

SHORT REPORT

Low genetic diversity of the intrinsic OXA-51-like class D carbapenemases among *Acinetobacter baumannii* clinical isolates in Greece

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SUMMARY

This study examined the geographical distribution and diversity of the intrinsic OXA-51-like class D carbapenemases among *Acinetobacter baumannii* clones recovered in three major Greek regions from 2000 to 2005. The *bla*_{OXA-66} allele was exclusively detected among clonally distinct *A. baumannii* isolates recovered in the regions of Thessaloniki and Larissa. This sequence was also the most widespread among *A. baumannii* isolates in Athens, while less frequent were *bla*_{OXA-69} and *bla*_{OXA-65} alleles. These findings highlight the high prevalence of a specific *bla*_{OXA-51-like} allele in Greece, possibly indicating that our *A. baumannii* clones might have originated from a common ancestor. However, the possibility that *bla*_{OXA-51-like} variants, with *bla*_{OXA-66} predominating, are widely disseminated among several unrelated *A. baumannii* strains cannot be excluded.

Acinetobacter baumannii today plays a significant role worldwide as a cause of outbreaks of infections among hospitalized patients, especially those in critical-care environments. The carbapenems, imipenem and meropenem, have been widely used to treat infections caused by multidrug-resistant *A. baumannii* clinical isolates but carbapenem resistance among these strains has increased in recent years [1]. Resistance is mediated through various combined mechanisms that include target inaccessibility or drug inactivation by β -lactamases with carbapenemase properties such as class B metallo- β -lactamases (MBLs) and several class D OXA-type enzymes [2]. The metallo-enzymes are rarely detected, while the OXA-type carbapenemases are widespread among *A. baumannii* from several geographic regions [3–5].

The latter enzymes initially formed two subgroups, namely OXA-23 and OXA-24 [3], but recently two novel subgroups of class D oxacillinases with carbapenemase properties, formed by OXA-51 variants and OXA-58, have been additionally described in *A. baumannii* [6, 7].

The OXA-51 subgroup of enzymes exhibits relatively weak hydrolytic activities, compared with other carbapenemases [8], but has been associated with carbapenem resistance in isolates with the insertion sequence *ISAbal* upstream of the oxacillinase gene [9]. *bla*_{OXA-51-like} alleles are chromosomally located and it has been confirmed that these sequences are intrinsic in most or all *A. baumannii* strains [4, 10, 11]. To date in the same cluster of enzymes, a large number of closely related variants have been described that include OXA-64 to -71, OXA-75 to -80, OXA-82 to -84, OXA-86 to -89, OXA-91, OXA-92, OXA-94 and OXA-95 (www.lahey.org/studies/other.asp#table1 [9–14]). *A. baumannii* is a predominant

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pathogen in Greece, particularly among immunocompromised patients and the OXA-51 subgroup has been commonly detected among selected clinical isolates with various susceptibility profiles to carbapenems [5, 15]. The purpose of the present study was to investigate the geographical distribution and the diversity of the *bla*_{OXA-51-like} alleles among *A. baumannii* clones in Greece.

In total, 150 isolates of *A. baumannii* were included randomly, all of which were collected between 2000 and 2005 from clinical samples of patients hospitalized in five hospitals in three major Greek regions (Athens, Thessaloniki and Larissa). Several of these isolates were retrieved from collections stored during previous studies [5, 15, 16]. The isolates were identified to species level by the Vitek 2 automated system (bioMérieux, Marcy l'Etoile, France), the API 20NE system (bioMérieux) and conventional biochemical tests.

The minimum inhibitory concentrations (MICs) of imipenem and meropenem were determined using an agar dilution method [17] with agar plates containing serially diluted antibiotics ranging from 0.25 to 512 mg/l respectively for each agent; the inoculum was 10⁴ c.f.u. per spot. The isolates were also tested by E-test MBL (AB Biodisk, Solna, Sweden) as well as the imipenem-EDTA double-disc synergy test (DDST) for MBL production [18]. *Pseudomonas aeruginosa* ATCC 27853 was used as control in susceptibility testing and a VIM-type carbapenemase-producing *A. baumannii* strain [5] was used as control for E-test MBL.

PFGE of *ApaI*-digested genomic DNA of *A. baumannii* isolates was performed with a CHEF-DRIII system (Bio-Rad, Hemel Hempstead, UK) according to previously described methods [19]. The interpreting criteria were described by Tenover *et al.* [20]. *ApaI* macrorestriction patterns were digitized and analysed using the Quantity One Software (Bio-Rad Laboratories Inc., Hercules, CA, USA) to calculate Dice coefficients of correlation and to generate a dendrogram by the unweighted pair-group method using arithmetic averages (UPGMA) clustering.

The isolates were screened by PCR for the *bla*_{OXA-51-like} genes using primers OXA-69A and OXA-69B that amplify a 975-bp product including the whole coding sequence of the gene [8], in order to discriminate the alleles by sequence analysis. Isolates that did not give a PCR product with these external primers were screened with the partially degenerate primers published previously [15] that were designed

to amplify the 825-bp ORF of all *bla*_{OXA-51-like} alleles. Amplicons were purified using ExoSAP-IT reagent (USB Corporation, Cleveland, OH, USA) and both strands were sequenced using the standard dideoxynucleotide method in an ABI Prism 377 DNA sequencer (PerkinElmer, Applied Biosystems Division, Foster City, CA, USA).

The UPGMA dendrogram of *ApaI*-digested genomic DNA grouped as many as 112 of the isolates into two major clusters; four additional clusters were detected each containing from two to 19 isolates, while the remaining seven isolates exhibited unique PFGE types (Fig.). The characteristics of the *A. baumannii* isolates of the study are presented in the Table. The imipenem and meropenem MICs ranged from 0.5 to 512 mg/l and from 0.25 to 256 mg/l, respectively. Both E-test MBL and DDST were negative in all isolates. PCR screening for *bla*_{OXA-51-like} gene with primers OXA-69A and OXA-69B was positive in all but seven of the 150 acinetobacters. The seven isolates that did not give a PCR product with these external primers, gave a positive signal with the degenerate primers. Sequencing analysis of the 150 *bla*_{OXA-51-like} amplicons revealed alleles that were identical to *bla*_{OXA-66} in all isolates from the regions of Thessaloniki (*n*=47) and Larissa (*n*=63), while *bla*_{OXA-66} (*n*=20) along with *bla*_{OXA-69} (*n*=18) and *bla*_{OXA-65} (*n*=2) alleles were detected in isolates from the Athens region. In total, *bla*_{OXA-66} was detected in 130 isolates that grouped into ten genotypes, *bla*_{OXA-69} in 18 isolates that grouped into three genotypes and *bla*_{OXA-65} in two isolates, each one belonging to an individual genotype.

Carbapenem-hydrolysing β -lactamases that belong to class D have been widely described over the last years [13]. The subgroup of class D carbapenemases formed by OXA-51 variants was first detected in distinct clones of *A. baumannii* from Argentina [6]; it shares very weak identities with other known oxacillinases and thus comprises a novel phylum among the OXA-type carbapenemases [3]. It is of interest that the OXA-51 cluster, in contrast with other class D carbapenemases, does not possess the tyrosine-to-phenylalanine substitution in the conserved Y-G-N motif of the class D protein [12]. So far, more than 20 class D β -lactamases, all of which belonged to the same OXA-51 subgroup, have been identified among *A. baumannii* clinical isolates from centres in Argentina, South Africa, Hong Kong, Spain, Singapore and Turkey [9–14]. The number of alleles in this subgroup seems to have extended constantly

Table. Genotypes, region of isolation, range of imipenem and meropenem minimum inhibitory concentrations (MICs) and *bla*_{OXA-51-like} alleles among the 150 *A. baumannii* isolates

PFGE type	No. of isolates	Region of isolation*			MIC		<i>bla</i> _{OXA-51-like} alleles detected
		A	T	L	Imipenem (mg/l)	Meropenem (mg/l)	
I	72	+	+	+	2–512	2–256	<i>bla</i> _{OXA-66} (<i>n</i> = 69), <i>bla</i> _{OXA-69} (<i>n</i> = 3)
II	40	+	+	+	1–64	0.5–256	<i>bla</i> _{OXA-66}
III	19	+			1–16	1–16	<i>bla</i> _{OXA-69} (<i>n</i> = 13), <i>bla</i> _{OXA-66} (<i>n</i> = 6)
IV	8			+	8–128	2–128	<i>bla</i> _{OXA-66}
V	2			+	16	4–8	<i>bla</i> _{OXA-66}
VI	2	+			64–256	8–16	<i>bla</i> _{OXA-69}
VII	1			+	16	16	<i>bla</i> _{OXA-66}
VIII	1			+	32	16	<i>bla</i> _{OXA-66}
IX	1			+	4	8	<i>bla</i> _{OXA-66}
X	1		+		1	1	<i>bla</i> _{OXA-66}
XI	1			+	16	8	<i>bla</i> _{OXA-66}
XII	1	+			0.5	0.25	<i>bla</i> _{OXA-65}
XIII	1	+			512	256	<i>bla</i> _{OXA-65}

* Region of isolation: A, Athens (*n* = 40); T, Thessaloniki (*n* = 47); L, Larissa (*n* = 63). The + sign refers to the region of isolation of each genotype.

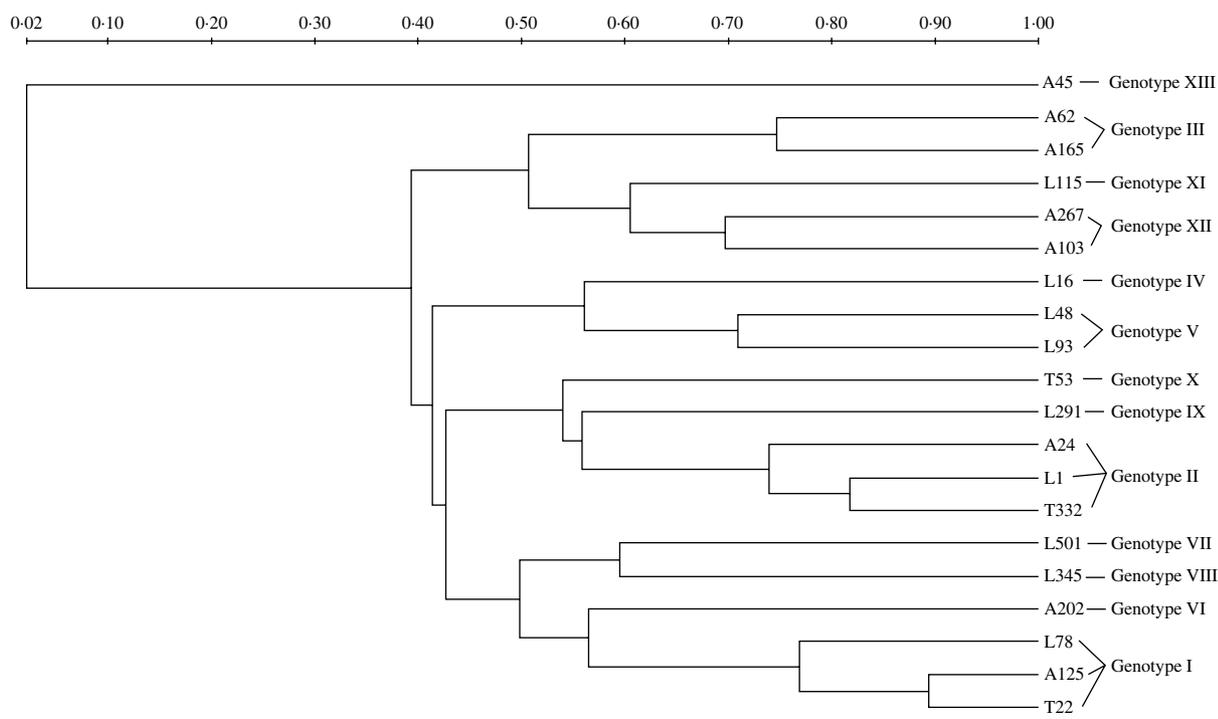


Fig. UPGMA dendrogram of *Apa*I-digested genomic DNA of representative isolates corresponding to all 13 genotypes included in the study. Clusters of possibly related isolates were defined at 70% similarity. Tags of each branch stand for the origin (A, Athens; L, Larissa; T, Thessaloniki) and the numbering of each isolate.

through silent or amino-acid shift mutations. The members of the cluster diverge by 1–15 amino-acid modifications and these intrinsic enzymes seem

to represent the largest and most diverse collection of class D carbapenemases in *A. baumannii* [8, 13, 21].

In the present study three *bla*_{OXA-51} variants were detected in different parts of the country. Interestingly, the *bla*_{OXA-66} allele was exclusive for acinetobacters that exhibited various levels of susceptibility to carbapenems and were recovered in two regions. It was also the prevalent *bla*_{OXA-51-like} allele among *A. baumannii* in the third region of the study. The *bla*_{OXA-66} sequence was integrated in almost all isolates of the two major PFGE-defined clones, while the same sequence was additionally detected in eight of the remaining 12 clonal types; less frequently detected were other *bla*_{OXA-51-like} alleles such *bla*_{OXA-69} and *bla*_{OXA-65}. It is of interest that the three *bla*_{OXA-51-like} alleles detected here are the most closely related at the genetic level within the OXA-51 subgroup [12, 13]. Initially, the *bla*_{OXA-66} allele was identified in individual isolates from Spain, Hong Kong and Singapore, while the *bla*_{OXA-69} allele was found in two isolates from Singapore and one from Turkey and the *bla*_{OXA-65} allele was found in clonal isolates from Argentina [12]. In a previous study conducted in Turkey, a higher diversity of *bla*_{OXA-51-like} alleles occurred among *A. baumannii* isolated in distinct hospitals [14]; the original *bla*_{OXA-51} sequence was the most frequently detected while *bla*_{OXA-66} was present only among imipenem-susceptible *A. baumannii* clones. In a French collection of *A. baumannii* strains, the latter sequence was identified along with six additional variants among imipenem-susceptible and -resistant isolates [8], and recently in Argentina seven *bla*_{OXA-51-like} alleles were found among representative *A. baumannii* isolates [10]. Our study brings more evidence to support the hypothesis that the *bla*_{OXA-51-like} gene is intrinsic to *A. baumannii*. It also highlights the high prevalence and spread of a specific allele, *bla*_{OXA-66}, which possibly indicates that our *A. baumannii* clones might have originated from a common ancestor and have undergone, as yet, limited dissemination. It might be expected that after a lengthy time period a common ancestor isolate could have evolved to different macrorestriction patterns following the accumulation of spontaneous mutations. However, the possibility that these few *bla*_{OXA-51-like} variants, with *bla*_{OXA-66} being predominant, are widely disseminated in several unrelated *A. baumannii* strains in Greece cannot be excluded and warrants further investigation.

DECLARATION OF INTEREST

None.

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