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Original Article

Correlation between antibiotic resistance and serum resistance in *Acinetobacter baumannii*

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Abstract: Objective: This study aimed to investigate the relationship between antibiotic resistance and serum resistance in clinical isolates of *Acinetobacter baumannii* (*A. baumannii*). Methods: The tested 67 clinical isolates were collected from several hospitals in China. Antibiotic resistance to 21 antibiotics from 9 antimicrobial categories was assessed by Kirby-Bauer disc diffusion or broth microdilution methods. Multilocus sequence typing (MLST) was used to group the *A. baumannii* isolates. At last, the *in vivo* mice model was used to detect the relationship between drug resistance and mortality from inoculation with *A. baumannii*. Results: Among all 67 isolates, 16 were defined as multidrug-resistant (MDR), and 46 were extensively drug-resistant (XDR). MLST grouped the *A. baumannii* isolates into 9 existing sequence types (STs). ST208 accounted for 44.8% (30/67) of the isolates, which belonged to clonal complex (CC) 92. The serum resistance testing showed that 53 out of 68 strains of *A. baumannii* (67 clinical isolates and reference strain ATCC19606) were highly resistant to killing by complement system in normal human serum (NHS). The comparison of the antibiotic resistance and serum resistant strains showed that antibiotic resistant isolates had stronger serum resistance than susceptible strains. Furthermore, mice infected by XDR isolates had higher mortality rate. Conclusion: Drug-resistant *A. baumannii* strains have stronger serum resistance. These results sounds alarming and should be considered in the clinical treatment of drug-resistant *A. baumannii*.

Keywords: *Acinetobacter baumannii,* antibiotic resistance, serum resistance, mortality

Introduction

Acinetobacter baumannii (*A. baumannii*) is one of the most important nosocomial opportunistic pathogens that is responsible for severe nosocomial infections including pneumonia, bloodstream, urinary tract, wound infections, and meningitis [1, 2]. The *A. baumannii* is marked by strong ability to acquire drug resistance and clone transmission. In the early 1970s, the infectious agent was susceptible to most antibiotics [3]. However in recent years, multidrug-resistant, extensively drug resistant, and even pan drug resistant *A. baumannii* have been detected worldwide [4, 5]. Strains resistant to antibiotics from at least one in three or more antimicrobial categories were defined as Multidrug-resistant *A. baumannii* (MDRAB). Bacterial isolates that indicated susceptibility to one or two categories of drugs were considered as Extensively Drug Resistant *A. baumannii* (XDRAB). Finally, strains resistant to all agents from all antimicrobial categories were defined as Pan Drug Resistant *A. baumannii* (PDRAB) [6].

The CHINET monitoring of bacterial epidemiology and resistance in China showed that from 2005 to 2014 the resistance rate of *A. baumannii* to carbapenem antibiotics increased from 31% to 66.7% [7]. Gao and colleagues [7] investigated changes in drug resistance of

2917 *A. baumannii* strains in nearly 20 hospitals in China. It was found that the prevalence of XDRAB increased from 11.1% in 2004 to 60.4% in 2014 [7]. Polymyxins used to be the last line of defense against MDRAB infection [8]. However, polymyxin resistant strains have been continuously found [8, 9]. The development of more severe multidrug resistance results in accumulation of difficulties that complicate clinical diagnosis and treatment.

Currently, the systematic and comprehensive investigation of *A. baumannii* resistance mechanism is indicated that the emergence of drug resistance is linked to the mutation or modification of specific genes that inhibit the entry of drugs into bacteria or change the drug targets [10]. An important virulence characteristic, the serum resistance depends on the ability of *A. baumannii* to with stand recognition and destruction by serum circulating complements. Several other virulence factors have been identified including specific characteristics of outer membrane protein A, lipopolysaccharide, capsular polysaccharide, phospholipase D, outer membrane vesicles, and penicillin binding proteins. These virulence factors play an important role in initiation and progression of bacterial infection in host cells via regulation of adhesion, invasion, biofilm formation, induction of apoptosis, and barrier function against the host immune system [11, 12]. Previous studies have shown that increased bacterial resistance may change bacterial virulence. Multiple studies evaluated relationship between drug resistance and virulence of *A. baumannii* [13]. It's generally accepted that there is a positive correlation between drug resistance and virulence [14, 15]. However, different opinions were also expressed [16, 17]. To date, the association of drug resistance and serum resistance in *A. baumannii* has not been reported.

In this study, to clarify potential correlation among various types of bacterial resistance, levels of antibiotic and serum resistance in 68 *A. baumannii* strains (67 clinical isolates and reference strain ATCC19606) was examined and analyzed.

Materials and methods

Bacterial strains and growth conditions

A total of 67 non-repetitive clinical isolates were collected from several hospitals in China and written informed consent was obtained from the participants of this study. The isolates were identified by conventional microbiological tests as well as using API 20NE assay [18-20]. The reference strain of *A. baumannii* (ATCC 19606) was used in this study. Strains were stored in glycerol stocks at -80°C, and were cultivated for further analysis in nutrient agar at 37°C overnight without shaking.

Antimicrobial susceptibility testing

The antibiotic resistance of each isolate was assessed using 21 drugs from nine antimicrobial categories including penicillins, β-lactamase/β-lactamase inhibitor combinations, cephems, carbapenems, aminoglycosides, tetracyclines, fluoroquinolones, folate pathway inhibitors, and lipopeptides [21, 22]. These antimicrobial agents and categories were chosen because of their wide epidemiological spread, significant worldwide clinical use against *Acinetobacter spp.*, and testing for MDR/XDR [6]. MIC to polymyxin B was assessed using broth micro dilution method. The inhibition zone diameter to 20 drugs were assessed using the standard disc diffusion method [15]. Interpretive breakpoints for susceptible, intermediate and resistant levels were defined according to the Clinical and Laboratory Standards Institute guidelines. Strains resistant to 0-2 antimicrobial categories of drugs were described separately and defined as non-MDR to facilitate analyses of the relationship between antibiotic and serum resistance [16].

Molecular typing by multi-locus sequence typing (MLST)

MLST was used to molecular typing of *A. baumannii* clinical isolates. Briefly, seven conserved housekeeping genes (gltA, gryB, gdhB, recA, cpn60, rpoD, and gpi) were amplified according to primers available at http://pubmlst.org/abaumannii/. After sequencing, the allelic numbers and sequence types (STs) were identified with databases online at http://pubmlst.org/databases/.

Serum resistance assays

Complement-mediated bactericidal assays were performed by measuring the change in bacterial titer over time in the presence of 90% active or inactive (heated at 56°C for 30 min) human serum at 37°C. An input bacterial titer

of approximately 1×10^5 CFU was used, then 1 hour later the titers were measured. Numbers of surviving bacterial were determined by plating two independent samples of serial 10-fold dilutions in duplicate or triplicate [23]. Experiments with each particular strain were repeated for a minimum of three times. The strain serum resistance was considered as weak when the number of surviving bacteria in the normal human serum (NHS) group was significantly lower than that of the heat-inactivated NHS (HI-NHS) group. Alternatively, serum resistance was considered strong [24].

Virulence assay in vivo

This experimental procedures with laboratory animals were approved by the Animal Care and Use Committee of Yangzhou University (approval ID: SYXK (Su) 2005-0005). Ninety six-week old male BALB/c mice (18-20 grams) were used for the pneumonia model. Mice were rendered transiently neutropenic by the intra-peritoneal injection of 0.15 mL Cyclophosphamid per 150 mg/kg body weight on days 3 and 4 before *A. baumannii* inoculation. The final inoculum was obtained after strain was grown to midlogarithmic phase. Inoculum was resuspended in 0.9% NaCl. The mice were anesthetized by intra-peritoneal injection of 2% sodium pentobarbital solution. Intra-tracheal instillation of *A. baumannii* was performed as previously described [25]. Briefly, the trachea was canulated with a needle and 50 μL of bacterial suspension containing 10^8 CFU/mL was deposited. The mice were closely monitored and mortality rates were recorded.

Statistical analyses

CFU values are expressed as mean ± standard deviation (SD). Serum resistance of strains were analyzed by the two-tailed, unpaired t test. Survival rates (%) were expressed as mean ± standard error of mean (SEM). Independent samples T-test was used for intergroup comparisons, specifically, for comparison of serum resistance among isolates of non-MDR, MDR, and XDR strains, or among isolates of non-resistant (susceptible + intermediate) and resistant strain to each tested antibiotic. Fisher's exact test was used to determine the significance of differences (in percentages) between strong and weak serum and drug resistances. Data analyses were performed using SPSS for Windows version 19.0 (SPSS Statistics, Inc. Chicago, IL, USA). $P<0.05$ was considered as statistically significant for all tests.

Results

Antimicrobial susceptibility testing

Among 21 tested agents, resistance to Ticarcillin/Clavulanic acid was most commonly observed (61, 91.04%), and followed by Piperacillin (60, 89.55%), Sulfisoxazole (59, 88.06%), Ciprofloxacin (57, 85.07%), Cefotaxime and Ceftriaxone (56, 83.58%), Gentamicin (54, 62.9%), ceftazidime (54, 80.60%), Piperacillin/Tazobactam and Gentamicin (53, 79.10%), Doxycycline (52, 77.61%), Amikacin (49, 73.13%), Tobramycin (48, 71.64%), Polymyxin B (39, 58.21%), Ceftazidime (36, 53.73%), Cefepime (33, 49.25%), Imipenem (32, 47.76%), Meropenem (25, 37.31%), Levofloxacin (23, 34.33%), Ampicillin/Sulbactam (19, 28.36%), and Gatifloxacin (3, 4.48%). Interestingly, all 67 clinical isolates used in this study were susceptible to Minocycline.

Among 9 categories, resistance to β-lactamase/β-lactamase inhibitor combinations was the most common (61, 91.04%), followed by Penicillins (60, 89.55%), Folate pathway inhibitors (59, 88.06%), Fluoroquinolones (57, 85.07%), Cephems (56, 83.58%), Tetracyclines (54, 80.60%), Aminoglycosides (53, 79.10%), Lipopeptides (39, 58.21%), and Carbapenems (33, 49.25%) (**Figure 1A**).

In the 67 clinical isolates used in this study, only 1 (1.49%) strain was susceptible to all 21 drugs. 4 strains (5.97%) were resistant to 1 category of antibiotic; these were classified as non-MDR in this study. The other 62 clinical isolates were resistant to at least 3 drug categories. 16 isolates (23.88%) were classified as MDR and 46 isolates (68.66%) were classified as XDR (**Figure 1B**).

Molecular typing

A total of 67 clinical *A. baumannii* isolates were typed by MLST analysis. A total of nine defined STs were identified. The proportion of ST208 was 44.8% (30/67), which was the major clonal type. The second place was shared by ST191 and ST195 (9 isolates each), followed by ST136 and ST457 (5 isolates each), ST540 and ST381

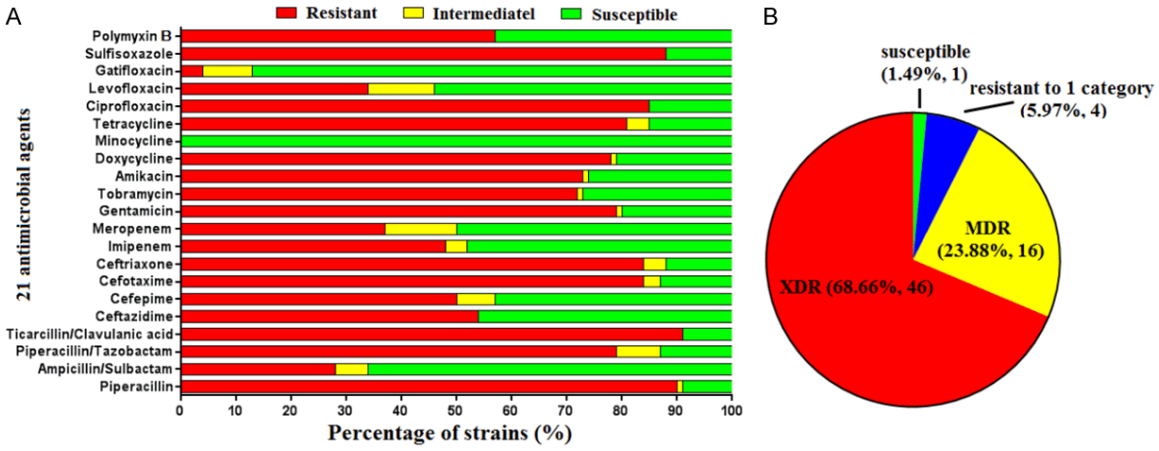

Figure 1. *A. baumannii* isolates susceptibility to antibiotics was examined in this study. A: The percentages of antibiotic resistant (red columns), intermediately resistant (yellow columns), and drug-susceptible (green columns) of 67 *A. baumannii* clinical isolates are shown. B: The percentages of non-MDR (green and blue part), MDR (yellow part) and XDR (red part) of 67 *A. baumannii* clinical isolates are shown.

(3 isolates each), ST730 (2 isolates), and ST541 (1 isolates). Except for ST541 and ST730, the other detected STs in our study were all clustered into CC92. The STs of XDR and MDR *A. baumannii* strains were all belong to CC92. The results suggested that CC92 might cause more widespread nosocomial infections than other strains can.

Serum resistance assays

The susceptibility of 68 *A. baumannii* isolates (67 clinical isolates and reference strain ATCC19606) was evaluated to complement-mediated killing in NHS. Strains in the midlogarithmic growth phase were incubated in NHS or HI-NHS as a control. As shown in **Figure 2**, after incubation for 1 hour, 15 out of the 68 strains, including ATCC19606, AB17, AB19, and AB21, demonstrated significantly lower numbers of surviving bacteria in the NHS group in comparison with the HI-NHS group. These strains were classified as weak serum-resistant strains. The other 53 strains were highly resistant to NHS; these were classified as strong serum-resistant strains.

Antibiotic resistant isolates had stronger serum resistance than susceptible strains

In order to explore whether there's any relationship between antibiotic and serum resistance, the composition of the serum-resistant groups with respect to antibiotic-resistant phenotypes was first analyzed. As shown in **Figure 3A**, the bacteria survival rate in serum indicates the serum resistance level. The 46 XDR strains had stronger serum resistance than 17 MDR strains ($P<0.05$). Among the 53 strong serum-resistant strains, the percentage of XDR isolates (38, 71.1%) was higher than that of 15 weak serum resistant strains (8, 53.33%) ($P=0.007$; **Figure 3B**).

Serum and antibiotic resistance was also analyzed, testing drugs from nine antimicrobial categories. Resistant isolates tested with drugs from 6 of the categories-Penicillins, Cephems, Aminoglycosides, Tetracyclines, Fluoroquinolones, and Folate pathway inhibitors-had higher survival rates in serum compared to non-resistant ones with a significant difference ($P<0.05$; **Figure 4**). As depicted in **Figure 5**, for Penicillins, β-lactamase/β-lactamase inhibitor combinations, Cephems, Aminoglycosides, Tetracyclines, Fluoroquinolones, and Folate pathway inhibitors, the percentages of resistant isolates with strong serum resistance were also much higher than that in the weak serum resistance strains ($P<0.05$).

Finally, the percentages of 21 antibiotic-resistant isolates with strong and weak serum resistance were calculated in order to determine whether serum resistance correlates with resistance to any particular antibiotic. As shown in **Figure 6**, for Piperacillin, Ticarcillin/Clavulanic acid, Cefotaxime, Ceftriaxone, Meropenem, Gentamicin, Tobramycin, Amikacin, Tetracycline, Ciprofloxacin, and Sulfisoxazole, the survival

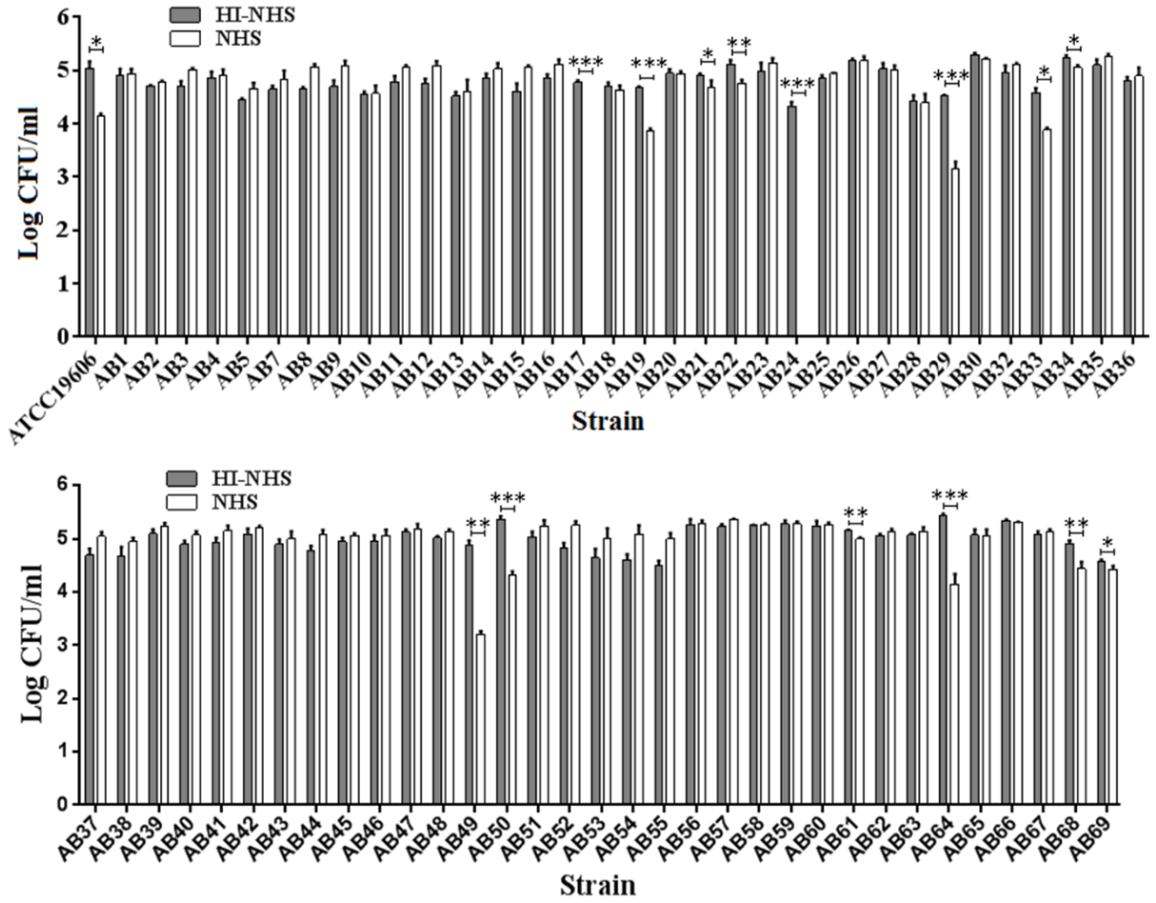

Figure 2. Serum sensitivity of *A. baumannii* strains was tested in NHS and HI NHS environments. The 68 *A. baumannii* strains (67 clinical isolates and reference strain ATCC19606) were incubated for 1 hour at 37 °C in the presence of NHS (white columns) or HI NHS (gray columns). Data represents mean ± SD of three independent experiments. The *p* values were determined using two-tailed, unpaired t-test.

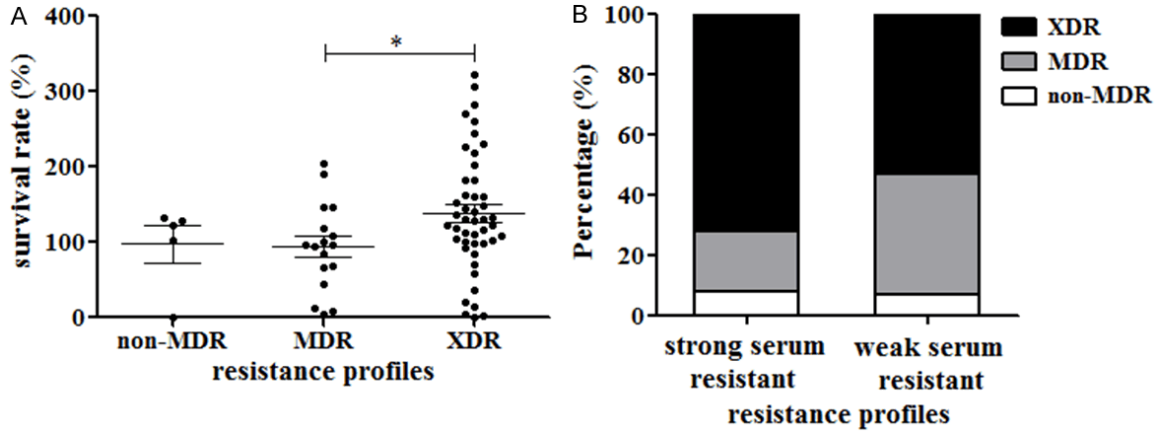

Figure 3. XDR isolates had stronger serum resistance. A: Relationship between antibiotic resistance phenotypes and serum resistance was tested in *A. baumannii strains*. Survival rate % corresponds to serum resistance level. XDR isolates had higher survival rate in serum. Data is presented as mean ± SEM of three independent experiments. The *p* values were determined using independent samples t-test. B: The percentages of isolates with antibiotic resistant phenotypes with strong and weak serum resistance are shown. Bacterial population that exhibited strong serum resistance also contained a larger proportion of XDR isolates. The *p* value was determined using Fisher's exact test.

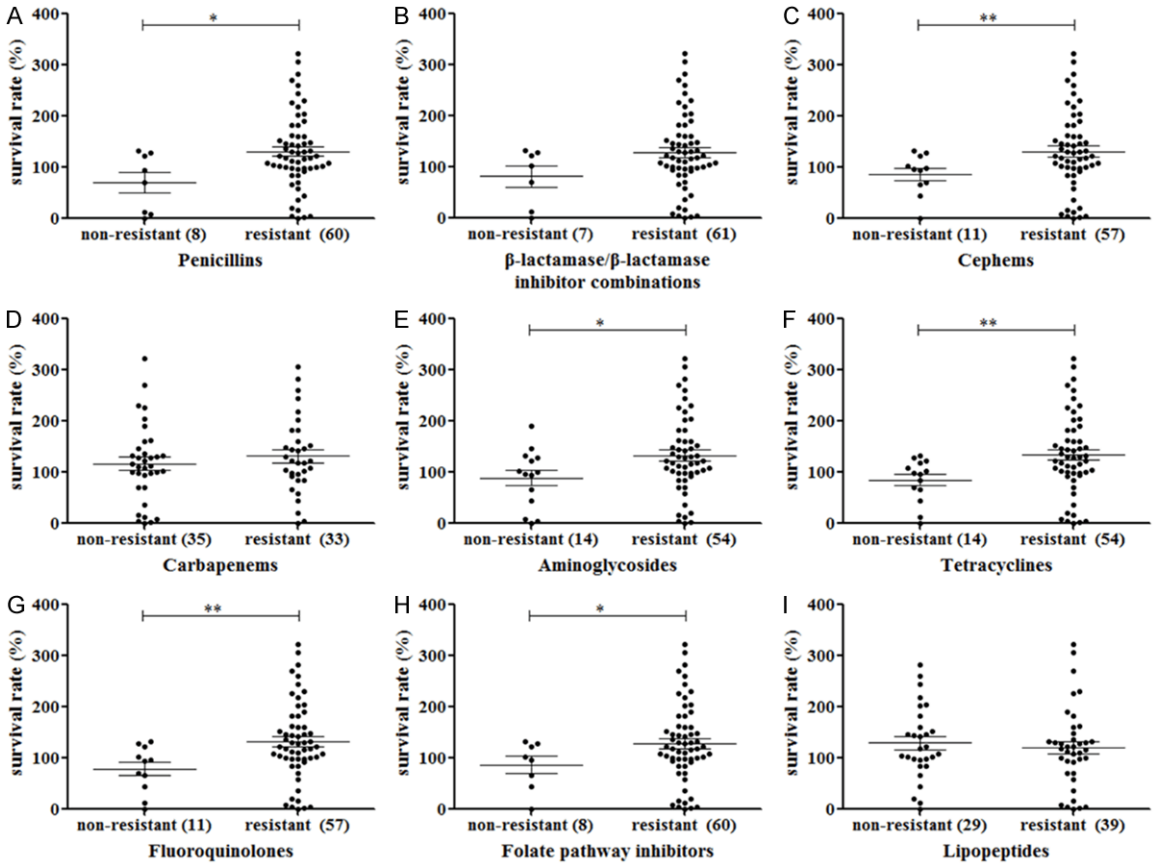

Figure 4. Relationship between serum resistance and the resistance of *A. baumannii* isolates to each of the nine antimicrobial categories. Survival rate (%) represents serum resistance level. For 6 categories (A, C, E-H), resistant isolates tended to have higher survival rates in serum than non-resistant (susceptible + intermediate) isolates. For β-lactamase/β-lactamase inhibitor combinations (B), Carbapenems (D), and Lipopeptides (I), no significant differences in serum resistance among resistant and non-resistant isolates were observed. Data represents the mean ± SEM of three independent experiments. The *p* values were determined using an independent samples T test.

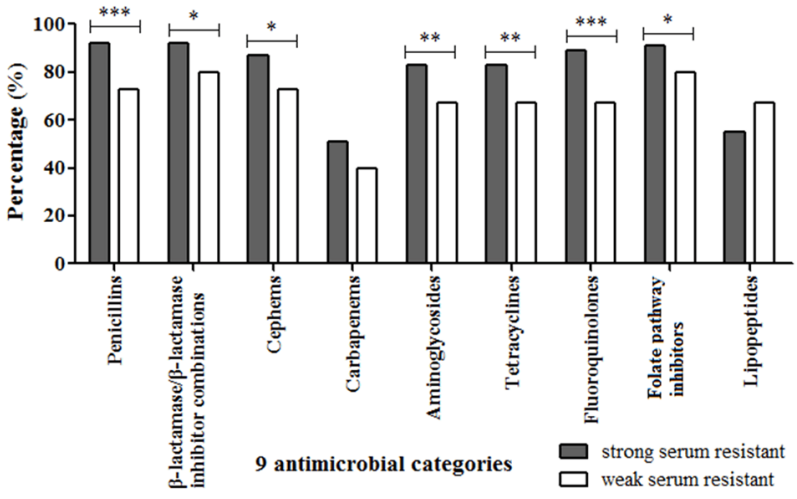

Figure 5. Percentages of isolates resistant to drugs from 9 antimicrobial categories with strong and weak serum resistance are shown. For 7 antimicrobial categories, the percentage of resistant isolates with strong serum resistance were much higher than those with weak serum resistance. No significant differences were found for Carbapenems and Lipopeptides. The *p* values were determined using a Fisher's exact test.

rates of 11 resistant isolates with strong serum resistance were much higher than those with weak serum resistance ($P<0.05$).

XDR isolate inoculation results in higher mortality

The 8-day survival curve following intra-tracheal inoculation is presented in **Figure 7A**. A total of 3 non-MDR (AB17, AB43, AB58), 3 MDR (ATCC19606, AB20, AB26), and 3 XDR (AB9, AB24, AB25) isolates were selected to infect 10 mice in each group, respectively. Control mice were inoculated with saline. No mice di-

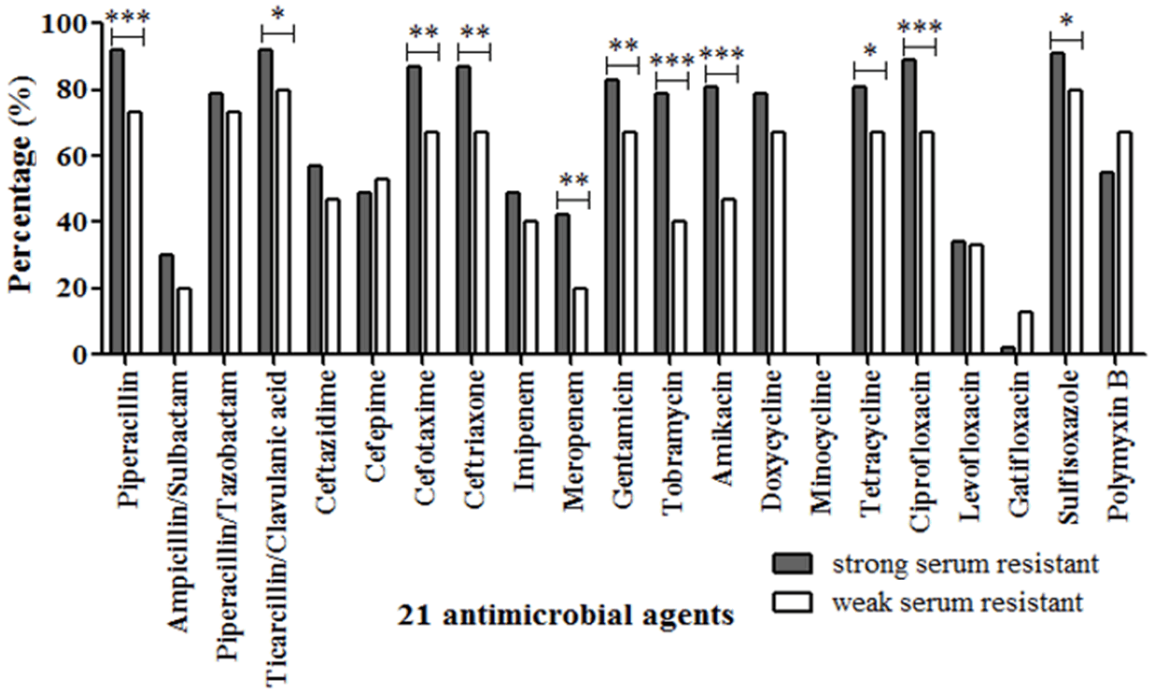

Figure 6. Percentages isolates tested for resistance to 21 antimicrobial agents with strong and weak serum resistance are shown. For 11 antimicrobial agents, the percentage of resistant isolates with strong serum resistance was significantly higher than that of those with weak serum resistance. The *p* values were determined using a Fisher's exact test.

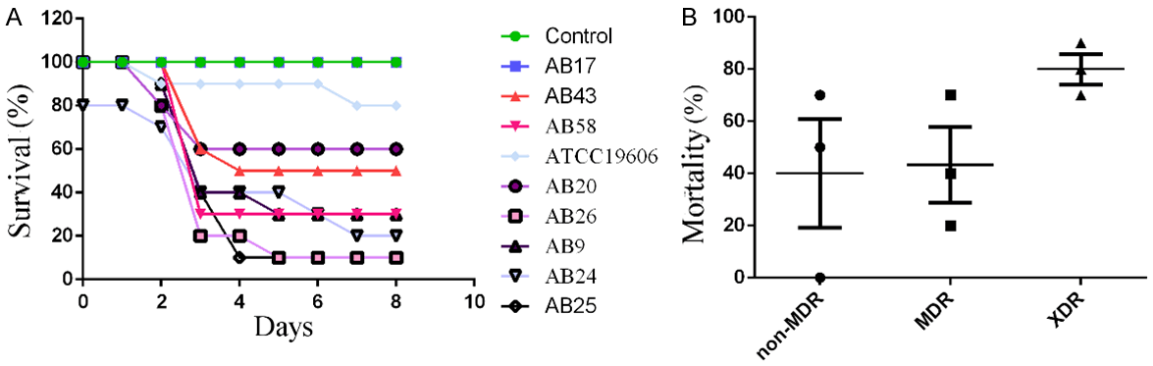

Figure 7. Survival and mortality rates associated with infection of non-MDR, MDR, and XDR strains in mice. A: The survival rates of mice infected by non-MDR (AB17, AB43, AB58), MDR (ATCC19606, AB20, AB26), and XDR (AB9, AB24, AB25) *A. baumannii* strains are shown. B: The mortality rate of mice infected by different antibiotic resistance phenotypes of *A. baumannii* within 7 days are shown. Data represents mean ± SEM of 3 groups. The *p* values were determined using an independent samples T test.

ed within 8 days. This showed that the damage caused to the mice by the procedure was almost insignificant. The death of mice in the experimental group was unrelated to the damage caused during the procedure. An acute onset of infection occurred on the third day after inoculation and the highest mortality for all experimental mice was observed on the third day. The final survival rates were as follows: AB17-100%, ATCC19606-80%, AB20-60%, AB43-50%, AB9 (AB26, AB58)-30%, AB24-20%, and AB25-10%. As shown in **Figure 7B**, the mortality of mice caused by XDR was higher than that caused by MDR and non-MDR isolates.

Discussion

The drug resistance of *A. baumannii* has increased severely in recent years. In this study, *A.*

baumannii ATCC19606 was used as the reference strain. The drug resistance tests showed that ATCC19606 is a multidrug resistant strain, which was consistent with the previous report [26]. The carbapenems antibiotics (imipenem and meropenem) are the main choices for the treatment of serious infections caused by *A. baumannii* [27]. Their observed resistance rates were 47.76% and 37.31%, respectively, which was lower than the results obtained by CHINET (62.4% and 66.7%) [8]. However, the drug resistance rate to polymyxin B was 58.21% in our study, which was much higher than the CHINET result (1.9%). In some cases, polymyxin B is the only choice for the treatment of multidrug resistant *A. baumannii* infection [28]. The proportions of MDR and XDR *A. baumannii* strains in this study were 23.88% and 68.66%, respectively. These data indicate that the resistance profile of *A. baumannii* in China was mainly represented by XDR, which poses more serious problems during clinical treatment. All the 67 *A. baumannii* clinical isolates tested in this study were sensitive to minocycline, which may be caused by regional factors. This data indicates that minocycline can be used as an effective drug for the treatment of *A. baumannii* infection in China.

MLST is a good tool for global and long-term epidemiological studies. In this study, CC92 was the most widely distributed *A. baumannii* clone in China, which is also the largest and most widespread CC in the world. More than 132 STs were belonged to CC 92, and in many countries of Asia, North America, Europe, and Oceania have detected these STs [29-31]. ST92 is the ancestral ST of CC 92 [31]. Before 2011, ST92 was the most prevalent strain in most regions [32]. This study confirmed that ST208 may be the most widespread strain in China at this time, and could be used as a severe epidemic marker.

ICU patients infected by *A. baumannii* suffer from severe nosocomial infections including pneumonia, urinary tract infections, bloodstream infections, meningitis, and wound infections [1]. The mortality rate caused by *A. baumannii* is higher than that of other diseases. The nature of bacteremia is associated with the ability of *A. baumannii* strains to resist with stand the effect of complement in normal human serum [19, 20]. The complement system is an essential and effective part of the innate immune system present in normal human serum. Some bacterial factors may provide opportunities for bacteria to escape the complement system. Due to the long coevolution of bacteria and its host, some of the most successful pathogens have developed effective mechanisms for attenuating or escaping complement attack [33]. A previous report showed that 8 (75%) out of 12 strains of *A. baumannii* could grow in serum [34]. Sanchez-Larrayoz et al. found that 12 (80%) out of 15 strains of *A. baumannii* were highly resistant to the NHS complement system [24]. Similarly, in our study, 53 (77.94%) out of the 68 strains of *A. baumannii* resisted the NHS system.

The mortality rate due to infection by *A. baumannii* within 30 days is as high as 61.6% [18]. Some strains are able to cause severe bacteremia because of their ability to resist the complement system in NHS [19, 20]. The association between antibiotic and serum resistance of *A. baumannii* strains was analyzed for the first time. XDR isolates had stronger serum resistance than MDR and non-MDR strains. The percentage of XDR isolates with strong serum resistance was higher than that of those with weak serum resistance, which was consistent with the study that the frequency of serum-resistant isolates was higher among ESBL-producing strains than among non-ESBL-producing strains [35]. Isolates resistant to 6 drug categories had higher survival rates in serum than non-resistant ones. The percentage of isolates resistant to 7 drug categories with strong serum resistance was much higher than that with weak serum resistance. Out of 21 antibiotics that were tested, the percentage of isolates resistant to 11 of them with strong serum resistance was much higher than that of those with weak serum resistance. As stated above, resistant strains have stronger serum resistance. The observed effect may be due to the acquisition of plasmids under antibiotic pressure by conjugation, which could increase the ability of strains to survive against serum [36]. These data suggest that the levels of drug resistance of *A. baumannii* and its level of serum resistance increase together.

When bacteria infects the host *in vivo*, it is exposed to a complex and diverse environment [37]. The complex immune response can't be completely replicated *in vitro*. Thus, the *in vivo* mice model was used to detect the relationship

between drug resistance and mortality from inoculation with *A. baumannii*. In this study, the mice pneumonia model was established by a tracheal intubation method. This resulted in a high success rate, low damage to mice, and high repeatability [38]. As *A. baumannii* is a low-virulence strain, the immune system in normal mice can inhibit the infection. Therefore, the cyclophosphamide was used before *A. baumannii* inoculation to develop neutropenia of short duration in order to facilitate the onset of the infectious process [25, 39]. During the onset of infection in mice, symptoms such as weight loss, decreased mobility, hair loss, and slow breathing were observed. The euthanized, diseased mice were dissected and the number of bacteria in the lungs, spleen, and blood were counted. The results show that approximately 2×10^{10} CFU/g of bacteria were detectable in the lung and approx. 1.5×10^{2} CFU/g were detectable in the spleen. However, nearly no bacteria was detected in the blood. This results were similar to those previously reported [40, 41]. The mortality rate of mice infected by XDR was much higher than that of those infected with MDR and non-MDR isolates. The results suggest that there might be a correlation between antimicrobial resistance and virulence. Hennequin's group reported that the virulence of clinical strains could be increased by the plasmid acquisition of transcriptional factors under antibiotic pressure [42]. For K. pneumoniae, acquisition of ESBL-encoding plasmids increase the virulence potential of the strains because sometimes the gene encoding ESBLs were located on plasmids also encoding virulence factors [36]. Thus, acquisition of antibiotic resistance plasmids by the bacterium could increase its virulence potential. However, further studies are necessary to be done to clarify the mechanisms between antimicrobial resistance and virulence in *A. baumannii*.

In conclusion, this study demonstrates the positive correlation between antibiotic and serum resistance of *A. baumannii* strains for the first time. Inoculation of the drug- and serum-resistant strains of *A. baumannii* resulted in high mortality in mice indicating adverse trend in bacterial evolution. Further detailed investigation of the mechanism of linkage between antibiotic and serum resistance is required to overcome complications with treatment of drug-resistant infection.

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Disclosure of conflict of interest

None.

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