

SHORT REPORT

Molecular analysis of imipenem-resistant *Acinetobacter baumannii* isolated from US service members wounded in Iraq, 2003–2008

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SUMMARY

Global dissemination of imipenem-resistant (IR) clones of *Acinetobacter baumannii*–*A. calcoaceticus* complex (ABC) have been frequently reported but the molecular epidemiological features of IR-ABC in military treatment facilities (MTFs) have not been described. We characterized 46 IR-ABC strains from a dataset of 298 ABC isolates collected from US service members hospitalized in different US MTFs domestically and overseas during 2003–2008. All IR strains carried the *bla*_{OXA-51} gene and 40 also carried *bla*_{OXA-23} on plasmids and/or chromosome; one carried *bla*_{OXA-58} and four contained *ISAbal* located upstream of *bla*_{OXA-51}. Strains tended to cluster by pulsed-field gel electrophoresis profiles in time and location. Strains from two major clusters were identified as international clone I by multilocus sequence typing.

Key words: *A. baumannii*, imipenem resistance, OXA-23.

The *Acinetobacter baumannii*–*A. calcoaceticus* complex (ABC) has emerged as a significant opportunistic pathogen in hospitals frequently associated with nosocomial outbreaks worldwide [1]. Colonization and infection with these organisms is increasingly common in trauma patients and patient care environments resulting from natural disasters or man-made conflict [2, 3]. Since the start of Operation Iraqi Freedom (OIF), US military medical treatment facilities (MTFs) have noted a marked increase in ABC infections in US service members injured on the battlefield. In particular, *Acinetobacter* isolates from

deployed patients have been shown to have a higher frequency of antibiotic resistance than those from non-deployed patients [4].

Multidrug-resistant *A. baumannii* are often resistant to almost all clinically used antibiotics and as a result carbapenems have become the most frequent treatment option for combating these infections. However, there are increasing reports of carbapenem resistance in *Acinetobacter* strains worldwide [1]. Carbapenem-hydrolysing class D β -lactamases (CHDLs) are the most often reported mechanisms of carbapenem resistance in *Acinetobacter* spp. [5] and four groups of CHDLs have been identified in imipenem-resistant (IR) *A. baumannii*, including intrinsic and chromosomally located OXA-51-like β -lactamases and acquired OXA-23-like, OXA-24/

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OXA-40-like and OXA-58-like β -lactamases [5]. IR-ABC bearing *bla*_{OXA-23} are the most common worldwide and this element is located in the AbaR4 island in the chromosome [6] although *bla*_{OXA-23} genes have also been found on plasmids [5]. Clonal spread and global dissemination of IR-ABC has been well documented in recent years [1] with correlation between strain genotype and antimicrobial phenotype, especially within the same region or MTF [7].

Genotyping by pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST) are frequently used for epidemiological investigations and surveillance of ABC outbreaks [7–9]. PFGE profiles of isolates from different facilities can be compared by the use of standardized methodology but MLST has been shown to give results consistent with other techniques originally used to define the three major widespread international clones I, II and III [9]. In the current study, we focused on characterization of IR-ABC strains from a wider collection of ABC isolates from US service members engaged in OIF and hospitalized in different US MTFs domestically and overseas during 2003–2008. We sought correlations between strain genotype and imipenem-resistance genotypic mechanism, and the epidemiological significance of imipenem resistance in this setting.

Of the total 298 ABC isolates, those collected during 2003–2004 were from US patients hospitalized at Landstuhl Regional Medical Center (LRMC) in Germany, Walter Reed Army Medical Center (WRAMC) in the USA, and the USNS Comfort in the Persian Gulf during *Acinetobacter* wound infection outbreaks in these facilities [2]. Isolates collected in 2006 were from the National Navy Medical Center (NNMC) [7] and those in 2008 were recovered directly from two US combat support hospitals (CSHs) in Iraq. We included ABC isolates previously published to make this retrospective study on imipenem resistance more complete. The sequenced *A. baumannii* US AB0057 strain from WRAMC and ACICU strain from Italy (kindly provided by Dr A. Carattoli) were used as the reference strains.

The species identity and antimicrobial susceptibility of the 298 isolates were confirmed by the the Phoenix NMIC/ID133 panel (Becton Dickinson, USA) and classification of susceptibility was according to Clinical and Laboratory Standards Institute guidelines [10]. PFGE analysis was conducted for all ABC isolates using *ApaI* endonuclease digestion as previously described [7, 8] and fragments were separated using a CHEF-DRII apparatus (Bio-Rad, USA). Gel

images were normalized against *Salmonella enterica* serovar Braenderup *XbaI* digests and analysed using BioNumerics 6.1 software (Applied Maths, Belgium). Isolates that shared $\geq 90\%$ similarity in profile were considered to be of the same PFGE type. MLST was performed on selected strains of representative PFGE types according to the Institut Pasteur scheme [9] (<http://www.pasteur.fr/recherche/genopole/PF8/mlst/Abaumannii.html>).

As several of the IR-*A. baumannii* strains had previously shown positive hybridizations with the *bla*_{OXA-23} gene probe [7], all 46 phenotypically IR strains and 10 imipenem-susceptible strains (as negative controls) were screened by PCR for this gene using the following primer pair: OXA-F1: 5'-CAACAATAAAAGCACTGTA-3' and OXA-23-R3: 5'-GATGTGTCATAGTATTCGTCG-3'. OXA-51-like, OXA-24-/OXA-40-like, OXA-58-like and OXA-143 genes as well as the OXA-51-like gene upstream flanking region were screened for using previously published primers [3, 11, 12]. Additionally, IR strains negative for functional CHDL genes were screened for class A carbapenemase genes SME, IMI, NMC and KPC and class B carbapenemase genes VIM, IMP, GIM, SPM and NDM-1 with previously designed primers [13–15]. Representative IR-*A. baumannii* strains of different PFGE types were also assayed by PCR for the AbaR4 resistance island. The primer pairs used for adjacent sequences to upstream and downstream regions of AbaR4 were designed using the AB0057 genome sequence [6]. The primer sequences were AbaR4-A-F: 5'-ACGAATGACTTCATCTT CAG-3', AbaR4-A-R: 5'-TGCATTAGGCAATGATTCAC-3' and AbaR4-B-F: 5'-ACTTATCCCAGTCCTCAACA-3', AbaR4-B-R: 5'-CTACATGCAGTCGACGAGGT-3'. Bacterial lysates generated by boiling for 5 min served as DNA template.

The location of the *bla*_{OXA-23} in representative IR-*A. baumannii* strains of different PFGE types was determined by Southern hybridization. Plasmid DNA and total genomic DNA were subjected to electrophoresis and transferred to nylon membranes by standard methods. The *bla*_{OXA-23} probe made from PCR product was labelled with alkaline phosphatase using Gene Images AlkPhos Direct labelling and detection system (GE Healthcare, USA) according to the manufacturer's instructions. Plasmid profiles of IR-*A. baumannii* strains were compared with their PFGE patterns to determine correlation between these two methods. Electrophoretic profiles of *EcoRI*/*ApaI* plasmid digests, with *HindIII* lambda DNA as

Table 1. *Multilocus sequence typing profile and epidemiological features of eight representative imipenem-resistant A. baumannii strains*

Strain	ST	Allelic profile	PFGE type	Isolation location	Year
NNMC-63	25	3-3-2-4-7-2-4	5	NNMC, USA	2006
NNMC-86	n.a.	3-2-2-2-2-1	11	NNMC, USA	2006
OIFC-190	1	1-1-1-5-1-1	7	WRAMC, USA	2003
IS-240	1	1-1-1-5-1-1	15	CSH, Iraq	2008
OIFC-054	3	3-3-2-2-3-1-3	2	WRAMC, USA	2003
OIFC-064	n.a.	3-3-2-5-4-1-4	16	WRAMC, USA	2003
NNMC-69	2	2-2-2-2-2-2	48	NNMC, USA	2006
IS-25	3	3-3-2-2-3-1-3	17	CSH, Iraq	2008

ST, Sequence type; n.a., not assigned; PFGE, pulsed-field gel electrophoresis type; NNMC, National Navy Medical Center; WRAMC, Walter Reed Army Medical Center; CSH, combat support hospital.

the molecular size marker, were applied to the Fragment Size Calculator (<http://www.basic.northwestern.edu/biotools/SizeCalc.html>) for determination of the plasmid size. The plasmid carrying *bla*_{OXA-23} was also transformed into an imipenem-susceptible *A. baumannii* strain by electroporation by standard methods [15].

Forty-six strains (15%) were identified as resistant to imipenem with minimum inhibitory concentration (MIC) ≥ 8 mg/l. Cluster analysis grouped the strains into 10 PFGE types, four of which (types 5, 7, 11, 15) accounted for 39 strains and exhibited an association by isolation time and location. PFGE type 7 was found only in 2003 strain collections but this type was isolated from different military hospitals, WRAMC (USA) and LRMC (Germany) (Fig. 1). PFGE types 5 and 11 were recovered only from NNMC in 2006 while type 15 occurred only in two CSHs in Iraq in 2008; the remaining six strains had unique PFGE types (Fig. 1). MLST was conducted on eight representative IR strains of PFGE types 5, 7, 11, and 15, and four unique types. Strains of PFGE types 7 and 15 fell in sequence type (ST) 1, PFGE type 5 in ST 25, and PFGE type 11 was unassigned (Table 1).

Forty (87%) of IR-*A. baumannii* strains yielded *bla*_{OXA-23} PCR amplicons (Fig. 1). Among these, 10 were of PFGE type 11, 17 in type 5, seven in type 7, and five in type 15. One strain of PFGE type 49, which was unrelated to the four predominant PFGE types, was also *bla*_{OXA-23} positive. Only one strain (OIFC-64) was positive for the OXA-58 gene but all strains (including imipenem susceptible) carried the intrinsic OXA-51-like gene. None of the strains harboured the OXA-24-/40-like gene but four (NNMC-2, NNMC-78, IS-25, CO-22), which were negative for OXA-23, OXA-58 and OXA-24 produced an ~ 1.2 kb amplicon with the IS*Aba1*-F and OXA-51-R

primers as described previously [12]. The only strain (OIFC-54) negative by PCR for the four functional CHDL genes was also negative for the novel CHDL OXA-143 gene, and class A and B carbapenemase genes. Only strains of PFGE type 7 and the reference control strain AB0057 (of the same PFGE type) harboured the *AbaR4* resistance island (data not shown).

Plasmid profiles appeared to correlate with PFGE genotypes. For example, strains of PFGE types 5 and 11 isolated from NNMC in 2006 gave similar plasmid profiles within each PFGE type although some variation in the number and plasmid size was observed. Similar results were seen in PFGE type 7 strains from LRMC and WRAMC isolated in 2003 and PFGE type 15 strains from CSHs in Iraq isolated in 2008. Southern blot analysis revealed that the OXA-23 gene probe hybridized with both chromosomal DNA and a large plasmid (~ 70 kb) from strain NNMC-86 of PFGE type 11 but hybridized only with chromosomal DNA from three other representative IR-*A. baumannii* strains tested (NNMC-79, NNMC-84, OIFC-190) of PFGE types 5, 7, and 15. Transfer of the plasmid into a susceptible *A. baumannii* recipient by electroporation conferred imipenem resistance in this strain.

The IR-*A. baumannii* strains isolated from different overseas and domestic MTFs (LRMC and WRAMC) in 2003, shared over 90% genetic similarity by PFGE. This finding is consistent with the fact that all injured US personnel from Iraq were first evacuated to LRMC in Germany and then transferred to other MTFs such as WRAMC in the USA in 2003 [2] thus providing a potential for cross-transmission of bacteria through environmental contamination of treatment facilities [16]. Isolates of PFGE type 15 from two separate CSHs in Iraq also appeared to be clonally disseminated in the local area. These results mirror recent

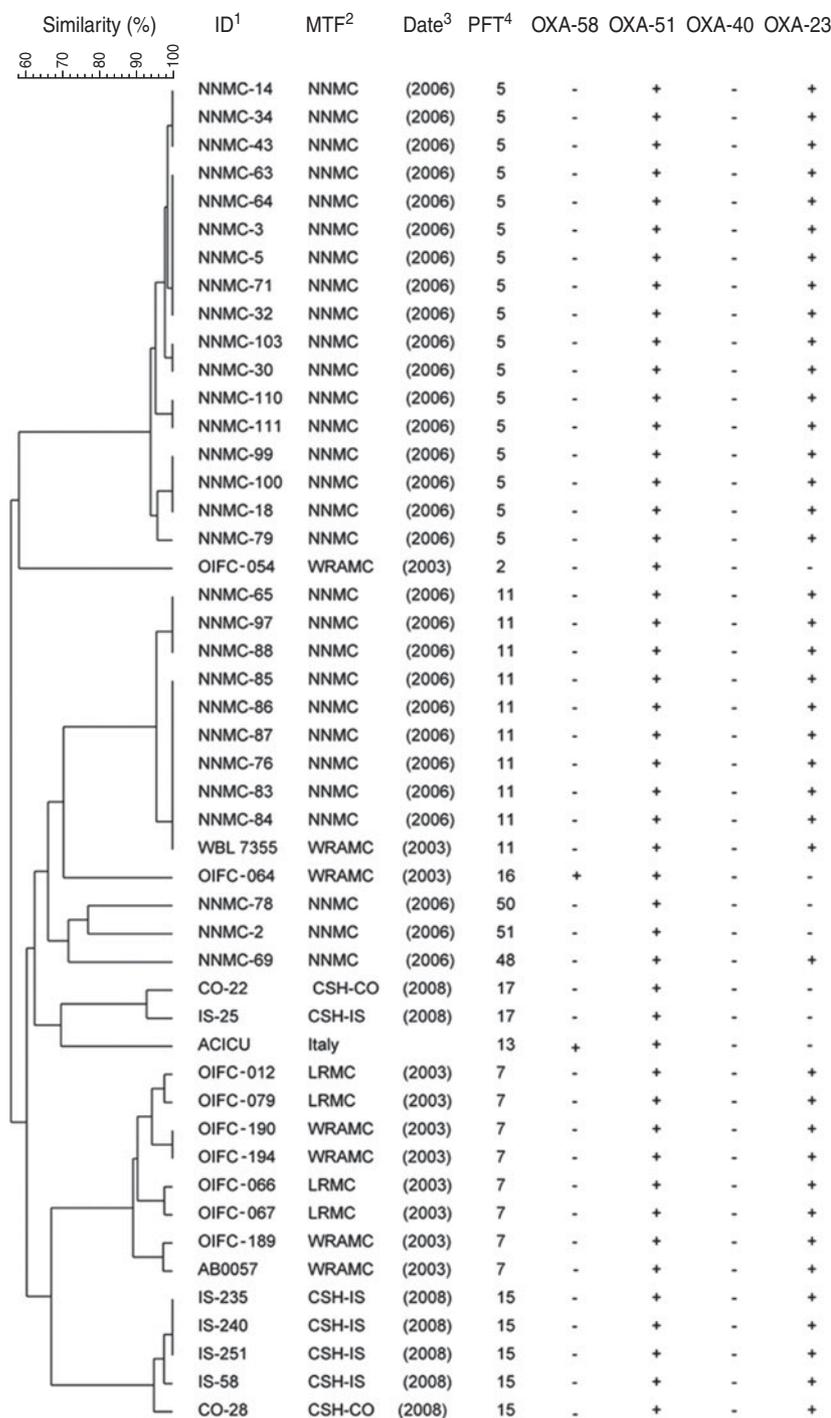


Fig. 1. Dendrogram based on pulsed-field gel electrophoresis (PFGE) pattern analysis and characteristics of imipenem-resistant *A. baumannii* strains. ¹ Identification number; ² military treatment facility; ³ year of isolation; ⁴ PFGE type. NNMC, National Navy Medical Center; WRAMC, Walter Reed Army Medical Center; CSH-CO/IS, combat support hospital in Cropper/Ibn Sina; LRMC, Landstuhl Regional Medical Center.

reports of clonal spread of *A. baumannii* OXA-23-mediated imipenem resistance among different cities in China and within medical centres worldwide [7, 17].

MLST has shown that *A. baumannii* from worldwide sources is a genetically compact species

comprised of three major international clones which are almost universally multiresistant to antimicrobials. These clones correspond to three clonal complexes each comprising a founder predominant genotype with occasional single locus variants [9].

MLST showed that representative strains of PFGE types 7 and 15 were of ST1 (international clone I) and PFGE types 2 and 17 of ST3 (international clone III). These results suggest that IR-*A. baumannii* in different MTFs and different time periods were both evolutionarily related and may be disseminated through nosocomial transmission. The latter scenario seems particularly plausible since the LRMC is located in Europe where the three international clones were first delineated, although the origin of the strains studied here (Iraq or Europe) remains controversial. The presence of other clones (ST25 and unassigned) of IR-*A. baumannii* also implies the independent circulation of unique strain populations in the NNMC. As expected from other studies PFGE demonstrated higher discriminatory power than MLST and was more effective at differentiating isolates from different locations and time periods. The correlation of plasmid profiles with PFGE supports the use of the former as a supplementary tool for molecular epidemiological studies of *A. baumannii*.

All 46 IR strains carried the OXA-51-like gene intrinsic to *A. baumannii* [12] but it is noteworthy that this gene has also recently been detected in *A. nosocomialis* [18]. PCR assays determined that the *bla*_{OXA-23} gene is probably responsible for most imipenem resistance (87%) in our historical strain collection. The successful transfer of this gene conferring imipenem resistance to a susceptible strain adds support to a plasmid location but as *Acinetobacter* spp. are naturally transformable [19] the latter mechanism cannot be ruled out. The five strains that were negative for OXA-23 and OXA-58 by PCR were also genetically distinct from all other IR strains based on PFGE; *bla*_{OXA-24} and *bla*_{OXA-143} genes were also absent from these strains. However, four of them yielded amplicons for the IS*Aba1* F and OXA-51 R primer pair, inferring that IS*Aba1* played a role in overexpression of OXA-51 for imipenem resistance. The lack of functional CHDL and carbapenemase genes in one of the isolates may indicate the possibility of multiple subtypes of genes not detectable using published primers or other mechanisms such as unique carbapenemase genes or efflux pumps. The apparent association of the AbaR4 resistance island with strains of PFGE type 7 and its absence from strains representative of other PFGE types could be interpreted that the island may be inserted at different locations in strains of different genetic backgrounds.

In conclusion, we found correlations between PFGE patterns, MLST types and imipenem resistance

in this group of *A. baumannii* strains isolated from US service members hospitalized in various US MFTs at different time periods which is suggestive of nosocomial dissemination within single, and between multiple, treatment centres. The CHDL gene, *bla*_{OXA-23}, appeared to be responsible for imipenem resistance in the majority of strains and its plasmid location enhances the possibility of horizontal transfer of this resistance mechanism.

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DECLARATION OF INTEREST

None

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