ORIGINAL ARTICLE

Investigation of the molecular epidemiology of *Acinetobacter baumannii* isolated from patients and environmental contamination

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The objective of this work was to investigate correlations between *Acinetobacter baumannii* isolates from neurosurgical intensive care unit patients and its environment. This is a prospective, observational study. The minimal inhibitory concentrations of antimicrobial agents against 27 clinical and 28 environmental isolates were determined by the agar dilution method. Molecular genotyping was performed by enterobacterial repetitive intergenic consensus PCR (ERIC-PCR), pulsed-field gel electrophoresis (PFGE) and multi-locus sequence typing (MLST). The presence of carbapenemase and metallo- β -lactamase genes were analyzed by specific PCRs and DNA sequencing. From the clinical *A. baumannii* isolates, 25.9% were found resistant to minocycline, 51.9% to cefoperazone-sulbactam, 59.3% to imipenem and 70% resistant to other antimicrobial agents. Environmental isolates were more sensitive compared with clinical isolates ($P < 0.05$). Twenty-seven clinical isolates comprised three ERIC-PCR genotypes, four major PFGE pulsotypes and five distinct MLST sequence types (STs) (ST208, ST368, ST191, ST195, ST540), all belonging to CC92 with only one locus (*gpi*) difference among them. Twenty-eight environmental isolates showed more diverse genetic types than clinical isolates and comprised six ERIC-PCR groups, nine PFGE groups and two main STs (ST208, ST229). Four clinical and 15 environmental isolates could not be identified by MLST and were assigned to non-clonal STs. We identified the presence of the *bla*_{OXA-23} carbapenemase encoding gene in most of the clinical (21/27) but fewer in the environmental isolates (3/28). The *A. baumannii* strains isolated from patients were genetically similar to the environmental strains, with CC92 members as the major fraction but with different antibiotic susceptibilities.

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INTRODUCTION

The Gram-negative bacterium *Acinetobacter baumannii* is one of the critical pathogens of hospital-acquired infections, including pneumonia, especially ventilator-associated pneumonia, meningitis, bacteraemia, urinary tract infections, surgical wounds, as well as soft tissue infections.¹ *A. baumannii* possesses an uncanny ability to survive in both moist and dry conditions for many weeks or even months, hence this organism is widely distributed in natural and nosocomial environments, human skin, as well as mucosal surfaces.² Outbreaks of *A. baumannii* infections have been reported in various geographic areas,³ and nosocomial transmissions have been linked to environmental contamination.⁴ Prolonged survival is likely a major factor for nosocomial transmission of this organism, especially in intensive care units (ICUs)^{1,5–7} and via health-care worker contamination.⁸ Carbapenem has been effective against *A. baumannii* over the past decade and has often been used as an alternative to treat otherwise multi-drug resistant *A. baumannii* infections, but the resistance rates against carbapenem have increased dramatically, due to its frequent

application in recent years. *A. baumannii* has been reported to be resistant to multiple antibiotics because of its ability to acquire resistance,⁹ which is mediated by bacterial expression of carbapenemases, including class D β -lactamases (OXA-type carbapenemases) and class B metallo- β -lactamases. It was reported worldwide that the majority of carbapenem-resistant *A. baumannii* isolates have at least one of the following genes: *bla*_{OXA-23}, *bla*_{OXA-24}, and *bla*_{OXA-58}.^{10–12} These acquired genes and *bla*_{OXA-51}, the intrinsic gene carried by *A. baumannii*, can be upregulated by insertion sequences located upstream of these genes; ISAbal is the most commonly detected.¹³ The existence of the OXA-23 subtype is the most common mechanism for *A. baumannii* resistance to the carbapenems.¹⁴ In nosocomial infections, genotyping has an important role in distinguishing the outbreak pathogens for epidemiological classifications, and common methods for pathogen genotype classifications are enterobacterial repetitive intergenic consensus PCR (ERIC-PCR), pulsed-field gel electrophoresis (PFGE) and multi-locus sequence typing (MLST).¹⁵

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In this study, we investigated the resistance rates and epidemiological characteristics of clinical and environmental *A. baumannii* bacteria isolated from a neurosurgical ICU and analyzed the mechanisms of carbapenem resistance.

MATERIALS AND METHODS

Bacterial strains

A total of 27 clinical strains were obtained from 27 individual patients of the neurosurgical ICU, Renji Hospital Shanghai, China from July to November 2012. Isolate sources included sputum (23), throat swab (1), urine (1) and drainage liquid (2). Environmental strains were isolated from 214 surface samples in the ward of neurosurgical ICU at the same period from which 28 (28/214, 13.1%) were *A. baumannii* positive, including bedside tables (17), bed rails (7), the touch pads of ventilator equipment (3) and door handles (1) but not the hands of health-care workers (0). The identification of all bacteria was performed by a Microscan WalkAway 96 plus automated system (Siemens Healthcare Diagnostics, Berlin, Germany). *Escherichia coli* ATCC25922 and *Pseudomonas aeruginosa* ATCC27853 were used as reference strains. All selected isolates were stored at -20°C and grown overnight on blood agar plates at 35°C before use.

Antimicrobial susceptibility testing

The MICs of antimicrobial agents against the isolates were determined by the agar dilution method. The antimicrobial agents, including amikacin, ciprofloxacin, gentamicin, ceftazidime, cefotaxime, cefoxitin, minocycline, sulfamethoxazole, meropenem, piperacillin, tazobactam, cefepime, imipenem, cefoperazone, sulbactam, colistin and tigecycline, were purchased from the Kejia Doping Test Equipment Company (Shanghai, China). The susceptibility breakpoints were interpreted as recommended by the Clinical and Laboratory Standards Institute (CLSI, 2010) except for tigecycline.¹⁶ For tigecycline, we used breakpoints recommended by the US Food and Drug Administration.

Enterobacterial repetitive intergenic consensus-PCR

A. baumannii genomic DNA was extracted as described previously.¹⁷ The primer pair ERIC1 (5'-ATGTAAGCTCCTGGGATTAC-3') and ERIC2 (5'-AAGTAAGTGAAGTGGGGTGAGCG-3') were used to amplify intervening fragments of ERIC in the genomic DNA. Amplification reactions were performed in a final volume of 25 μl . Each reaction system contained 2.5 μl of $10\times\text{PCR}$ buffer, 2 μl dNTP mixture, 0.125 μl Taq enzyme, 0.5 μl each primer ($10\mu\text{mol l}^{-1}$) and 1 μl DNA template, added to 25 μl of ddH₂O. Amplification reactions were carried out in an ABI 2700 thermal cycler (Applied Biosystems, Carlsbad, CA, USA) with initial denaturation (5 min at 94°C), followed by four cycles of denaturation (1 min at 94°C), annealing (1 min at 26°C) and extension (1 min at 72°C) and 40 cycles of denaturation (30 s at 94°C), annealing (30 s at 40°C) and extension (1 min at 72°C), with a final extension at 72°C for 10 min. Amplified products of each sample were subjected to electrophoresis in 1.2% agarose gel containing ethidium bromide.

Pulsed-field gel electrophoresis

The PFGE procedures were carried out according to a previously described method.¹⁸ Strains were digested with proteinase K (Takara Biotechnology Co. LTD., Dalian, China), and chromosomal DNA was digested with *NotI* (Takara Biotechnology Co. LTD.). The PFGE was run in a CHEFMapper™ system (Bio-Rad Laboratories, Inc., Berkeley, CA, USA), with pulses ranging from 5 to 20 s at 14°C with 6V cm^{-1} for 19 h. The DNA band profiles were detected after staining with ethidium bromide and photographed with the Image Lab software (Bio-Rad). Criteria for pulsotyping were the location and the number of different fragments as described previously.^{19,20} The exact same profiles were considered as the same type, 1- to 3-band differences as subtypes of the same type and >3 different bands as another type.

Multi-locus sequence typing

MLST was performed based on analyses and comparison of internal fragment sequences from seven housekeeping genes (*gltA*, *gyrB*, *gdhB*, *recA*, *cpn60*, *gpi* and *rpoD*).²¹ Amplification reactions were carried out in an ABI 2700 thermal

cycler (Applied Biosystems) with 30 cycles of denaturation (30 s at 94°C), annealing (30 s at 54°C) and extension (1 min at 72°C) after 5-min denaturation at 94°C and followed by a final extension at 72°C for 10 min. All PCR products were purified and sequenced with the assistance of JIE LI Bio-Technologies (Shanghai, China). The sequences of these seven housekeeping genes were analyzed by means of the PubMed database (<http://pubmlst.org/abaumannii/>). The sequence type (ST) was designated according to the allelic profiles in the database. The eBURST algorithm (version 3; <http://eburst.mlst.net/>) was used to assign STs to clonal complexes (CCs) and to assess the genetic relationship with definition of groups sharing alleles at ≥ 6 of the 7 loci.²²

PCR amplification of carbapenemase genes

The genes of the molecular Ambler class B enzymes (*bla*_{IMP-type}, *bla*_{VIM-type}) and class D enzymes (*bla*_{OXA-23-like}, *bla*_{OXA-24-like}, *bla*_{OXA-51-like} and *bla*_{OXA-58-like}) were analyzed by PCR. PCR primers were designed using the Primer 3.0 software (Primer PREMIER, Palo Alto, CA, USA) (Table 1). Amplification reactions were carried out in a final volume of 25 μl with 2.5 μl of $10\times\text{PCR}$ buffer, 2 μl dNTP mixture, 0.125 μl rTaq enzyme, 0.5 μl of each primer ($10\mu\text{mol l}^{-1}$) and 1 μl DNA template and added ddH₂O in a total volume of 25 μl . Amplification reactions were carried out in a ABI 2700 thermal cycler (Applied Biosystems) with an initial denaturation step (5 min at 94°C) followed by 30 cycles of denaturation (30 s at 94°C), annealing (30 s at $54\text{--}56^{\circ}\text{C}$) and extension (45 s at 72°C) and a final extension at 72°C for 7 min. Amplified products of each sample were subjected to electrophoresis in 1.2% agarose gel containing ethidium bromide. After TANON gel imaging, PCR product purification and DNA sequencing were performed by JIE LI Bio-Technologies. The resulting *bla*_{OXA-23} was analyzed with the corresponding *bla*_{OXA-23} sequence in GenBank (CP003847.1)

Statistical analyses

Statistical analyses of antimicrobial susceptibility results were carried out by the WHONET5.4 software provided by the World Health Organization. Resistance rates for each antibiotic drug were compared between clinical *A. baumannii* and environmental *A. baumannii*, by Chi-square or Fisher's exact test. *P*-values <0.05 were considered as significant.

RESULTS

Antimicrobial susceptibility testing

In the present study, a total of 55 *A. baumannii* isolates, including 27 clinical isolates and 28 environmental isolates, were selected from the neurosurgical ICU of our hospital. The resistance characteristics of these isolates are summarized in Table 2. All isolates were susceptible to colistin and tigecycline. Among 27 clinical isolates, all (100%) were resistant to trimethoprim-sulfamethoxazole, and $>80\%$ were resistant

Table 1 Sequences of primers designed for this study

Primer	Sequence	Product length
<i>bla</i> _{OXA-23-like}	P1 5'-GCTCTAAGCCGCGCAAATAC-3'	123 bp
	P2 5'-GACCTTTTCTCGCCCTTCCA-3'	
<i>bla</i> _{OXA-24-like}	P1 5'-ACGGACTGGCCTAGAGCTAA-3'	262 bp
	P2 5'-AACACCCATTCCCATCCAC-3'	
<i>bla</i> _{OXA-51-like}	P1 5'-AACGCTTCCATTAGCCCAA-3'	152 bp
	P2 5'-GAGGCTGAACAACCATCCA-3'	
<i>bla</i> _{OXA-58-like}	P1 5'-GGTCTACAGCCATTTCCAG-3'	483 bp
	P2 5'-ATGGCAGCATTTAGACCGA-3'	
<i>bla</i> _{VIM-1-like}	P1 5'-GAAGACTCTCATCGAGCGG-3'	310 bp
	P2 5'-GTGCTTTGACAACGTTGCT-3'	
<i>bla</i> _{VIM-2-like}	P1 5'-TCCACGCATTTTCATGACGA-3'	285 bp
	P2 5'-AGACGTGCGTGACAACATCAT-3'	
<i>bla</i> _{IMP-1-like}	P1 5'-CACTTGGTTTGTGGAGCGTG-3'	263 bp
	P2 5'-GTCCCGGCGCTGGATAAAAA-3'	

Table 2 Susceptibility analysis of *Acinetobacter baumannii* in this study

Antimicrobial agents	Clinical isolates (n = 27)		Environmental isolates (n = 28)		χ^2	P
	MIC ₉₀ (mg l ⁻¹)	R %	MIC ₉₀ (mg l ⁻¹)	R %		
Piperacillin	> 256	85.2	256	28.6	65.32	<0.01
Piperacillin/Tazobactam	256	81.5	32	10.7	100.87	<0.01
Ceftazidime	128	88.9	128	28.6	75.02	<0.01
Cefotaxime	256	81.5	128	21.4	72.30	<0.01
Cefepime	128	85.2	32	14.3	100.54	<0.01
Cefoxitin	> 256	100	256	92.9	5.43	<0.05
Imipenem	32	59.3	1	7.1	61.43	<0.01
Meropenem	64	74.1	1	3.6	104.61	<0.01
Amikacin	> 256	70.4	> 256	28.6	34.95	<0.01
Gentamicin	> 256	85.2	> 256	35.7	51.24	<0.01
Ciprofloxacin	> 256	85.2	256	32.1	58.13	<0.01
Trimethoprim-sulfamethoxazole	> 256	100	> 256	50	66.67	<0.05
Cefoperazone-sulbactam	32	51.9	0.5	3.6	16.15	<0.01
Minocycline	16	25.9	8	10.7	7.73	<0.01
Tigecycline	2	0	0.5	0		
Colistin	0.5	0	0.25	0		

Abbreviations: MIC₉₀, MIC for 90% of the organisms; R %, percent resistant.

to cephalosporins (ceftazidime, cefotaxime, cefepime and cefoxitin), ciprofloxacin and gentamicin. The majority of *A. baumannii* strains were carbapenem resistant (CRAB), with 59.3% against imipenem and 74.1% against meropenem. Minocycline had the lowest resistance rate of 25.9%. In contrast, environmental isolates were more susceptible to all antimicrobial agents mentioned above, compared with clinical isolates. The resistance rates against imipenem, meropenem, cefoperazone-sulbactam and minocycline were 7.1, 3.6, 3.6 and 10.7% respectively, whereas resistance rates were 20–50% against other antimicrobial agents, except for cefoxitin (92.9%). Statistical analysis of resistance rates against each antibiotic drug revealed significant differences between clinical and environmental isolates ($P < 0.05$).

Molecular typing

Molecular typing is by PFGE, ERIC-PCR and MLST.

Pulsed-field gel electrophoresis

Molecular typing by PFGE identified four major clinical groups (A, B, D, E) and nine major environmental groups, which were termed A–I. Twenty-three clinical isolates were classified in PFGE group A, only one isolate was clustered in PFGE group B, two isolates were in PFGE group D and one isolate in group E. The 28 environmental isolates showed more diverse genetic types than the clinical isolates, but 15 isolates were also PFGE type A, which was the major fraction (Table 3).

Enterobacterial repetitive intergenic consensus PCR

With ERIC-PCR analyses, the 27 clinical *A. baumannii* isolates were classified into three major groups a(20), b(6) and c(1), whereas environmental isolates yielded six groups (a–f). Fourteen environment strains showed identical DNA fingerprinting pattern as type a strains, four isolates were type b, two isolates were type c and others were different in the size of > 3 bands and were designated as type d(4), e (2) and f(2) (Table 3). The grouping of ERIC-PCR results did correlate only partly with the PFGE clustering.

Multi-locus sequence typing

According to the MLST results, 27 clinical *A. baumannii* isolates were grouped into five existing STs (ST208, 1-3-3-2-2-97-3; ST368, 1-3-3-2-2-140-3; ST191, 1-3-3-2-2-94-3; ST195, 1-3-3-2-2-96-3; ST540, 1-3-3-2-2-160-3) and four non-clonal STs (Table 3). ST208, accounting for 66.7% (18/27), was the major clone that spread in the neuro-surgical ICU of our hospital, followed by ST540 (2/27, 7.4%) and one each of ST368, ST191 and ST195. Clonal relation analysis showed that ST208, ST368, ST191, ST195 and ST540 belonged to the CC92 lineage, with only one locus (*gpi*) different among these STs, accounting for 85.2% (24/27) of all clinical isolates. Furthermore, the MLST results of the clinical isolates showed that CC92 corresponded to the group A obtained by PFGE. Molecular typing of environmental isolates by MLST identified two existing STs, ST208 and ST229, with nine and four isolates respectively, but a significant proportion (54%) had to be assigned as non-clonal STs (Figure 1).

PCR amplification for carbapenemase genes

The two groups of strains were analyzed for their carbapenem resistance genes. *bla*_{OXA-51} is intrinsically found in *A. baumannii* and was detected in all strains. Twenty-one of the 27 clinical isolates carried *bla*_{OXA-23}, whereas only 3 of the 28 environmental isolates were *bla*_{OXA-23} positive. From the 27 clinical isolates, 20 were carbapenem non-susceptible *A. baumannii* (CNSAb) and 7 were carbapenem-susceptible *A. baumannii* strains. All of the clinical CNSAb, as well as 1 CNSAb strain, carried the *bla*_{OXA-23} gene. In addition, two of the three environmental CNSAb, as well as one CNSAb strain, were OXA-23 positive. However, there were no *bla*_{IMP-type}, *bla*_{VIM-type}, *bla*_{OXA-24-like} and *bla*_{OXA-58-like} genes among the isolates.

DISCUSSION

In recent years, *A. baumannii* has emerged as a successful pathogen of various troublesome nosocomial infections, with the ability to resist various kinds of antibiotics.²³ As observed in other Asian countries,²⁴ our study revealed that these strains were resistant to various kinds of antimicrobial agents, as a result of carbapenemase expression levels, mainly including class B metallo- β -lactamases and class D OXA-type

Table 3 Molecular typing results of clinical and environmental *Acinetobacter baumannii* strains and the distribution of OXA-23 enzymes

No. of strains	PFGE	ERIC-PCR (No.)	MLST (No.)	No. of CSAb		No. of CRAB	
				OXA-23(+)/OXA-23(−)		OXA-23(+)/OXA-23(−)	
Clinical strains							
23	A	a(16) b(6) c(1)	ST208 (17) ST368 (1) ST191 (1) ST195 (1) ST540 (2) ND (1)	1	6	16	0
1	B	a(1)	ND (1)	0	0	1	0
2	D	a(2)	ST208 (1) ND (1)	0	0	2	0
1	E	a(1)	ND (1)	0	0	1	0
Environmental strains							
15	A	a(12) b(1) e(2)	ST208 (9) ST229 (3) ND (3)	1	11	2	1
2	B	f(2)	ND (2)	0	2	0	0
1	C	c(1)	ND (1)	0	1	0	0
2	D	a(1) d(1)	ST229 (1) ND (1)	0	2	0	0
3	E	b(2) d(1)	ND (3)	0	3	0	0
2	F	a(1) d(1)	ND (3)	0	2	0	0
1	G	b(1)	ND (1)	0	1	0	0
1	H	d(1)	ND (1)	0	1	0	0
1	I	c(1)	ND (1)	0	1	0	0

Abbreviations: CRAB, carbapenem resistant; CSAb, carbapenem-susceptible *A. baumannii*; ERIC-PCR, enterobacterial repetitive intergenic consensus PCR; MLST, multi-locus sequence typing; ND, not determined; PFGE, pulsed-field gel electrophoresis.

carbapenemases.²⁵ Colistin and tigecycline were the main choices for the treatment of infection caused by CRAB. Although colistin-resistant strains have been discovered in some area, our survey showed that colistin retained its activity against these CRAB isolates carrying *bla*_{OXA-23} have been discovered worldwide and are prevalent in Asian regions.^{16,22,26–28} Our study indicated that *bla*_{OXA-23} was the most prevalent CHDL (carbapenem hydrolyzing class D β -lactamase) gene and was most frequently found in CRAB isolates collected in our neurosurgical ICU. Many study indicated that the dissemination of *bla*_{OXA-23} is attributed to transposons, including Tn2006, Tn2007 and Tn2008, while clonal spread had a minor role.²⁹ Tn2008 was recognized as the major transposon carrying *bla*_{OXA-23} in China.³⁰ However, the spread of *bla*_{OXA-23} in our hospital need to be validated.

In our study, 13.1% of the 214 environmental samples collected in our neurosurgical ICU were *A. baumannii* positive, which was higher compared with previous reports in which the environmental surfaces surrounding patients in ICUs were 2.3% (513 samples), 7.3% (151 samples) and 9.8% (479 samples) *A. baumannii* positive.^{4,31,32} The sites commonly contaminated in our ward were bedside tables frequently touched by both the health-care workers and patients.

PFGE has been considered as the standard for typing of many bacterial species owing to its superior reproducibility and high discriminatory power³³ and has been carried out for typing of organisms in local epidemiological studies, such as *A. baumannii*,^{34,35} *Escherichia coli*²⁰ and *Klebsiella pneumoniae*.³⁶ PFGE can be used for investigating bacterial relations after clinical outbreaks by generating distinct patterns of restricted chromosomal DNA

fragments. According to our study, the clinical isolates had more clone types than the environmental isolates, with group A being the most prominent type.

ERIC-PCR uses amplification of intervening fragments between highly conserved ERIC sequences by means of consensus primers.³⁷ ERIC-PCR is a rapid, simple and low-cost method for molecular typing, which has been applied previously for clinical *A. baumannii* isolate differentiation. Our ERIC-PCR results showed that clone a is the common genotype with 74.1% in clinical strains (20/27) and 50% in environmental isolates (14/28). The vast majority of the PFGE A strain also appeared as a common strain in ERIC-PCR classifications (clinical samples 23A vs 16a and environmental samples 15A vs 12a), while other classifications did not match well.

MLST, based on analyses of nucleotide sequences from internal fragments of multiple housekeeping genes, has been considered as an unambiguous typing method for global and long-term epidemiological studies.¹⁸ Our MLST results, based on comparative sequence analyses of conserved regions in 7 housekeeping genes of the 27 clinical *A. baumannii* isolates, led to the clonal ST208, ST368, ST191, ST195 and ST540 groups and 4 non-clonal STs. ST208 (18/27, 66.7%) was the major clone that spread in the neurosurgical ICU infections of our hospital, followed by ST540 (2/27, 7.4%). Clonal relation analysis showed that ST208, ST368, ST191, ST195 and ST540 belonged to CC92 with only one locus (*gpi*) difference among these STs, accounting for 85.2% (24/27) and reflecting the global predominance of the CC92 *A. baumannii* type.¹⁶ Fifteen environmental isolates of PFGE group A were assigned to ST208 (9/28, 32.1%), ST229 (3/28,

Figure 1 Phylogenetic tree of MLST. A full color version of this figure is available at *The Journal of Antibiotics* journal online.

10.7%) and a distinct allele profile (3/28, 10.7%). Based on all of the above data, a large proportion of the environmental isolates (53%) and four clinical isolates (14.8%) in this study were assigned to non-clonal STs. As expected, most of the non-clonal STs were found to be susceptible isolates and most OXA-23(+) clinical strains belonged to CC92.³⁸

Genotype analysis were carried out by molecular typing (ERIC-PCR, PFGE, MLST), and we found that there was a certain similarity between clinical and environmental isolates, but a general clonal transmission as a result of environmental contamination could not be identified, probably because of the significant differences in antimicrobial susceptibilities. However, the identification of environmental contamination suggested a potential risk for nosocomial transmission, but in this study we analyzed only a limited number of samples from both patients and the environment, hence, further research is necessary.

In summary, colistin and tigecycline can be the last treatment option for CRAB. However, its efficacy, toxicity and resistance are further concerns. Patients' isolates were genetically similar to environmental isolates, with a major clone corresponding to CC92. The clonal spread of isolates producing *bla*_{OXA-23} carbapenemase might be the major factor that resulted in the high carbapenem resistance rate of *A. baumannii* infections in our neurosurgical ICU, yet this was not due to a high rate of resistant environmental isolates.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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