Original Article

Molecular Epidemiology and Characterization of Genotypes of *Acinetobacter baumannii* Isolates from Regions of South China

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SUMMARY: The aim of this study was to analyze the molecular epidemiologic characteristics of *Acinetobacter baumannii*. A total of 398 isolates were collected in 7 regions of South China from January to June of 2012. Drug sensitivity was tested toward 15 commonly used antibiotics; thus, 146 multi-drug-resistant strains (resistant to more than 7 drugs) were identified, representing 36.7% of all isolates. Pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST) were used for molecular subtyping. According to the PFGE results (with a cutoff of 70% similarity for the DNA electrophoretic bands), 146 strains were subdivided into 15 clusters, with cluster A being the largest (33.6%, distributed in all districts except Jiaying). Cluster B was also widespread and included 14.4% of all strains. In addition, MLST results revealed 11 sequence types (ST), with ST208 being the most prevalent, followed by ST191 and ST729. Furthermore, 4 novel alleles and 6 novel STs were identified. Our results showed that multi-drug-resistant *A. baumannii* in South China shares the origin with other widespread strains in other countries. The nosocomial infections caused by *A. baumannii* have been severe in South China. Continuous monitoring and judicious antibiotic use are required.

INTRODUCTION

Acinetobacter baumannii, a gram-negative bacterium and a widespread conditional pathogen, is widespread in nature and in hospitals. Its strong ability to develop drug resistance and cloning spread have made it an important pathogen of nosocomial infections, especially in ventilator-associated pneumonia, bacteremia, and urinary tract infections (1). In recent years, nosocomial infections caused by *A. baumannii* have become more and more severe, and *A. baumannii* infections are more prevalent than *Pseudomonas aeruginosa* infections (2). Although an increasing number of studies worldwide have been focused on *A. baumannii*, there are hardly any good treatments of *A. baumannii* infections because of the diverse pathogenic genotypes and compli-

cated drug resistance mechanism. *A. baumannii* strains can evolve into ones with multi-drug-resistance through gene mutations, and can acquire exogenous genetic elements such as plasmids, integrons, transposons, and islands that contain multi-drug-resistance genes. Therefore, most antimicrobial drugs become ineffective against *A. baumannii* because of its multi-drug-resistance (3). It is sometimes called “the MRSA of gram-negative bacilli” (4).

The key strategy for control of the spread of *A. baumannii* is to determine the spread process, to cut off the bacterial transmission routes, and to prevent drug-resistant strains from spreading in the community. It will be helpful to elucidate the molecular characteristics and drug resistance profiles of epidemic strains. Strains from different hospitals and regions usually have different genotypes and different drug resistance profiles. In this study, strains from 7 districts of South China were collected, and pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST) were used for molecular subtyping. The MLST database of internationally widespread clones (<http://pubmlst.org/abau-mannii/>) can aid in the identification of the route of global dissemination and the genetic background of an *A. baumannii* strain. Our work may pave the way for effective prevention and treatment of nosocomial infections caused by *A. baumannii*.

Received November 28, 2014. Accepted June 8, 2015.
J-STAGE Advance Publication July 10, 2015.

DOI: 10.7883/yoken.JJID.2014.544

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MATERIALS AND METHODS

Collection and Identification of Bacterial Strains: A total of 398 nonrepeated *A. baumannii* isolates were obtained from 19 hospitals of 7 districts in South China from January to June of 2012. The isolated microorganisms were assumed to be of nosocomial origin because they were isolated from patients 48 h after they were admitted to a hospital or later. All isolates were identified by means of a Vitek 32 microorganism auto-analysis system (BioMerieux Corporate, Lyon, France) in combination with sequencing of 16S ribosomal DNA amplified by polymerase chain reaction (PCR).

Antibiotic sensitivity testing: The minimum inhibitory concentrations (MICs) of 15 antimicrobial agents were determined by the standard agar dilution method. The sensitivity data were interpreted according to the guidelines of the Clinical and Laboratory Standards Institute 2012 (5). *Escherichia coli* ATCC 25922 served as a control.

PFGE: This method was used for genotyping as described previously (6). In brief, *A. baumannii* DNA samples were digested with *ApaI* (TaKaRa, Dalian, China) for 4 h at 37°C. At the same time, DNA of *Salmonella enterica* serovar Braenderup H9812—which was used as a size standard—was digested with *XbaI* (TaKaRa). The DNA fragments were separated on a CHEF-Mapper system (Bio-Rad, Hercules, CA, USA) for 20 h at 6 V/cm and 14°C, with a pulse angle of 120°C and pulse duration from 5 to 20 s. Images were captured by means of the Gel Doc System (Bio-Rad). The unweighted pair group method with arithmetic means (UPGMA) was used for cluster analysis using the BioNumerics software, version 4.0 (Applied Maths, Sint-Martens-Latem, Belgium). The R software, version 3.1.1 (<http://www.r-project.org/>), was used to construct the PFGE cluster distribution in the different districts.

MLST: This analysis of *A. baumannii* involved 7 conserved housekeeping genes (*gltA*, *gryB*, *gdhB*, *recA*, *cpn60*, *rpoD*, or *gpi*) according to protocols available in

the *A. baumannii* MLST database (<http://pubmlst.org/abumannii/>). The annealing temperatures were set to 44°C for *gpi* and at 55°C for other genes. Each PCR product was purified using a commercial PCR product kit (TaKaRa) before sequencing. The allelic numbers and sequence types (STs) were identified by means of the *A. baumannii* MLST database. The clustering of related STs (defined as clonal complexes; CCs) was analyzed in the software on the eBURST website (<http://eburst.mlst.net>). A phylogenetic tree was constructed in the Splits Tree Software.

RESULTS

Antibiotic resistance of the isolates: The antibiotic sensitivity test showed that our *A. baumannii* isolates were strongly drug-resistant. A total of 146 strains were resistant to 7 of the 15 commonly used antibiotics. Most strains were resistant to β -lactams, aminoglycosides, quinolones, and tetracycline antibiotics, with the highest resistance rate (96.6%) toward ampicillin. The resistance rates for the third-generation cephalosporins were all above 60.0% except for cefoperazone/sulbactam (23.3%). In addition, isolates in this study showed higher sensitivity to cefoperazone/sulbactam than to carbapenems, with the resistance rates 23.3% and 29.4%, respectively (Table 1).

PFGE: A total of 146 multi-drug-resistant strains from 7 districts of South China were identified by PFGE. We obtained bands with sizes ranging from 30 to 500 kb. The number of electrophoretic bands was between 18 and 25. Gel images were input into BioNumerics, and a phylogenetic tree was built for cluster analysis. A restriction map of 126 pulsotypes was constructed when a cutoff of similarity was set to 100% (Fig. 1). When UPGMA was used for cluster analysis with the similarity cutoff of 70%, 146 strains were grouped into 15 clusters, and most strains (87.0%) were in 8 PFGE clusters (clusters A, B, C, D, E, F, G, and H). The strains in the largest cluster (cluster A with 49 strains, 33.6% of total) came from all 6 regions (except the Jiax-

Table 1. Drug resistance rates of 146 multi-drug *Acinetobacter baumannii* for common antibiotics

Antibiotic	CLSI breakpoint interpretation			MIC (μ g/mL)		
	%S	%I	%R	MIC ₅₀	MIC ₉₀	Range
1. Ampicillin	2.0	1.4	96.6	>512	>512	1->512
2. Piperacillin	23.3	10.3	66.4	256	>512	0.25->512
3. Meropenem	41.8	28.8	29.4	8	32	<0.125-128
4. Aztreonam	36.3	4.8	58.9	32	64	0.25->512
5. Cefoperazone/sulbactam	58.2	18.5	23.3	16	64	1-256
6. Cefotaxime	16.4	2.7	80.8	256	512	2->512
7. Cefepime	28.8	4.8	66.4	32	128	1-512
8. Ceftriaxone	8.9	10.3	80.8	256	512	2->512
9. Ceftazidime	20.5	1.4	78.1	64	128	1->512
10. Gentamicin	21.9	2.7	75.3	256	>512	0.25->512
11. Amikacin	37.0	0.0	63.0	64	>512	0.5->512
12. Ciprofloxacin	27.4	2.0	70.6	64	128	<0.125-512
13. Tetracycline	15.1	2.7	82.2	256	>512	1->512
14. Chloramphenicol	4.8	6.8	88.4	128	256	2->512
15. Trimethoprim/sulfamethoxazole	11.0	0.0	89.0	>512	>512	1->512

Fig. 1. Cluster map of PFGE results for 146 multiple-drug-resistant *Acinetobacter baumannii* strains. The bacterium chromosome DNA was digested with *Apa*I.

ing district) and were also the most prevalent bacterial pathogens in these areas. The second largest cluster was cluster B: 21 strains which made up 14.4% of the total.

In this work, we compared PFGE cluster profiles among the different geographic areas (Fig. 2). It turned out that most areas shared more than 1 PFGE cluster, while almost each cluster (clusters A, B, C, D, F, and G) appeared in different districts. Strains from Wenzhou had the most PFGE clusters (10) with clusters A (33.3%) and B (30.8%) being the most prevalent. There were 5 clusters in the Hangzhou district, and clusters A (41.7%) and B (25.0%) were the most prevalent ones. In addition, the distribution of the PFGE types tended to cluster regionally. All strains in clusters H and E were from Shaoxing (6/6) and Jiaxing (10/10), respectively, and most strains in cluster C (11/17) were from Ningbo (Fig. 1).

The relation analysis of the PFGE typing results and drug resistance profiles showed that there were several different resistance profiles for the same PFGE type and

vice versa. Therefore, there was no correlation between PFGE types and drug resistance. Furthermore, 139 strains resistant to ampicillin shared most PFGE types (120 pulsotypes), and they were distributed across all 15 clusters.

MLST: On the basis of the above PFGE results, a total of 40 strains from the dominant clusters were selected for the MLST analysis. As a result, 11 STs were identified (Table 2 and Fig. 3). ST208 was the most prevalent ST, accounting for a half of isolates (20/40) typed. It was found in 5 of the 7 areas. The second place was shared by ST191 and ST729 (4 isolates each), followed by ST541 (3 isolates). ST208, ST191, ST381, ST195, ST540, and ST368 belong to EUII and CC 92. There were 5 singleton STs—ST541, ST727, ST728, ST729, and ST730—which could not be categorized according to any of the 3 major pan-European clonal lineages based on MLST.

Six novel STs (ST540, ST541, ST727, ST728, ST729, and ST730) and 4 new alleles (*gdhB142*, *recA68*,

Fig. 2. (Color online) PFGE profiles of clonal relationship among 146 multi-drug-resistant *Acinetobacter baumannii* isolates from different districts.

Fig. 3. (Color online) EBURST results of 765 STs in Pubmlst *Acinetobacter baumannii* database with 11 STs in this study. Each circle represents a ST; STs with blue color are the ancestors of corresponding group; lines in the circle represent the possible evolution relationships; the size of circle reflects the number of strains; STs with red color have already been included in database while STs with pink color are found only in this study; the black ones exist in database but are not found in the study.

Table 2. STs and allele numbers of *Acinetobacter baumannii* isolates

CC	ST	Allelic profile	Total of isolates (%)	Isolate
92	ST208	1-3-3-2-2-97-3	20 (50.0)	LS2, LS4, LS10, LS14, LS23, HZ12, SX1, SX3, SX9, SX29, WZ2, WZ38, WZ39, WZ41, JH2, JH5, JH6, JH7, JH12, JH15
92	ST191	1-3-3-2-2-94-3	4 (10.0)	HZ33, WZ4, WZ32, JH3
singleton	ST729	22-15-3-2-4-169-2	4 (10.0)	SX15, SX16, SX17, SX23
singleton	ST541	39-65-43-30-25-24-28	3 (7.5)	NB1, NB3, NB4
92	ST381	1-81-3-2-2-16-3	2 (2.5)	JX6, JX17
92	ST195	1-3-3-2-2-96-3	2 (5.0)	HZ2, NB6
92	ST540	1-3-3-2-2-160-3	1 (2.5)	HZ7
92	ST368	1-3-3-2-2-140-3	1 (2.5)	SX20
singleton	ST727	27-70-128-68-2-16-29	1 (2.5)	NB11
singleton	ST728	39-65-142-30-2-97-28	1 (2.5)	NB18
singleton	ST730	22-15-13-69-4-62-2	1 (2.5)	JX15

LS, Lishui; HZ, Hangzhou; JX, Jiaying; SX, Shaoxing; WZ, Wenzhou; JH, Jinhua; NB, Ningbo.

recA69, and *gpi69*) were identified. ST727 has a new allele (*recA68*), which represents a G/A mutation at position 208 of the *recA2* locus. ST728 has a new allele (*gdhB142*), which is more closely related to *gdhB93*. ST729 has a new allele (*gpi69*), which represents a T/C mutation at position 6 of the *gpi62* locus. ST730 has a new allele (*recA69*): a T/C mutation at position 344 of the *recA12* locus. The novelty of the STs indicated that this bacterium's genome underwent rapid evolution.

The resistance profiles of the strains (i.e., different STs) were analyzed. Strains in CC 92 (20 strains) showed greater resistance to piperacillin, cefotaxime, cefepime, ceftazidime, ciprofloxacin, and tetracycline than other strains (i.e., STs) did. This finding indicated that CC 92 might cause more widespread nosocomial infections than other strains can.

DISCUSSION

A. baumannii, a conditionally pathogenic bacterium, can cause infections in the elderly and in children, especially in the physically weak population. It was reported that *A. baumannii* with multi- or even pan-drug resistance is widespread worldwide (4). The main reason for the high prevalence of multi-drug-resistant strains is the abuse of antimicrobial drugs in clinical practice and in agriculture. Results of MIC assays in the present work revealed a serious situation. Most strains were resistant to β -lactams, aminoglycosides, quinolones, and tetracycline antibiotics, with high MICs (Table 1). β -lactams are the most widely used antibiotics in clinical practice and the bacterial resistance to this kind of antibiotics was found to be very high in the present study. At present, the first-line antibacterial agents for *A. baumannii* infection are still mostly imipenem, meropenem, and cefoperazone/sulbactam. Some studies showed that a combination of several drugs is effective against *A. baumannii* infections (7). The sensitivity of *A. baumannii* to cefoperazone/sulbactam may be increased with a higher dose. Therefore, the combination cefoperazone/sulbactam may be effective against nosocomial *A. baumannii* infections.

It is important to analyze the correlations among pathogens at the molecular level to understand their molecular epidemiology. Tracking the source and route

of pathogens should help to control the spread of multi-drug-resistant strains. Establishing a PFGE typing database for clinically isolated multi-drug-resistant strains may help to characterize newly isolated strains quickly and efficiently via comparison of their PFGE types with the database. In this study, we found that 146 multi-drug-resistant *A. baumannii* strains from 7 districts in South China could be subdivided into 15 PFGE clusters, with strains of clusters A and B prevalent in 6 of these 7 districts. This result indicates that clone transmission may occur in these districts.

MLST has been successfully applied to analysis of molecular epidemiology (8). It has been reported that bacteria isolated from different regions of the world can share a few common genetic characteristics (9). Our study also showed that ST208 is the most widespread strain in South China, and the results of eBURST indicated that ST208 belongs to the CC 92, the largest and most widespread CC in the world. CC 92 contains more than 132 STs, and these STs have been detected in many countries in Asia, Europe, Oceania, and North America (10–13). Our work also showed that the prevalent strains (according to our data) share a high degree of homology with other major epidemic strains in the world.

Previous phylogenetic analyses of the strains of *A. baumannii* showed that the ancestral ST of CC 92 is ST92, and all other STs of CC 92 evolved from ST92 (14,15). ST92 was the most prevalent strain in most regions before ST208 had become more and more dominant by 2011 (16,17). Fu et al. (18) confirmed that in South China, members of CC 92 were the most prevalent STs in 2005, including ST92, ST75, and ST90. In 2012, Deng et al. (19) showed that ST208 and ST191 are the most prevalent strains among the *A. baumannii* strains isolated in the First Affiliated Hospital of Zhejiang University, South China. Our study confirms those reports: ST208 is the dominant strain of *A. baumannii*. ST208, ST92, ST90, ST75, ST138, ST191, ST195, ST540, and ST368 differ only in the housekeeping gene *gpi*. Only one point mutation (T/C) at position 3 of *gpi* differentiates ST208 and ST92. Between ST208 and ST75, there is a T/C substitution (at position 3) and 2 G/A substitutions (at positions 189 and 195) in *gpi*. MLST analysis has played a crucial role in studies on the

molecular evolution and epidemiology of bacteria.

In just 2 decades, *A. baumannii* became an important pathogenic bacterium prevalent in intensive care units (20). In particular, the emergence of multi-drug-resistant strains has placed a severe social and economic burden on patients, hospitals, and nations. Variations in climate, environment, equipment, sterilization procedures, diseases, and treatments in different regions have led to diverse clinical features, drug resistance profiles, and prognoses of *A. baumannii* infections. Therefore, it is important to characterize the clinical characteristics and drug resistance profiles of *A. baumannii* in various geographic areas during various periods in order to prevent or treat such infections more efficiently.

Acknowledgments This work is supported by the Natural Science Foundation of Zhejiang Province, China (LY14C060005); the Science and Technology Foundation of National Health and Family Planning Commission of China (WKJ2012-2-032); the National Natural Science Foundation of China (81401702); Technology Innovation Team of Zhejiang Province, China (2010R50048-13); Science and Technology Foundation of Wenzhou City, China (H20110010, Y20110101, Y20120126, Y20140422); Science and Technology Foundation of Wenzhou Municipal Health Bureau (2011A021), and the Education Foundation of Zhejiang Province, China (Y201327704).

Conflict of interest None to declare.

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