; hence, they are noted as new singletons (two ST194 and one ST196). However, this is the first report of the molecular typing of OXA-23 and OXA-58 in our region.

Conclusion

Our findings noticeably emphasise that the rate of carbapenem resistance is astounding, where 81 % of our multidrug-resistant (MDR) *Acinetobacter baumannii* clinical isolates carry OXA-66 and OXA-69 genes that belong to the worldwide lineage 2 (WW2, clonal complex CC2) and WW1 (CC1), respectively. Moreover, the identification of the novel sequence types ST194–ST197 isolates represents a local genetic diversity that needs further investigation, as well as the molecular epidemiology of carbapenem-resistant *A. baumannii*, which has become an endemic pathogen within the Middle Eastern countries.

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