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Original Paper

Whole-Genome Analysis of an Extensive Drug-Resistant *Acinetobacter Baumannii* ST195 Isolate from a Recipient After DCD Renal Transplantation in China

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Key Words
Dcd transplantation • *Acinetobacter baumannii* • Drug-resistant • Whole-Genome Analysis

Abstract
Background/Aims: Infection with *Acinetobacter baumannii* was emerging as one of the leading causes of mortality after donation after cardiac death transpalantion. ***Methods:*** We reported a case of a recipient who underwent DCD renal transplantation and later got infected by A.baumannii. Etests were done to verify the susceptibility test results in clinic. Whole-genome analysis was applied to investigate the resistant mechanism at gene level. ***Results:*** The pathogen was isolated from his draining liquid the day after the surgery, and susceptibility test reavealed that it was sensitive to tigecycline. However, the isolate obtained from the draining liquid became tigecycline-resistant after fifteen-day administration of tigecycline. The Susceptibility tests showed that the pathogen recovered from tigecycline resistance and became intermediated to tigecycline. Whole-Genome analysis revealed the genetic

H. Jiang and L. Cao contributed to and H. Jiang, J. Chen, and J. Wu supervised this work equally.

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level change leading to tigecycline resistance and we identified the location of mutation by comparing the whole genome sequence of the isolates. Three loci were figured out which may contribute to drug resistance, including genes encoding HTH domain protein, MFS transporter and AdeS. ***Conclusion:*** Understanding the genetic characteristics associated with drug resistance mechanism and antimicrobial profiles of pathogen is important in controlling infection outbreak and preventing serious complications and gives a new insight into the development of antimicrobial agents.

Introduction

Acinetobacter baumannii is a non-fermenting Gram-negative bacterium, widely distributed in soil, water, hospital environment and human skin, of which some can be an opportunistic pathogen in human and others just acting as colonizing or environmental organisms. With a strong capacity for clonal transmission and acquirement of drug-resistance gene, multidrug-resistant *A.baumannii* (MDRAB), extensively drug resistant *A.baumannii* (XDRAB) and pan drug resistant *A.baumannii* (PDRAB) emerged and became worldwide prevalent [1]. As one of the ESKAPE pathogen (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species) [2], it is responsible for a large amount of nosocomial infections in China.

Data from CHINET (a surveillance network of drug resistant bacteria in China) showed that the clinical isolation rate of *A.baumannii* strains in 17 university hospitals was 11.11% (8769/78955) in 2014, ranking first in non-fermenting Gram-negative bacilli and third in all identified isolates. In addition, XDR strains are mainly identified among *A.baumannii* isolates and *klebsiella pneumonia* isolates [3]. Infections caused by *A.baumannii* are common in critical patients which also acts as one of the leading causes of mortality, and are often accompanied with other bacterial infection and/or fungal infection [4, 5]. With increasing reports of outbreaks and antibiotic resistance [6-11], treatment of infection induced by *A.baumannii* becomes a challenge in clinic practice.

Infection with multiresistant bacteria has become a leading cause of mortality in donation after cardiac death (DCD) renal transplant recipients. Patients in intensive care unit (ICU), as the main source of DCD donors, are susceptible to serious infection. Long-term use of high dose antibiotics leads to a high risk of developing nosocomial multiple drug-resistant (MDR) bacteria infection and extensive drug-resistant (XDR) bacteria infection. *A.baumannii* is one of the most common pathogens associated with infection after renal transplantation. Development of resistance to carbapenems leads to the dilemma of options for antimicrobial treatment in clinical practice. Besides, increasing occurrence of tigecycline resistance is emerging due to antimicrobial overuse.

Cases acquiring tigecycline resistant *A.baumannii* infection after renal transplantation have rarely been reported, yet events are emerging in clinical practice. Gail E. Reid et al. first reported a case of a 53-year-old female kidney and liver transplantation recipient who developed a multidrug-resistant *Acinetobacte baumannii* urinary tract infection in which tigecycline resistance occurred after a short term of tigecycline exposure [12]. However, lack of genomic information and molecular biological data of the isolate in this report makes it difficult to trace the details of mutagenesis.

Herein we reported a case of DCD renal transplant recipient who was infected by MDRAB after surgery in the Kidney transplant ward. With wide application of whole genome sequencing technique, we were able to identify the origin of infection and infer the putative transmission route with support of other laboratory data of the outbreak [13]. In this study, we made further investigation using WGS to identify the location of mutations involved in the development of tigecycline resistance with the intention to provide evidence

supporting clinical countermeasure. Furthermore, as variants of drug-resistant bacteria are increasing, new mechanism of drug resistance should be considered in antibiotics research and development to prevent spread of superbacteria.

Materials and Methods

Isolates and Antimicrobial susceptibility testing. The 3 *A.baumannii* isolates (AB0915, AB0929, AB1006) were from drainage fluid samples of a DCD renal transplantation recipient. Sample collection was done from September 2014 to October 2014 in kidney transplant ward of kidney disease center, the First Affiliated Hospital, College of Medicine, Zhejiang University. Etest of tigecyline was applied on ISO medium (Oxoid) using etest strips (bioMérieux). The minimum inhibitory concentrations (MICs) of tigecycline was determined according to the manufacture's instruction. The results were interpreted on the basis of the FDA criteria for Enterobacteriaceae (http://www.fda.gov/downloads/animalveterinary/safetyhealth/antimicrobialresistance/nationalantimicrobialresistancemonitoringsystem/ucm453387.pdf) or EUCAST criteria for Enterobacteriaceae, since CLSI and EUCAST do not suggest breakpoints for tigecycline against *A. baumannii*.

Pulsed-field gel electrophoresis (PFGE). PFGE was performed as described previously [13]. Briefly, Genomic DNA was digested with ApaI restriction enzyme (Sangon, Shanghai, China). *S.enterica* serovar Braenderup H98912 was used as the molecular size marker [14]. The electrophoresis condition was 6.0V/cm with an initial switch time of 5s to a final switch time of 35s and an alternating pulses of 120° angle at 14°C in 0.5×Tris-borate-EDTA buffer for 22hrs.

Whole genome sequencing (WGS), Mutation Detection and Gene identification. The three *A.baumannii* isolates were whole-genome sequenced. Briefly, genomic DNA was extracted and WGS was performed using next-generation sequencing via 2×125bp paired-end sequencing (Illumina, San Diego, CA, USA). Sequence contigs were mapped to de novo assembly and further analyzed to detect location of the single nucleotide variants between isolates using CLC Genomics Workbench7.0 (CLCbio, Denmark). False SNVs were excluded via self-mapping. ST was calculated using CGE server (https://cge.cbs.dtu.dk/services/MLST/). Genes were identified by searching a nucleotide database using a nucleotide query with BLAST. Homologs of the corresponding gene products were analyzed using BLASTX. Amino Acids change was figured out by DNAman. The WGS data of the isolates were deposited to NCBI genebank (Bioproject: PRJNA322764). The accession number was SAMN05178517 for AB1006, SAMN05178483 for AB0929, and SAMN05178265 for AB0915.

Results

Case report

A 39-year-old man underwent DCD kidney transplantation for end-stage renal disease. The donor was infected by *Klebsiella pneumoniae* in the lung and *Pseudomonas aeruginosa* in blood, both of which were tigecycline sensitive according to the results of antimicrobial susceptibility tests. A 100-mg loading dose of intravenous tigecycline was given to the recipient in case of postoperative infection on the same day of the surgery (D0), followed by 50mg every 12 hours. Also a 50-mg dose of caspofungin was used for prevention of infection against fungi. Subsequently the patient experienced an XDRAB local infection (AB0915) at drainage site the next day after the transplantation (D1). The results of antimicrobial susceptibility testing can be found in our previous study [13]. The patient's serum WBC and neutrophil count increased. The isolate was sensitive to tigecycline, so the previous antimicrobial therapy containing tigecycline continued with the addition of imipenem-cilastatin and levofloxacin. The patient suffered tenderness and ecchymosis in skin around the tube and experienced prolonged fever and diarrhea.

Fifteen days after the surgery (D15), a drainage fluid culture was acquired and grew tigecycline-resistant *A.baumannii* (AB0929), with similar drug resistant pattern of other

antibiotics to the previous drainage isolate of *A.baumannii*. Tigecycline was discontinued and a 1.0-g dose of cefoperazone-sulbactam every 6 hours (×2 days) and a 0.5-g dose of sulbactam every 8 hours (×2 days) were administered, followed by 1.0-g every 12 hours and 1.5-g every 8 hours respectively.

Interestingly, culture of drainage fluid grew *A.baumannii* (AB1006) intermediate to tigecycline on the twenty second day after the transplantation (five days after tigecycline discontinuation, D22). The therapy with cefoperazone-sulbactam and sulbactam successfully resolved the infection.

On the other hand, the patient also received immunosuppressive therapy, since he underwent renal transplantation. The dose of methylprednisolone was adjusted on the basis of the state of *A.baumannii* infection and renal function. MMF and FK506 was also given orally subsequently in addition to the immunosuppressive therapy. The patient was discharged after the resolution of infection, and accepted MMF, FK506 and prednisone as maintainance therapy for anti-rejection treatment.

The patient was hospitalized for a total of 52 days and his renal function recovered when he discharged (Fig. 1).

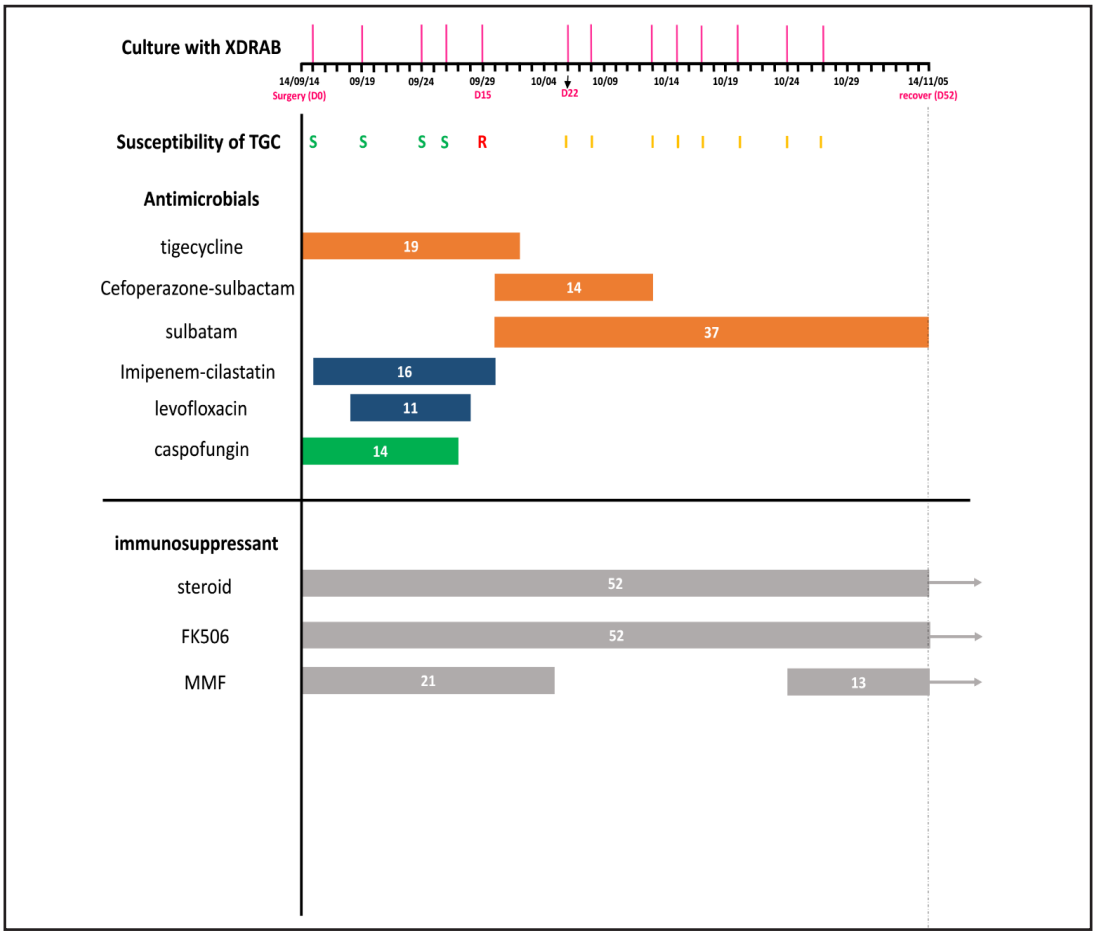

Fig. 1. Timeline of events in antimicrobial and immunosuppressive therapy and positive cultures of A.baumannii. The length of chromatic bars represents for the duration of antimicrobial administration and grey bars stands for immunosuppressive therapy. The dates of positive culture are marked with vertical bars on the top of timeline. The day on which transplantation was done is labelled D0 and so forth. A 100-mg loading dose of intravenous tigecycline was given to the recipient on D0 with the administration of a 50-mg dose of caspofungin. Tigecycline was discontinued after the culture of tigecycline-resistant A.baumannii and the administration of cefoperazone-sulbactam and sulbactam were begun.

Pulsed-field gel electrophoresis (PFGE)

The 3 *A.baumannii* isolates (AB0915, AB0929, AB1006) were obtained from the biobank of the First Affiliated Hospital, College of Medicine, Zhejiang University. PFGE showed that AB0929 was distinguished from AB0915 by one band as described in the previous research (data not shown) [13].

Antimicrobial susceptibility testing

The antimicrobial susceptibility testing results of the patient who underwent DCD transplantation was determined by automated system, VITEK® 2 (bioMérieux) in the laboratory of the hospital, when he was hospitalized (Fig. 2A). Susceptibility testing of AB0915 conducted by VITEK® 2 revealed the inhibition zone diameter of tigecycline was 22mm, suggesting sensitive according to the manufacture's construction. The *A.baumannii* isolate obtained from drainage fluid kept sensitive until D15 when the susceptibility testing revealed the MIC of AB0929 was 48ug/ml, suggesting tigecycline-resistant. AB1006 turned intermediate to tigecycline with the MIC of 24ug/ml.

According to the suggestion of Clinical and Laboratory Standards Institute (CLSI), Etest was recomended to confirm the MIC for XDRAB so as to provide more valuable information for clinical practice. The MIC values of the three isolates from the three susceptibility periods (sensitive-resistant-intermediate) were 1.5ug/ml, 12ug/ml and 1.5ug/ml

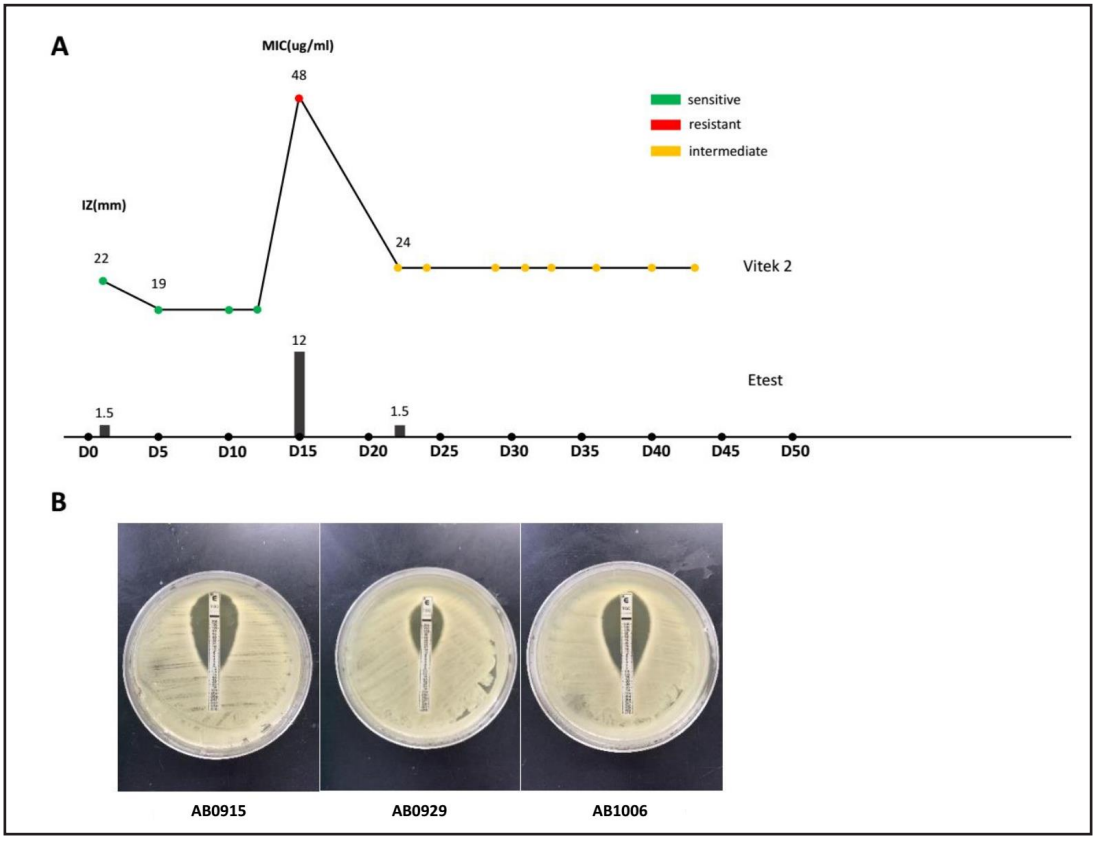

Fig. 2. A. Results of antimicrobe susceptibility testing determined by automated system and etest. The broken line graph in the upper part consists of two parts which were determined by two different programmes of VITEK® 2. Suscepitibility to tigecycline of first four points was represented in the form of inhibition zone (mm), but the points hereafter was represented in the form of MIC (ug/ml). The vertical bars in the bottom represent the MIC value obtained by etest. B. Results of etest. Etest was done to verify the susceptibility to tigecycline in the three clinical isolates of A.baumannii. The MIC (ug/ml) value was determined on the basis of the manufacture's instruction.

respectively (Fig. 2B). Interpretation of susceptibility testing results of tigecycline against *A.baumannii* has been controversial. Actually, the less specific breakpoints suggestion of FDA for Enterobacteriaceae (susceptible:2mg/L; resistant:8mg/L) or the European Committee on Antimicrobial Susceptibility Testing (EUCAST) criteria for Enterobacteriaceae (susceptible:1mg/L; resistant:2mg/L) is widely used by researchers so far [15].

WGS and Comparative Genome Analysis

Whole-genome sequencing of the 3 single *A.baumannii* isolates was performed, in hopes of infering the mechanism of resistance to tigecycline by figuring out the difference in the single nucleotide variants (SNVs). Comparative Genome Analysis revealed that there are 2 SNVs between isolate AB0929 and AB1006, 1 SNV between isolate AB0915 and AB1006, 3 SNVs between isolate AB0929 and AB0915 (Fig. 3). All three isoaltes belonged to multilocus sequence typing ST195. The BLASTX results revealed that the variant found between isolate AB0915 and AB1006 was associated with helix-turn-helix (HTH) domain protein and the 2 SNVs found between isolate AB0929 and AB1006 were associated with the major facilitator superfamily (MFS) transporter and AdeS respectively. Reasonably, the 3 SNVs between isolate AB0929 and AB0915 were just located in the same genes as the three genes mentioned above, of which the one related to AdeS probably played the most important role in the transition of antimicrobial susceptibility. The mutation occurring in AdeS gene led to amino acid substitution at 329th locus (Glu→Ala) in the encoded product (Fig. 4).

Discussion

Genes related to antimicrobial resistance of *A.baumannii* can be intrinsical or acquired by transformation. The main resistance mechanisms identified in nosocomial strains of acinetobacter include degradation of drugs by enzymes (β-lactamases, enzymes inactivating aminoglycosides), permeability alterations of outer membrane, efflux pumps, modification or defence of antibiotic targets [16], among which efflux pumps are the most relevant

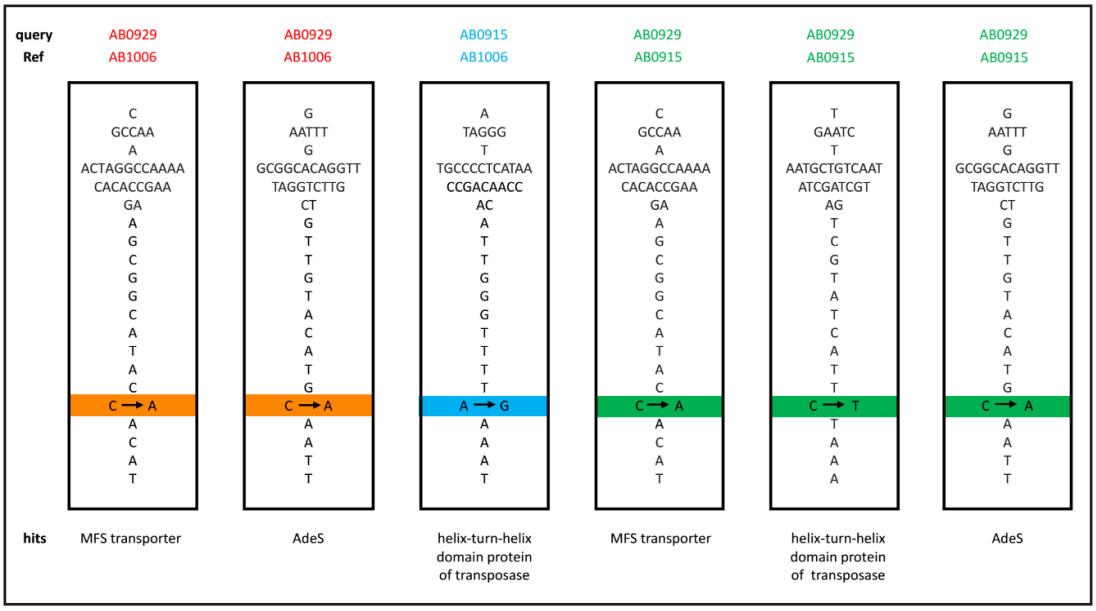

Fig. 3. Mutations detected in the three clinical A.baumannii isolates. Each row corresponds to a genomic site with a SNV detected by genome comparion between every two isolates using CLC Genomics Workbench. SNVs are indicated by colored shading. The "hits" column indicates within which coding region of a gene the detected SNVs lie.

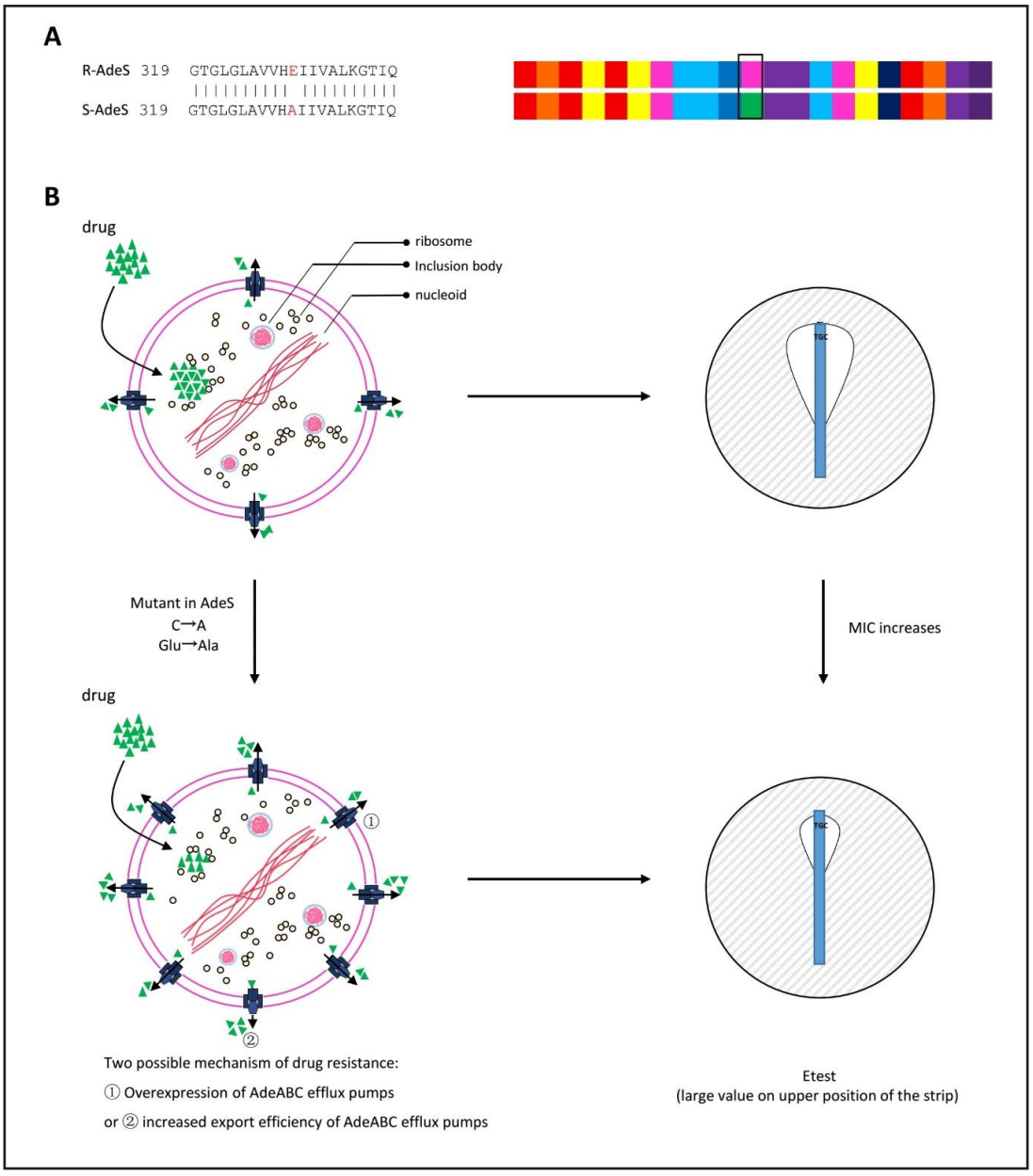

Fig. 4. A. The change of AdeS in amino acid sequence. The 329th Glu in the encoded product of AdeS was putatively substituted by Ala as a result of the mutation. Each color represents for an amino acid. B. Mechanism of tigecycline resistance in A.baumannii isolate AB0929. Tigecycline works as a protein synthesis inhibitor which can bind to the 30S ribosomal subunit of bacteria, thus preventing its interaction with aminoacyl-tRNA and disturbing the synthesis of protein in bacteria. Once the concentration of tigecycline in bacteria decreases, the interaction between the drug and the ribosome will be weakened, leading to the loss of efficacy. The mutation in AdeS occurring in AB0929 caused the putative substitution of the Ala for Glu at 329 locus and might lead to functional change in the gene product, thus resulting in overexpression or increased export efficiency of AdeABC efflux pump. The MIC value increased since more drugs were pumped out of the bacteria.

mechanism in tigecycline-resistant bacteria according to current reports. Resistance-nodulation-division (RND) family was one of the five classes of efflux pumps mostly reported. The RND superfamily, especially AdeABC, AdeFGH and AdeIJK, which display a broad range

of substrates (tigecycline included), have been widely reported to be associated with MDR in *A.baumannii*. AdeABC is the most relevant RND family member involved in tigecycline-resistance of clinical isolates [17].

Herein WGS was used to assess genome dynamics in the 3 *A.baumannii* isolates and understand the decreased and reversed susceptibility to tigecycline at genetic level. The mutation involved in this study was located in a sensor kinase namely AdeS [18]. AdeRS is a two-component system (TCS) located upstream from AdeABC genes. The three genes of AdeABC are cotranscripted and strictly regulated by the TCS AdeRS. Overexpression of efflux pumps is considered to be responsible for reduced accumulation of antimicrobial agents and leads to an increase in MIC (the lowest inhibiting concentration of an antimicrobial agent to an micriobe) compared to that for the primary strain. Increase in MIC induced by such mutants is usually 2 to 8 fold higher than that for the susceptibile strain and less than 100-fold which is deemed to result from either expression of antibiotic inactivating enzymes or changes in antimicrobial target protein [19].

Several amino acid alterations in the three RND superfamily members of *A.baumannii* have been described in efflux pumps overexpression. Mutations associated with the TCS AdeRS have been reported to be responsible for overexpression of the AdeABC and tigecycline-resistance [20-22]. Yet the signals remain unclear and the regulating mechanism is still under investigation. We reported a mutation leading to amino acid substitute at 329th locus (Glu→Ala) of the encoded product in AdeS. However, its further influence on expression or export efficiency of AdeABC and tigecycline susceptibility still needs to be investigated, in consideration that researches also implicated no significant correlation between overexpression of AdeABC system and alterations in the TCS AdeRS [20, 21, 23, 24].

The correlation between other efflux pump superfamilies and tigecycline nonsusceptibility in *A.baumannii* has not been clearly understood. Though TetA, TetB, TetM are thought to play a role in the tetracycline resistance mechanism [25], yet no sufficient data is available to prove its impact on the decreased susceptibility of glycylcyclines [26]. Hood [27] and Hua [28] reported that the MFS transporters were upregulated in response to tigecycline, implying that they might play a role in antimicrobial activity of tigecycline. Also, DNA-binding HTH domain-containing protein genes were upregulated in the presence of tigecycline [28], but no laboratory data is available to demonstrate its influence on tigecycline susceptibility of clinical *A.baumannii* isolates. Studies have discovered other mechanisms involved in tigecycline resistance as well, including TetX1 gene [29], SAM-dependent methyltransferase [30], 1-acyl-sn-glycerol-3-phosphate acyltransferase [31], and outer membrane proteins [32].

We pay more attention to the clinical consequence that the mutant-induced resistance brings in MDR bacterial infection. Treatment with tigecycline, probably leading to increasing MIC [24], might be regarded as a kind of stress response. In this study, we suppose that tigecycline susceptibility of *A.baumannii* could be influenced by multifactors. Mutations in response to tigecycline stress might play an vital role, especially those associated with drug efflux pumps. We suppose that the mutation in efflux pump gene might make a great contribution to the tigecycline nonsusceptibility, either because of an increased expression or export efficiency of AdeABC efflux pump. The intracellular concentration of tigecycline was lowered and *A.baumannii* became less susceptible to it. Furthermore, automated system, as a widely applied susceptibility testing method, has been questioned for its accuracy in determining the susceptibility to antimicrobials of clinical isolates [33-35]. It is also an important issue needed to be considered by clinicians in deciding the anti-infection therapeutic regimen, especially for those induced by multi-resitant bacteria, to ensure the treatment is effective and efficient. Since antimicrobial agents active against MDRAB and XDRAB are limited, we cannot emphasize too much the importance of investigating the drug-resistant mechanism and developing comprehensive intensive infection control strategy, the former facilitating the development of new antimicrobial agents.

Disclosure Statement

All authors have no potential conflicts of interest to disclose.

Abbreviations

WGS (Whole-Genome Analysis); MDRAB (Multidrug-resistant A.baumannii); XDRAB (Extensively Drug resistant A.baumannii); PDRAB (Pan Drug resistant A.baumannii); ESKAPE (pathogen, Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, and Enterobacter species); XDR (Extensively Drug-Resistant); MDR (Multiple Drug-Resistant); ICU (Intensive Care Unit); MMF (Mycophenolate Mofetil); FK506 (Tacrolimus); CLSI (Clinical and Laboratory Standards Institute); PFGE (Pulsed-Field Gel Electrophoresis); EUCAST (European Committee on Antimicrobial Susceptibility Testing); MIC (Minimum Inhibitory Concentration); SNVs (the Single Nucleotide Variants); HTH (Helix-Turn-Helix); MFS (Major Facilitator Superfamily); RND (Family, Resistance-Nodulation-Division Family); ABC (Superfamily, the ATP-Binding Cassette Superfamily); TCS (Two-Component System).

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Luxi did PFGE, comparative genome analysis and the manuscript writing. Qixia, Bingjue and Miao Chen read medical records and collected clinical data. Lihui, Tingting, Guangjun and Rending performed strains isolation and storage. Yanfei conducted the susceptibility test. Yan supervised computational work. Yunsong gave advice on experiment conduction. Yingying, Jian and Chaohong prepared the figures with guidance from Yan. Yucheng and Shi helped with Luxi experiments. Junwen supervised the manuscript writing. Jianghua contributed to the design. Hong and Jianyong conceived and supervised the study, designed and interpreted experiments and supervised the manuscript writing. Chen, Wu and Jiang jointly supervised this work. All authors reviewed the manuscript.

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