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Original Article

Cross-sectional and longitudinal studies on antimicrobial susceptibility profiles and the genomic diversity of *acinetobacter baumannii* isolates from senile patients

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Abstract: Background: To date, little data is available on the antimicrobial susceptibility profiles and molecular characteristics of Acinetobacter baumannii clinical isolates from senile patients. Herein, cross-sectional and longitudinal studies on antimicrobial susceptibility and genomic diversity were conducted to investigate the susceptibility patterns and clonal relatedness of *A. baumannii* isolates in our gerontal wards. Methods: Cross-sectional analysis was performed on 170 non-repetitive *A. baumannii* isolates recovered from senile patients (aged 61-99 years, mean = 86.8) over a 2-year period (2012-2013). The longitudinal study examined 77 repetitive *A. baumannii* isolates recovered from 8 senile patients (aged 87-98 years, mean = 92) with long-time hospitalization. Results: The majority of the 170 isolates (128/170, 75.3%) were non-susceptible to carbapenems (CRAB), and all CRAB were extensively drug-resistant (XDR) or multidrug-resistant (MDR), which were spread evenly over the different departments. The isolates belonged to 36 pulsotypes, as determined by pulsed-field gel electrophoresis. Groups I to IV (containing 119, 4, 1, and 2 isolates, respectively) were major epidemic strains with similar clonal relatedness (similarity >80%); 98.4% (124/126) of which were CRAB with the XDR phenotype. In the longitudinal study, all 77 isolates were XDR. A comparison of pulsotypes was performed for each patient. All isolates clustered in Group I except for one isolate, which belonged to a new group. Conclusions: Extensive drug-resistance of *A. baumannii* was more serious in gerontal wards than in regular wards. Clone dissemination was the most important type of XDR strains spread and the XDR clones were responsible for long-term infection of *A. baumannii*. Therefore, more preventive measures should be reinforced in gerontal wards.

Keywords: *Acinetobacter baumannii*, hospital-acquired infections, extensive drug-resistance, molecular epidemiology, senile patients

Introduction

Acinetobacter baumannii is a Gram-negative microorganismand one of the most common bacterial species responsible for hospital-acquired infections (HAIs) [1, 2], particularly in long-term care settings such as the intensive care unit (ICU) and gerontal wards [3, 4]. It is found ubiquitously in human skin, perineum, and the digestive system of hospitalized patients [5, 6], with the ability to colonize and survive in patients, medical devices and different environments based on its intrinsic characteristics [7, 8]. With the prevalence of multidrug resistance, *A. baumannii* infection in hospitals is increasing and poses a serious challenge worldwide [9, 10]. Investigations of the clonal relatedness of *A. baumannii* in local settings can monitor outbreaks and the epidemiology of this opportunistic pathogen, establishing the foundation for an effective infection control program. Senile patients are at high risk of HAIs because of their weakening immunity and long-term hospitalization. Previous studies have focused on the clonal relatedness of *A. baumannii* in general patients but not among senile patients specifically. *A. baumannii* is a severe problem in our gerontal wards, as it is one of the most common bacterial species associated with HAIs, and has always been regarded as a

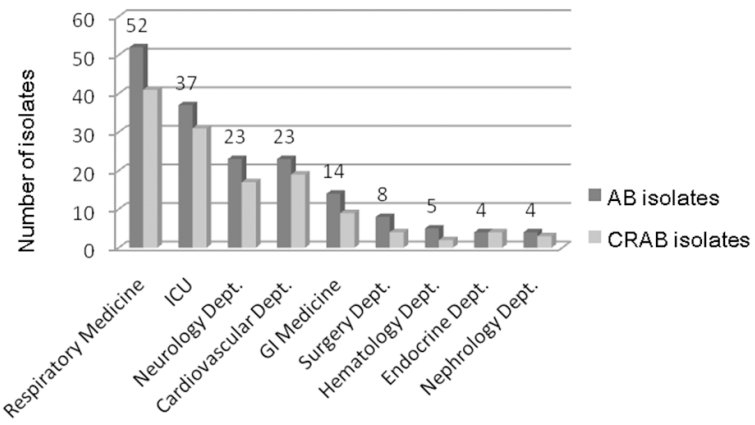

Figure 1. Distribution of 170 non-repetitive AB isolates and 128 CRAB isolates in different departments.

durable infection that is difficult to eradicate, especially in critical or severe patients. The cross-sectional and longitudinal studies on antimicrobial susceptibility and genotying described herein were performed to investigate the clonal relatedness of *A. baumannii* clinical isolates in our local gerontal wards.

Material and methods

Subjects and bacterial strains

Subjects include in the cross-sectional study were hospitalized senile patients (aged 61-99, mean = 86.8) with clinical infections that occurred more than 48 h after admission. Our hospital is tertiary and there are at total of 500 beds in the gerontal wards. A total of 170 non-repetitive *A. baumannii* isolates were obtained from clinical specimens over 24 months (January 2012 to December 2013). *A. baumannii* isolates were identified using Vitek II compact automated systems and confirmed by the presence of intrinsic $bla_{\text{OXA-51-like}}$ [11]. Only one isolate was collected from each patient in the cross-sectional study. The vast majority (153/170, 90%) of the isolates were recovered from sputa or respiratory tract secretions. The remaining 18 isolates were from urine (13/170, 7.6%), catheter (2/170, 1.2%), bile (1/170, 0.6%), and drainage (1/170, 0.6%). The distribution of isolates from different departments is illustrated in **Figure 1**.

Subjects of the longitudinal study included 8 senile patients (aged 87-98, mean = 92) who had been hospitalized for a long time (>1 yr) and were selected at random. All repetitive *A. baumannii* isolates (n = 77) were collected from each patient during their hospital stay (from May 2012 to December 2013, the collection time was 5.1 months for each patient, on average). Seventy-one isolates were recovered from sputa or respiratory tract secretions, and 6 isolates were recovered from urine (5 came from the same patient and 1 came from another patient). Eight patients were from the Respiratory Department (4 cases), Cardiovascular Department (1 case), GI Medicine (1 case), and ICU (1 case).

No experiments were carried out on humans or animals; rather, all experimental subjects were bacteria. The clinical samples were taken as part of standard patient care and therefore no ethical approval was required for their usage.

In vitro antimicrobial susceptibility test

All isolates were tested using the Kirby-Bauer (K-B) method of disk diffusion according to the recommendations of the Clinical and Laboratory Standards Institute (CLSI) [12] to determine their susceptibilities to amikacin (30 μg), ciprofloxacin (5 μg), trimethoprim/sulfamethoxazole (25 μg), gentamicine (10 μg), imipenem (10 μg), meropenem (10 μg), piperacillin (100 μg), polymyxin B (300 unites), levofloxacin (5 μg), minocycline (30 μg), cefepime (30 μg), ceftazidime (30 μg), piperacillin-tazobactam and cefoperazone/sulbactam (75/10 μg) (OXOID, UK). K-B results were interpreted according to the breakpoints established by the CLSI (the cefoperazone breakpoint for *enterobacteriaceae* was used for cefoperazone/sulbactam as the interpretive standards for *Acinetobacter spp.*) [12]. *Escherichia coli* ATCC25922 and *Pseudomonas aeruginosa* ATCC27853 were used as control strains for susceptibility testing. Resistance to imipenem or meropenem was defined as carbapenem resistance *A. baumannii* (CRAB), and carbapenem sensitivity was defined as CSAB.

Table 1. Initial diagnosis of 170 non-repetitive patients

Systems (n = total)	Initial diagnosis	Number of patients
Respiratory system (89)	Pneumonia	51
	Pulmonary carcinoma	18
	COPD	14
	Bronchitis	6
Cardio-cerebrovascular disorders (41)	Coronary heart disease	16
	Cerebral hemorrhage, embolism	13
	Arrhythmia	9
	Myocardial infarction	3
Nervous system (15)	Brain Trauma, cephalophyma	6
	Parkinson's disease	3
	Intracranial space-occupying lesions	3
	Alzheimer disease	3
Digestive system (15)	Gastrointestinal tumor	7
	Acute cholecystitis, Gastritis	4
	Upper gastrointestinal hemorrhage	2
	Intestinal Obstruction	2
Urinary system (4)	Chronic renal disease	2
	Hyperplasia of prostate	1
	Renal carcinoma	1
Other (6)	Leukemia, Fibromatosis, Fracture	6

COPD, chronic obstructive pulmonary diseases.

Pulsed-field gel electrophoresis (PFGE)

PFGE was employed to determine the clonal relatedness of the isolates. Plugs containing bacterial pellets were prepared prior to Apal (Biolabs) enzymatic digestion. The procedures were conducted as described previously [13]. *Saimonella* H9812 was used as a molecular marker and digested with Xba I (Biolabs). Electrophoresis was carried out using a CHEF-DR II apparatus (Bio-Rad Laboratories, CA, USA) in 0.5x TBE buffer at 14°C. The initial and final times of electrophoresis ranged from 5 to 20 seconds, and the total run time was 19 h. The gel was stained with ethidium bromide and viewed under ultraviolet (UV) light. Analyses were performed using BioNumerics software version 6.6.4.0 and the Unweighted Pair Group Means Method (UPGMA), as described by Seifert, to determine the isolates' pulsotypes [13]. Pulsotypes were defined as isolates with PFGE band patterns of 85% similarity or more according to previous criteria [14, 15].

Results

Clinical data analysis

All 170 senile patients in the cross-sectional study had multiple fundamental illnesses related to multiple organs, and most of the patients had basic diseases such as diabetes (58/170, 34.1%) and cancer (62/170, 36.5%), which were regarded as being related to infection. Firty-eight patients had fbeen treat with tracheal intubation and assisted respiration on a ventilator. The initial diagnosis of each patient is listed in **Table 1**. Regarding treatment, 7 patients had not used any antibiotics because they had little symptoms of infection; those 7 isolates were therefore regarded as colonizers. The remaining 163 patients had used one to four antibiotics while treating the infection. The most commonly used medicines were broad spectrum antibiotics such as β-lactamase inhibitor combinations, carbapenems and fluoroquinolone with a long period of treatment (**Table 2**). On average, nearly half of the patients (77/163, 47.2%) had used dual antimicrobial combination.

In the longitudinal study, all 8 patients had repeated infection symptoms including prolonged fever, increased leukocyte or abundant sputum, etc. They had been treated with antimicrobial therapy for a long time, and multifarious antibiotics were resorted to. As the initial diagnosis, 6 patients had pneumonia, 1 patient had pulmonary carcinoma and 1 patient had

Table 2. Frequency of antibiotic treatment of 163 non-repetitive patients

Antimicrobial class (total frequency)	Antibiotic	Number of patients	Dose	Average period of treatment (day)
β-lactamase inhibitor combinations (174)	Cefoperazone/sulbactam	55	1.5 g/bid-tid	12.8
	piperacillin/tazobactam	47	4.5 g/bid-tid	10.4
	Cefoperazone/tazobactam	38	2.25 g/bid	10.1
	Ceftazidime/tazobactam	25	2.4 g/bid-tid	10.9
	Ceftriaxone/tazobactam	6	2 g/bid	13.2
	Cefotaxime/tazobactam	3	2.25 g/bid	9.0
Carbapenems (108)	Meropenem	83	0.5-0.75 g/tid	13.9
	Imipenem	13	0.5 g/tid	10.7
	Biapenem	12	0.3 g/bid-tid	10.5
Fluoroquinolone (88)	Levofloxacin	41	0.3-0.5 g/qd	11.6
	Moxifloxacin	27	0.4 g/qd	12.9
	Ciprofloxacin	20	0.2 g/bid	10.6

qd, one time a day; bid, twice a day; tid, three times a day.

Table 3. Comparison of non-susceptible rates to 13 kinds of antibiotics among CRAB, CSAB and different pulsotypes

Antibiotic	Non-susceptible to antibiotic/no. (% of isolates)				
	Total (n = 170)	CRAB (n = 128)	CSAB (n = 42)	Group I~IV (n = 126)	Group V~XXXVI (n = 44)
AK	125 (73.5)	118 (92.2)	7 (16.7)	119 (94.4)	6 (13.6)
CIP	133 (78.2)	127 (99.2)	6 (14.3)	126 (100)	7 (15.9)
FEP	131 (77.1)	126 (98.4)	5 (11.9)	126 (100)	5 (11.4)
CN	128 (75.3)	123 (96.1)	5 (11.9)	123 (97.6)	5 (11.4)
IMP	128 (75.3)	128 (100)	0 (0)	124 (98.4)	4 (9.1)
LEV	130 (76.5)	127 (99.2)	3 (7.1)	126 (100)	4 (9.1)
MEM	129 (75.9)	128 (100)	1 (2.4)	124 (98.4)	5 (11.4)
MH	102 (60.0)	101 (78.9)	1 (2.4)	100 (79.4)	2 (4.5)
PRL	148 (87.1)	128 (100)	20 (47.6)	125 (99.2)	23 (52.3)
SXT	124 (72.9)	119 (93.0)	5 (11.9)	120 (95.2)	4 (9.1)
CAZ	122 (81.8)	114 (89.1)	8 (19.0)	115 (91.3)	7 (15.9)
TZP	136 (80.0)	128 (100)	8 (19.0)	126 (100)	10 (22.7)
CSF	123 (72.4)	121 (94.5)	2 (4.8)	120 (95.2)	3 (6.8)

AK, amikacin; CIP, ciprofloxacin; FEP, cefepime; CN, gentamicin; IMP, imipenem; LEV, levofloxacin; MEM, meropenem; MH, minocycline; PRL, peracillin; SXT, trimethoprim-sulfamethoxazole; CAZ, ceftazidime; TZP, piperacillin/Tazobactam; CSF, cefoperazone/sulbactam.

brain trauma. In total, 6 patients had been treat with tracheal intubation and assisted respiration on a ventilator. The outcomes revealed that 3 patients died, 3 patients had symptoms of infection that could not be controlled for a long time, and only 2 patients improved.

Phenotypic resistance

About three-quarters of the 170 non-repetitive *A. baumannii* isolates (128/170, 75.3%) were CRAB and non-susceptible to carbapenems (non-susceptible to both imipenem and meropenem), which is higher than the 63.5% carbapenem resistance rate of *A. baumannii* in the 2012 report of CHINET surveillance of antibiotic resistance in *A. baumannii* isolates in China [16]. Many isolates were resistant to diverse antibiotics containing aminoglycosides, fluoroquinolone, cephalosporins, carbapenems, etc. Rates of non-susceptibility to different antimicrobial agents were commonly >70%, except for minocycline, which had the lowest rate of 60.0% (**Table 3**). Most CRAB isolates (125/128, 97.7%) were extensively drug-resistant (XDR), and 3 were multidrug resistant (MDR). The discrimination between XDR and MDR was based on the standardized international terminology described by Magiorakos [17]. Moreover, 128 CRAB isolates displayed complete resistance to piperacillin, piperacillin/tazobactam and meropenem. The rates of non-susceptibility to other antimicrobial agents were nearly 90%. Only minocycline was relatively low, at 78.9% (**Table 3**). It is worth mentioning that

Table 4. Comparison of non-susceptible rates to 13 kinds of antibiotics in major departments

Antibiotic	Non-susceptible to antibiotic/no. (% of isolates)				
	Respiratory Medicine (n = 52)	ICU (n = 37)	Neurology Dept. (n = 23)	Cardiovascular Dept. (n = 23)	GI Medicine (n = 14)
AK	37 (71.2)	29 (78.4)	18 (78.3)	18 (78.3)	9 (64.3)
CIP	40 (76.9)	31 (83.8)	19 (82.6)	20 (87.0)	9 (64.3)
FEP	39 (75.0)	32 (86.5)	17 (73.9)	19 (82.6)	9 (64.3)
CN	37 (71.2)	32 (86.5)	18 (78.3)	18 (78.3)	9 (64.3)
IMP	40 (76.9)	31 (83.8)	16 (69.6)	19 (82.6)	9 (64.3)
LEV	40 (76.9)	31 (83.8)	18 (78.3)	19 (82.6)	9 (64.3)
MEM	40 (76.9)	33 (89.2)	16 (69.6)	19 (82.6)	9 (64.3)
MH	31 (59.6)	26 (70.3)	15 (65.2)	13 (56.5)	9 (64.3)
PRL	43 (82.7)	35 (94.6)	20 (87.0)	21 (91.3)	9 (64.3)
SXT	34 (65.4)	31 (83.8)	18 (78.3)	19 (82.6)	8 (57.1)
CAZ	38 (73.1)	27 (73.0)	16 (69.6)	19 (82.6)	7 (50.0)
TZP	41 (78.8)	33 (89.2)	19 (82.6)	19 (82.6)	9 (64.3)
CSF	38 (73.1)	30 (81.1)	14 (60.9)	19 (82.6)	9 (64.3)

AK, amikacin; CIP, ciprofloxacin; FEP, cefepime; CN, gentamicin; IMP, imipenem; LEV, levofloxacin; MEM, meropenem; MH, minocycline; PRL, peracillin; SXT, trimethoprim-sulfamethoxazole; CAZ, ceftazidime; TZP, piperacillin/Tazobactam; CSF, cefoperazone/sulbactam.

these CRAB isolates had a relatively high rate of intermediate susceptibility to minocycline (50/128, 39.1%) and cefoperazone/sulbactam (37/128, 28.9%) at the same time. The distribution of CRAB isolates in different hospital departments is illustrated in **Figure 1**. We can see that the proportion of CRAB to AB isolates in each department was similar. Accordingly, there were no significant differences in phenotypic resistance of isolates in several major departments (**Table 4**). In addition, the 170 patients were divided into CRAB and CSAB groups. The ratio of patients with mechanical ventilation in the CRAB group (53/128, 41.4%) was much higher than that in the CSAB group (3/42, 7.1%) ($P<0.01$, chi-square test).

In the longitudinal study, all 77 isolates showed the XDR phenotype. Most isolates were non-susceptible to amikacin (74/77, 96.1%), cefoperazone/sulbactam (76/77, 98.7%), ciprofloxacin (76/77, 98.7%), gentamicin (74/77, 96.1%), minocycline (63/77, 81.8%), and trimethoprim-sulfamethoxazole (74/77, 96.1%), and all were resistant to imipenem, meropenem, levofloxacin, cefepime, piperacillin, ceftazidime and piperacillin/tazobactam.

PFGE

In the cross-sectional study, the 170 non-repetitive isolates were assigned to 36 pulsotypes, as determined by PFGE (**Figure 2**). Approximately three-quarters (126/170, 74.1%) of the isolates clustered into four groups with high similarity (>80%), showing similar clonal relatedness. Group I contained 119 isolates (119/170, 70%), and Group II to IV contained 4, 1 and 2 isolates, respectively. Nearly all isolates from Group I to IV (124/126, 98.4%) were CRAB with the XDR phenotype (**Table 3**). The remaining 44 isolates (44/170, 25.9%) were unrelated to the four groups and were categorized into 32 profiles, which had relatively distant relatedness. Nearly all isolates from Group V to XXXVI were susceptible to different antibiotics (**Table 3**).

In the longitudinal study, 77 isolates came from 8 patients designated A to H. The quantities of isolates from patients A-H were 10, 12, 10, 10, 9, 9, 9, and 8, respectively. Comparisons of pulsotypes were made for each patient. The results showed that all isolates had similar clonal relatedness and clustered into one group (group I) as mentioned before, except for one isolate (no. 256) from patient F, which was classified into a new group (**Figure 3**). For each senile patient, it meant that almost all XDR *A. baumannii* isolated repeatedly during the period of infection belonged to the same clone, indicating that XDR *A. baumannii* is difficult to eradicate completely.

Discussion

A. baumannii is famous for its seemingly endless capacity to acquire antimicrobial resistance and plays an important role in causing hospital infections. Over three-quarters of *A. baumannii* isolates in this study were MDR, XDR or even pandrug-resistant (PDR), indicat-

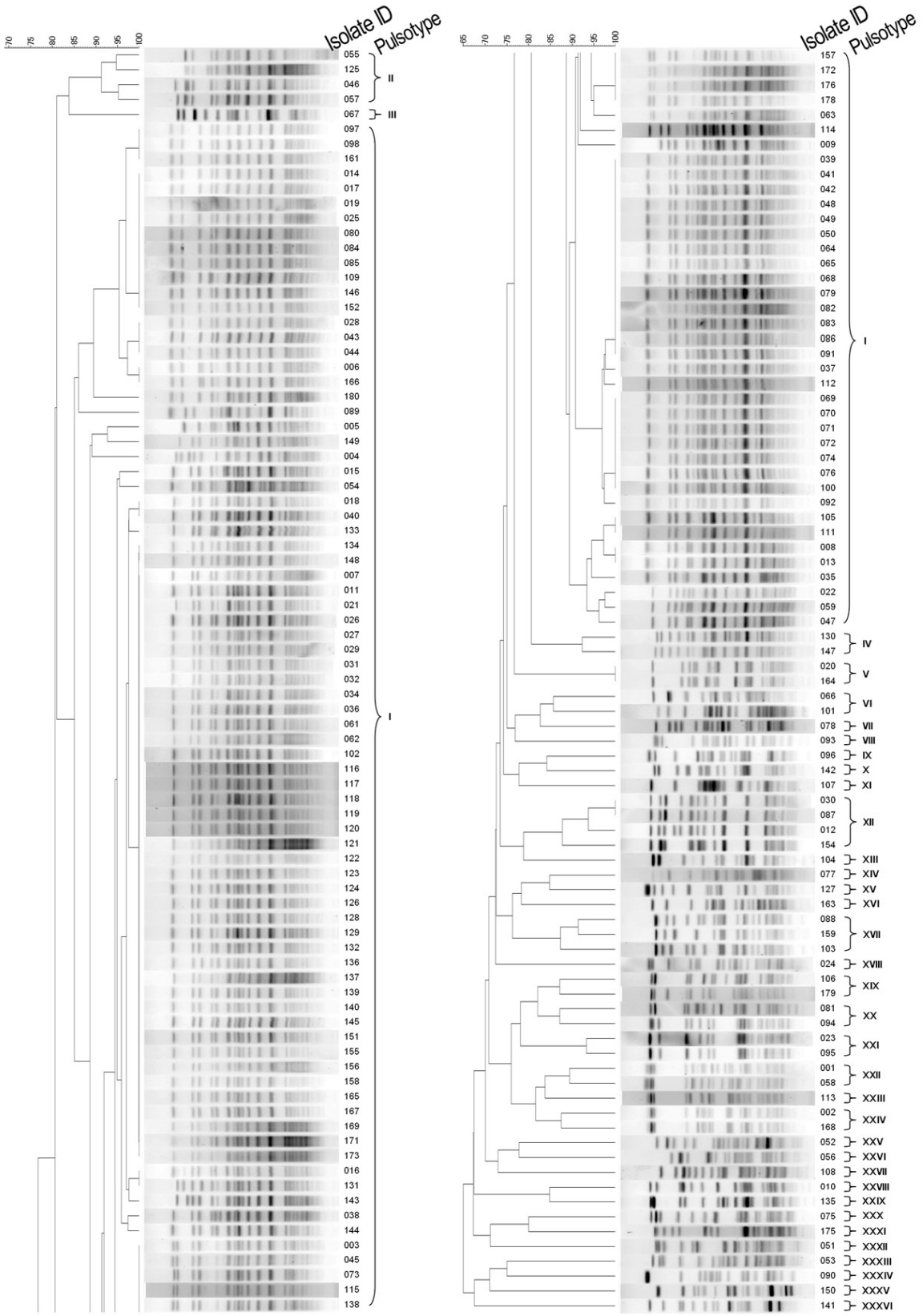

Figure 2. PFGE patterns of 170 non-repetitive A. baumannii isolates. Dendrogram was generated by BioNumerics software, using the unweighted pair-group method of arithmetic averages (UPGMA).

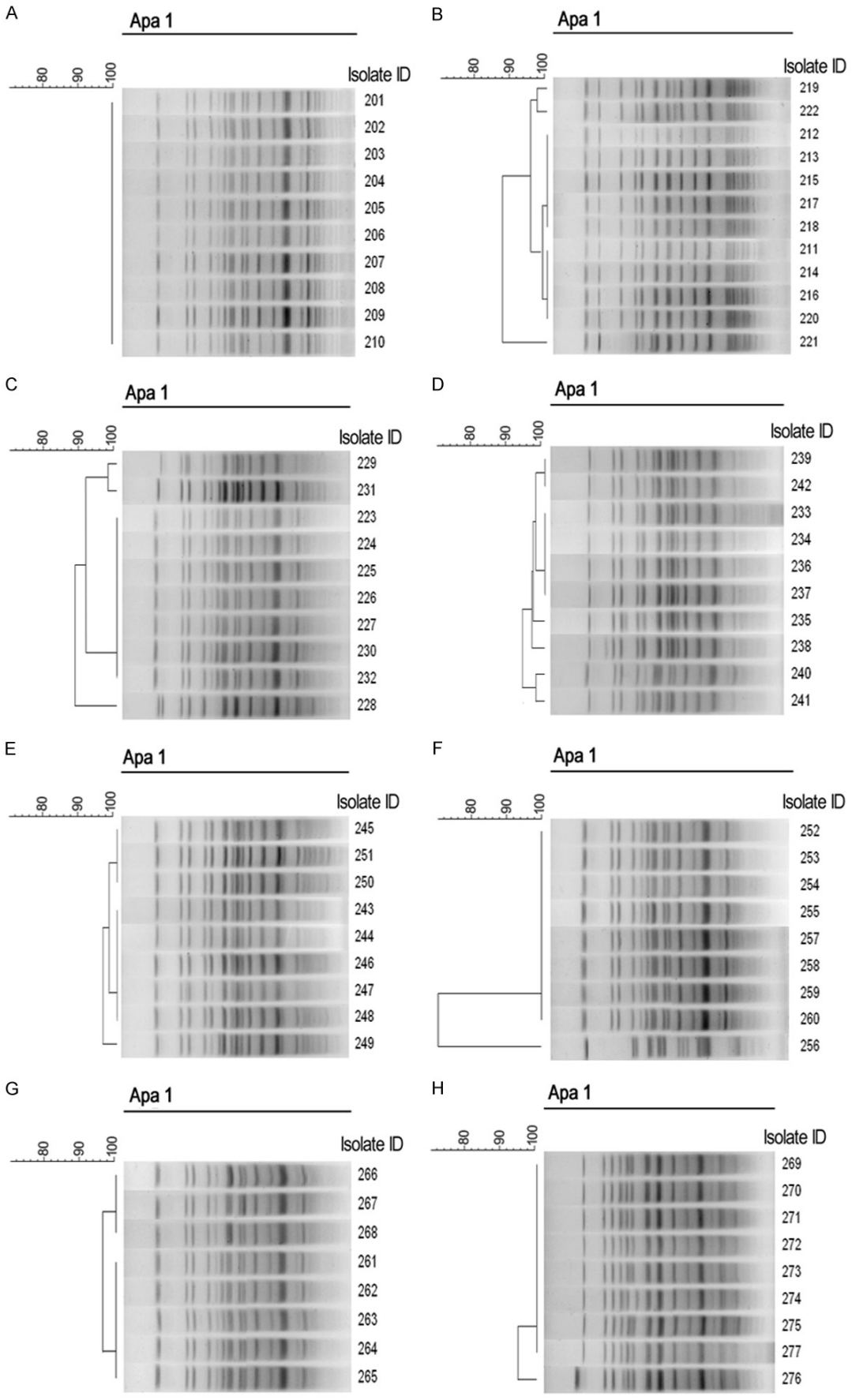
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Figure 3. PFGE patterns of 77 repetitive A. baumannii isolates came from 8 patients. A. Isolates from patient A; B. Isolates from patient B; C. Isolates from patient C; D. Isolates from patient D; E. Isolates from patient E; F. Isolates from patient F; G. Isolates from patient G; H. Isolates from patient H. Dendrogram was generated by UPGMA.

ing that our gerontal wards are overrun by resistant strains and that senile patients are a susceptible population to resistant strains of *A. baumannii*. There were 58 patients who freceived tracheal intubation, and the ratio of patients with mechanical ventilation in the CRAB group was much higher than that in the CSAB group, indicating that mechanical ventilation is an important risk factor for infection of resistant strains; this finding is consistent with previous research [18].

When treatment is needed, the choice of antimicrobial agent will be severely limited by resistance. MDR isolates may possess a number of mechanisms conferring resistance to the same class of antibiotics, adding to the difficulty of finding suitable therapeutic agents. Carbapenems are often the drug of choice because they have better antibacterial activity against Gram-negative bacilli. However, with the widespread usage of this type of drug, the likelihood of CRAB detection is increasing, which can be demonstrated clearly in the isolates of our gerontal wards. In our cross-sectional analysis, 75.3% of 170 non-repetitive isolates were CRAB, and the resistance rates to other antimicrobial agents were mostly over 70%. For CSAB, 42 isolates were susceptible to nearly all antimicrobial categories, but with regard to CRAB, all 128 isolates were XDR and even PDR. The resistance rate is higher than the average level of China, which is considered to be closely related to the drug use conditions in our gerontal wards. An analysis of case history revealed that the highest frequency of usage among antimicrobial agents was β-lactamase inhibitor combinations, followed by carbapenems and fluoroquinolone. The widespread usage of these potent and broad spectrum antibiotics may lead to a significant increase of resistant strains [19]. The same situation has occurred in other places and several reports have shown that the usage of broad spectrum antibiotics affected normal flora and induced MDR *A. baumannii* [20]. The lack of new methods of treatment for resistant strainshas sparked considerable interest in the use of dual or even triple antimicrobial combinations [21]. From this study, we conclude that the preferable choice of treatment in our gerontal wards is minocycline combined with cefoperazone/sulbactam, as these CRAB isolates exhibited a relatively higher rate of intermediate susceptibility to these drugs.

Gene homology is of great significance to the surveillance of bacterial infection and outbreak of hospital infections. Meanwhile, it can provide useful information for infection control. By means of PFGE typing, 170 non-repetitive isolates were assigned to 36 pulsotypes, and our gerontal wards revealed dissemination of XDR clone strains from Group I to IV, which had a very close relationship. Group I was the predominant epidemic strain, with the absolute advantage of 119 isolates. The number of isolates from Groups I to IV was 126 (124 were CRAB, accounting for 96.9% of the total 128 CRAB isolates). The high prevalence of Group I with a strong ability of extensive drug resistance, indicates that the serious drug resistance phenomenon of *A. baumannii* in our gerontal wards can be attributed to the same clone strain, and Group I appears to be an important lineage mediating the spread of extensive drug resistance. The epidemic strains were not limited to certain departments; it was could be found in all departments tested and existed continuously throughout the 2 years of investigation, indicating that the clone strains led to widespread dissemination, regardless of time or space.

In our longitudinal research, we found that each of the 8 patients had a long infection period of XDR *A. baumannii* and all isolates were clustered into Group I, except for one isolate, which belonged to a new group. This indicated that the XDR epidemic strain was really an obstinate problem in our gerontal wards and once the senile patients with long-term hospitalization get infected with XDR *A. baumannii*, it is difficult to eradicate the pathogen completely, despite the massive use of antibiotics over a long time. Most of the 8 patients had poor outcomes, indicating that infection with an XDR strain was often associated with longer duration of infection and a grim prognosis. Moreover, 6 of the 8 patients had received mechanical

ventilation treatment, which could benefit the adhesion and survival of XDR strains over a long period of time. This may be another important factor for longtime XDR strain infection in addition to the basic factors of older age and lower immunity.

Acinetobacter can survive for a long time under dry circumstances in locations such as hospital beds, the atmosphere, armrests, pillows, bedding, stethoscopes, and white coats. With the frequent consultation of doctors among different departments, the contaminated hands of medical staff and stethoscopes have become an important medium of dissemination. There is a serious clonal dissemination of XDR *A. baumannii* in our gerontal wards, and it seems that the epidemic strains cannot be effectively controlled. Horizontal infection control measures such as environmental cleaning and hand hygiene to avoid person-to-person transmission should be reinforced to prevent XDR *A. baumannii* from spreading. Further research should focus on the factors that play important roles in the XDR mechanism of epidemic strains, as well as determining the optimal combination of antibiotics to achieve a better curative effect.

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Disclosure of conflict of interest

None.

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