Genetic characterization and *in vitro* activity of antimicrobial combinations of multidrug-resistant *Acinetobacter baumannii* from a general hospital in China

FANG CHEN[1], LING WANG[1], MIN WANG[1], YIXIN XIE[1], XIAOMENG XIA[2], XIANPING LI[1], YANHUA LIU[1], WEI CAO[1], TINGTING ZHANG[1], PENGLING LI[1] and MIN YANG[1]

Departments of [1]Laboratory Medicine and [2]Obstetrics and Gynecology, The Second Xiangya Hospital, Central South University, Changsha, Hunan 410011, P.R. China

Received October 1, 2016; Accepted October 26, 2017

DOI: 10.3892/ol.2017.7600

Abstract. The present study aimed to develop a rational therapy based on the genetic epidemiology, molecular mechanism evaluation and *in vitro* antibiotic combinations activity in multidrug-resistant *Acinetobacter baumannii* (MDRAB). MDRAB was screened by the Kirby-Bauer method. The random amplified polymorphic DNA technique was used to establish genetic fingerprinting, and a series of resistance genes were detected by polymerase chain reaction. Antimicrobial agents including amikacin (AK), cefoperazone/sulbactam (SCF I/II), meropenem (MEM), minocycline (MINO) and ciprofloxacin (CIP) were used to determine the minimum inhibitory concentrations (MICs) and interactions between antibiotics by the broth microdilution method and chequerboard assays. In total, 34 MDRAB strains were isolated and classified into 8 phenotypes A-H, according to their general drug susceptibilities. A total of 4 major genotypes (I-IV) were clustered at 60% a genotypic similarity threshold. High positive rates of β-lactamase *TEM-1*, topoisomerase IV, oxacillinase (*OXA*)-23, AdeB family multidrug efflux RND transporter *adeB*, β-lactamase *AmpC*, class 1 integrons (*Int-1*), 16S rRNA methylase *rmtA*, phosphotransferase *aph*(3), 16S rRNA methyltransferase *armA* were presented to exceed 90%, acetylyltransferase *aac(3)-I*, *aac(6'-I*, *ant(3'')-I*, 16S rRNA methylase *rmtB*, oxacillinase *OXA-24* and metallo-β-lactamase *IMP-5* genes demonstrated positive rates of 29.4-85.29%, while *adeRS* two-component system was not observed in any strain. MEM+SCF I or SCF II primarily exhibited synergistic effects. AK+SCF I, AK+SCF II, MINO+SCF I, MINO+SCF II, MINO+CIP and MINO+MEM primarily presented additive effects. AK+CIP demonstrated 70.59% antagonism. The antibacterial activity of SCF I was superior compared with that of SCF II. The results indicated the polyclonal genetic epidemiological trend of MDRAB in the Second Xiangya Hospital, and verified the complexity of genetic resistance. In addition, combinations suggested to be efficacious were MEM+SCF I and MEM+SCF II, which were more effective compared with other combinations for the management of MDRAB infection.

Correspondence to: Dr Min Wang, Department of Laboratory Medicine, The Second Xiangya Hospital, Central South University, 139 Middle Renmin Road, Changsha, Hunan 410011, P.R. China
E-mail: wangmin0000@csu.edu.cn

Key words: multidrug-resistant *Acinetobacter baumannii*, resistance genes, *in vitro* antimicrobial activity, antibiotic interaction

Introduction

Acinetobacter baumannii, a leading nosocomial pathogen, has been identified to induce serious infections and high mortality rates in intensive care units (ICUs) (1,2). Multidrug-resistant *Acinetobacter baumannii* (*A. baumannii)* (MDRAB), with resistance to at least three classes of antibiotics among cephalosporins, carbapenems, β-lactamases, aminoglycosides and quinolones (3), is only susceptible to certain agents such as tigecycline, and polymyxins due to intrinsic and acquired resistance (4,5). In addition, several pandrug-resistant (PDR) *A. baumannii* strains with resistance to almost all available antibiotics have been identified in previous decades (6,7).

Abuse of broad-spectrum antibiotics has been demonstrated to be a major cause for the development of drug resistance of *A. baumannii*. At present, a number of genes responsible for drug resistance have been identified though long-term studies on the mechanisms of bacterial resistance (7-10). Production of β-lactamase is suggested to be associated with the bacterial resistance to penicillin, cephalosporins and carbapenems (9). At present, four major categories have been available for the β-lactamase protein-encoding genes, including narrow-spectrum β-lactamase, extended-spectrum β-lactamase (*ESBLs*), metallo-β-lactamases (*MBLs*) and oxacillinase (OXA)-type carbapenemases (10). Bacterial resistance to aminoglycoside usually results in the production of aminoglycoside-modifying enzymes. Aminoglycoside resistance genes, including acetyltransferase (*aac*), phosphotransferase (*aph*) and adenylyltransferase (*aad*), have been frequently identified in MDRAB (11). For example,

MDRAB has been demonstrated to acquire antimicrobial resistance genes via class 1 integrons (*Int-1*), which contain single or multiple gene cassettes (12). Carbapenemase and aminoglycoside resistance genes were localized within *Int-1* (13). In addition, 16S rRNA methylases may confer resistance to aminoglycosides (14). The increased production of fluoroquinolone-resistant *A. baumannii* was demonstrated to be markedly associated with spontaneous mutations in the quinolone resistance-determining regions (QR-DRs) in DNA gyrase (*gyrA*) or topoisomerase IV (*parC*) (15).

MDRAB remains a challenge for the clinical management of life-threatening infections, including bacteremia, pneumonia and wound infections. At present, MDRAB is a lethal threat to public health due to the lack of effective antimicrobial agents available. Currently, combination therapy has been considered to be a promising method for the management of infection. Previous studies revealed that tigecycline and polymyxins were active against MDRAB (4,5), but their application is inhibited due to high toxicity and low commercial availability in China. Consequently, the effective combinations of clinical drugs may be an improved choice for treating MDRAB infection. In the present study, the genotypes and encoding resistance genes of MDRAB were determined. Based on the phenotypic analysis, the gene structure and molecular determinants that confer alternative MDRAB phenotypes were investigated. Furthermore, five drugs were used to evaluate the *in vitro* activity of various antibiotic combinations against MDRAB, in order to provide reliable data to support novel clinical combination therapies.

Materials and methods

Bacterial isolates. A total of 34 consecutive and non-repetitive MDRAB strains were identified using the MicroScan WalkAway-96 system (Siemens AG, Munich, Germany) from the Second Xiangya Hospital (Changsha, China) between February 2011 and May 2011. Kirby-Bauer antibiotic susceptibility testing (K-B test) was utilized to determine the susceptibility to several clinically significant antimicrobial agents. MDRAB was defined as the presence of resistance to at least three classes of antibiotics, including: Cephalosporins, carbapenems, β-lactamases, aminoglycosides and quinolones. In the present study, a total of 34 strains were collected (Table I), 30 of which were identified from sputum samples from patients with nosocomial pneumonia, referring to criteria for radiologically-confirmed pneumonia occurring ≥48 h after hospitalization in non-intubated patients (16), 3 were isolated from wound secretion and one was isolated from fluid drainage. The samples were primarily collected from the ICU Respiratory and Cardiothoracic Surgery departments. The interpretation of susceptibility test and breakpoints was performed according to the Clinical and Laboratory Standard Institute (CLSI) criteria (17). The present study was approved by the Ethics Committee of The Second Xiangya Hospital, Central South University (Changsha, China). Written informed consent was obtained from all patients.

Random amplified polymorphic DNA (RAPD) genotyping and detection of drug resistance genes by polymerase chain reaction (PCR). Bacterial DNA was extracted and purified by using Tiangen UltraClean Microbial DNA Isolation kit (Tiangen Biotech Co., Ltd., Beijing, China) according to the manufacturer's instructions. MDRAB genotyping was conducted using RAPD (Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA) with an *AP2* primer (5-GTTTCGCTCC-3) designed by Primers Express software (version 2.0; Applied Biosystems; Thermo Fisher Scientific, Inc.) and synthesized by Sangon Biotech Co., Ltd. (Shanghai, China), as previously described (18). The genes encoding resistance, including β-lactamase *TEM-1*, *AmpC*, metallo-β-lactamase *IMP-5*, oxacillinase *(OXA)-23*, *OXA-24*, acetylyltransferase *aac(3)-I*, *aac(6')-I*, *ant(3'')*-I, 16S rRNA methyltransferase *armA*, 16S rRNA methylase *rmtA*, *rmtB*, phosphotransferase *aph-(3)*, AdeB family multidrug efflux RND transporter *adeB*, *adeRS* two-component system, *Int-1* and *ParC* genes, were detected by PCR. All primers were designed based on the sequences in GenBank (19) using Primer Express software v2.0 (Applied Biosystems; Thermo Fisher Scientific, Inc., Foster City, CA, USA) (Table II). The PCR total reaction volume of 20 μl, containing 10 μl 2X TaqMan PCR master mix, 1 μl forward and reverse primers (10 μmol/l), 7 μl ddH_2O and 1 μl DNA template. The amplification conditions of the target genes were presented in Table III. Following amplification, 3 μl of products were electrophoresed on a 1.5% agarose gel (Oxoid; Thermo Fisher Scientific, Inc.) and visualized using ethidium bromide (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) for 20 min at a voltage of 150 V.

Antimicrobial agents and minimum inhibitory concentration (MIC) assays. Antimicrobial agents used in the present study were amikacin (AK), cefoperazone/sulbactam [SCF, SCFI 1:1 (cefoperazone:sulbactam) and SCFII 2:1 (cefoperazone:sulbactam)], meropenem (MEM), minocycline (MINO) and ciprofloxacin (CIP). MIC assays were performed in 96-well microtiter plates by the broth microdilution method, according to the CLIS protocol (17). Bacteria were cultured in 10% horse blood agar (Oxoid; Thermo Fisher Scientific, Inc.) for 20-24 h until cells reached the exponential phase. The inoculums were adjusted with fresh Cationic adjustment of Mueller-Hinton Broth [Oxoid; Thermo Fisher Scientific, Inc.; CAMHB, containing Ca^{2+} (10-25 mg/l) and Mg^{2+} (10-12.5 mg/l)] to produce solutions with ~5x10^5 colony forming units (CFUs)/ml in a final volume of 100 μl. Subsequently, the bacteria were cultured using various concentrations of drugs: AK and SCF, 256, 128, 64, 32, 16, 8 μg/ml; MEM and MINO, 64, 32, 16, 8, 4, 2 μg/ml; CIP, 16, 8, 4, 2, 1, 0.5 μg/ml, for 18-20 h at 37˚C. The average MIC (MIC_G), the concentration that inhibited 50% of growth (MIC_{50}) or 90% of strains (MIC_{90}) were calculated. All tests were performed in triplicate, and growth and sterility controls were conducted simultaneously.

Chequerboard assay. A chequerboard assay was used to determine the potential interactions between antibiotics. In each assay, a combination of two antibiotics randomly chosen from the total five was used, and the range of drug concentration was identical to the MIC assays. The drugs in the 96-well plates were diluted with CAMHB by checkerboard method as previously described (20). The broth microdilution plates were inoculated with each MDRAB isolate (initial

Table I. Characteristics of study isolates.

NO[x]	PT	Date of strain separated	Ward	Clinical antimicrobial susceptibility													
				TZP	CAZ	SCF	ATM	IPM	AK	FEP	MEM	TIM	CIP	SXT	CTX	LEV	CN	SAM
1[AI]	51M	3/9	3	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
2[AI]	13M	3/9	4	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
3[BI]	46M	3/23	1	R	R	I	R	R	R	R	R	R	R	R	R	R	R	R
4[AI]	64M	3/22	10	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
5[AI]	34M	3/11	8	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
6[BI]	1M	3/17	3	R	R	I	R	R	R	R	R	R	R	R	R	R	R	R
7[CI]	65M	3/12	1	R	R	R	R	R	R	R	R	R	R	R	R	I	R	R
8[AI]	7M	5/14	4	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
9[DI]	88F	3/31	6	R	R	S	R	R	R	R	R	R	R	R	R	R	R	R
10[BI]	33F	3/28	3	R	R	I	R	R	R	R	R	R	R	R	R	R	R	R
11[BII]	25F	3/30	3	R	R	I	R	R	R	R	R	R	R	R	R	R	R	R
12[BII]	48F	3/28	1	R	R	I	R	R	R	R	R	R	R	R	R	R	R	R
13[BII]	30F	3/28	1	R	R	I	R	R	R	R	R	R	R	R	R	R	R	R
14[BII]	47F	4/2	2	R	R	I	R	R	R	R	R	R	R	R	R	R	R	R
15[BII]	55M	4/21	3	R	R	I	R	R	R	R	R	R	R	R	R	R	R	R
16[BIV]	25M	4/21	1	R	R	I	R	R	R	R	R	R	R	R	R	R	R	R
17[AII]	63M	3/20	1	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
18[BI]	72F	3/23	1	R	R	I	R	R	R	R	R	R	R	R	R	R	R	R
19[CI]	57M	3/12	1	R	R	R	R	R	R	R	R	R	R	R	R	I	R	R
20[AI]	78M	3/18	1	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
21[AII]	41M	3/10	4	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
22[EII]	80F	3/30	1	R	R	I	R	R	R	R	R	R	R	R	R	R	I	R
23[FI]	46M	4/5	4	R	R	I	R	R	R	I	R	R	R	R	R	R	R	R
24[BII]	43M	4/3	9	R	R	I	R	R	R	R	R	R	R	R	R	R	R	R
25[AII]	55M	4/5	5	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
26[AII]	55M	4/6	5	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
27[BII]	57F	4/7	2	R	R	I	R	R	R	R	R	R	R	R	R	R	R	R
28[AI]	70F	4/7	2	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
29[DII]	69M	4/5	2	R	R	S	R	R	R	R	R	R	R	R	R	R	R	R
30[GII]	87F	2/28	1	R	S	R	R	R	R	R	R	R	R	R	R	R	S	S
31[BIII]	56M	2/28	1	R	R	I	R	R	R	R	R	R	R	R	R	R	R	R
32[BI]	6F	2/28	7	R	R	I	R	R	R	R	R	R	R	R	R	R	R	R
33[BI]	27M	3/4	1	R	R	I	R	R	R	R	R	R	R	R	R	R	R	R
34[HI]	79M	2/28	1	R	S	I	R	R	R	R	R	R	R	R	R	R	R	R

PT, patient age and sex. Wards: 1, Intensive care unit; 2, Department of Respiratory; 3, Cardiothoracic surgery; 4, Orthopedics; 5, Neurology; 6, Geriatric Ward; 7, Department of Minimally Invasive Surgery; 8, Neurosurgery; 9, Urology; 10, Digestive system department. Antimicrobials: TZP, piperacillin/tazobactam; CAZ, ceftazidime; SCF, cefoperazone/sulbactam; ATM, aztreonam; IPM, imipenem; AK, amikacin; FEP, cefepime; MEM, meropenem; TIM, ticarcillin/clavulanic acid; CIP, ciprofloxacin; SXT, trimethoprim/sulfamethoxazole; CTX, cefotaxime; LEV, levofloxacin; CN, gentamicin; SAM, ampicillin/sulbactam. NO[x]: The isolates were classified into 8 phenotypes (A-H) according to their susceptibility to the tested clinical antimicrobials; A, resistant to all the aforementioned drugs; B, only intermediate to SCF; C, only intermediate to LEV; D, only susceptible to SCF; E, intermediate to SCF and CN; F, intermediate to SCF and FEP; G, susceptible to CAZ, CN and SAM; H, susceptible to CAZ and intermediate to SCF; R, resistance; I, intermediary; S, sensitivity.

Table II. Primers of resistance genes.

Target genes	Primer sequences (5'-3') Forward	Reverse	Size, bp
TEM-1	TTCGTGTCGCCCTTATTC	ACGCTCGTCGTTTGGTAT	512
IMP-5	CTACCGCAGCAGAGTCTTTG	AACCAGTTTTGCCTTACCAT	587
OXA-23	TGTCATAGTATTCGTCGTT	TTCCCAAGCGGTAAA	453
OXA-24	TTTGCCGATGACCTT	TAGCTTGCTCCACCC	175
AmpC	CGACAGCAGGTGGAT	GGTTAAGGTTGGCATG	510
aac(3)-I	ACCTACTCCCAACATCAGCC	ATATAGATCTCACTACGCGC	158
aac(6')-I	TATGAGTGGCTAAATCGA	CCCGCTTTCTCGTAGCA	395
ant(3")-I	TGATTTGCTGGTTACGGTGAC	CGCTATGTTCTCTTGCTTTTG	284
armA	GGGGTCTTACTATTCTG	TTCCCTTCTCCTTTC	503
rmtA	CCTAGCGTCCATCCTTTCCTC	AGCGATATCCAACACACGATGG	315
rmtB	ATGAACATCAACGATGCCCTC	TTATCCATTCTTTTTTATCAAGTATAT	756
aph(3)	ATACAGAGACCACCATACAGT	GGACAATCAATAATAGCAAT	234
adeB	GTATGAATTGATGCTGC	CACTCGTAGCCAATACC	1,000
adeRS	CTCAGACTCCCGTGATCATGTTG	CGTAAGTCTTCGACTAAGTGAGA	1,115
Int-1	GCACCGCCAACTTTC	CCTTGATGTTACCCGAGA	433
ParC	CTGAACAGGCTTACTTGAA	AAGTTATCTTGCCATTCG	200

Table III. Polymerase chain reaction conditions of target genes.

Target genes	Initialdenaturation (˚C, min)	Amplification Denaturation (˚C, sec)	Annealing (˚C, sec)	Extension (˚C, sec)	Cycles (n)	Final extension (˚C, min)
AP2	95, 6	95, 45	33, 45	72, 120	45	72, 10
TEM-1	94, 5	94, 60	55, 60	72, 50	30	72, 7
IMP	94, 5	94, 60	55, 60	72, 50	30	72, 7
OXA-23	94, 5	94, 30	48, 30	72, 35	30	72, 10
OXA-24	94, 5	94, 30	48, 30	72, 35	30	72, 10
AmpC	94, 5	94, 30	50, 30	72, 50	30	72, 10
aac(3)-I	94, 5	94, 30	55, 30	72, 30	35	72, 10
aac(6')-I	94, 5	94, 30	55, 30	72, 30	35	72, 10
ant(3")-I	94, 5	94, 30	55, 30	72, 30	35	72, 10
rmtA	93, 2	93, 20	55, 30	72, 30	30	72, 5
aph (3)	93, 2	93, 20	55, 30	72, 30	30	72, 5
armA	94, 5	94, 30	47, 30	72, 35	30	72, 10
rmtB	94, 5	94, 30	55, 30	72, 60	30	72, 10
adeB	95, 5	95, 30	53, 60	72, 60	30	72, 7
adeRS	95, 5	95, 30	53, 40	72, 60	30	72, 7
Int-1	94, 5	94, 30	53, 30	72, 60	30	72, 10
ParC	94, 4	94, 30	53, 30	72, 40	30	72, 7

concentration of bacteria was 0.5 McFarland) for 18-24 h at 37˚C, to yield ~5x10^5 CFU/ml in a 100 μl volume. The effect of the combinations was analyzed through measuring the fractional inhibitory concentration index (FIC) with the following formula: $FIC_A + FIC_B$, where FIC_A was the ratio of MIC of drug A in combination compared with that of drug A used alone, and FIC_B was the ratio of MIC of drug B in combination compared with that of drug B used alone. The interaction was defined as synergy (FIC ≤0.5), addition (0.5< FIC ≤1), indifference (1< FIC ≤2) or antagonism (FIC >2), respectively.

Results

Antimicrobial susceptibility. The MDRAB phenotype was determined according to the susceptibility results. In total, 34 strains were isolated, among which 11 strains (29.41%) were PDR *A. baumannii* with resistance to almost all clinically significant agents. All the strains were resistant to piperacillin/tazobactam, imipenem, meropenem, amikacin, cefotaxime, ticarcillin/clavulanic acid and ciprofloxacin, while only 14 strains (41.18%) were

resistant to cefoperazone/sulbactam. The isolates were also resistant to other tested drugs including ceftazidime (97.06%), aztreonam (97.06%), trimethoprim/sulfamethoxazole (97.06%), cefepime (94.12%), gentamicin (94.12%), ampicillin/sulbactam (94.12%) and levofloxacin (94.12%). The strains were classified into 8 phenotypes (A-H) based on the resistance to the aforementioned primary clinical drugs (Table I).

Genotypic diversity and molecular determinants for MDR. To detect the extent of genotypic diversity of the tested MDRAB, RAPD was performed and the fingerprint images were analyzed by NTsys 2.10e (Exeter Software; Exeter Publishing Ltd., East Setauket, NY, USA) using dice similarity index for cluster analysis and unweighted pair group average for dendrogram construction. The isolates were clustered into four major genotypes (I-IV) at 60% genotypic similarity threshold (Fig. 1).

Table IV summarizes the distribution of resistance genes in the strains. *TEM-1* and *ParC* were identified in all strains. A total of seven genes demonstrated high positive rates, including *OXA-23* (97.06%), *AdeB* (97.06%), *AmpC* (94.12%), *Int-1* (94.12%), *rmtA* (94.12%), *aph(3)* (91.18%) and *armA* (91.18%). The other genes, including *aac(3)-I*, *aac(6'-I*, *ant(3'')-I*, *rmtB*, *OXA-24* and *IMP-5*, demonstrated positive rates of 29.41, 32.35, 76.47, 41.18, 85.29 and 64.71%, respectively.

MIC and the interaction of drug combinations. The antibiotic susceptibility levels, expressed as MIC of AK, SCF I, SCF II, MEM, MINO and CIP, were preliminarily determined for the 34 MDRAB isolates. The distribution of MIC_{50}, MIC_{90} and MIC_G are presented in Table V. The majority of isolates were resistant to CIP (91.18%), SCF II (91.18%), amikacin (85.29%), SCF I (82.35%) and MEM (73.53%), while less isolates (5.88%) were resistant to MINO.

A chequerboard assay was performed with random combinations of two drugs (Table V). Fig. 2 demonstrates the percentage of isolates inhibited at various MIC of antibiotics when use alone or in combination. The majority of the drug combinations exhibited superior inhibitory effects compared with each used alone. All the combinations demonstrated synergism, with the exception of CIP with MINO. MEM in combination with SCF I or SCF II primarily demonstrated synergy, while the combination of AK+SCF I, AK+SCF II, MINO+SCF I, MINO+SCF II, MINO+CIP, and MINO+MEM primarily exhibited additive effects. Concurrently, the combination of AK+CIP demonstrated evidence of antagonism (Fig. 3).

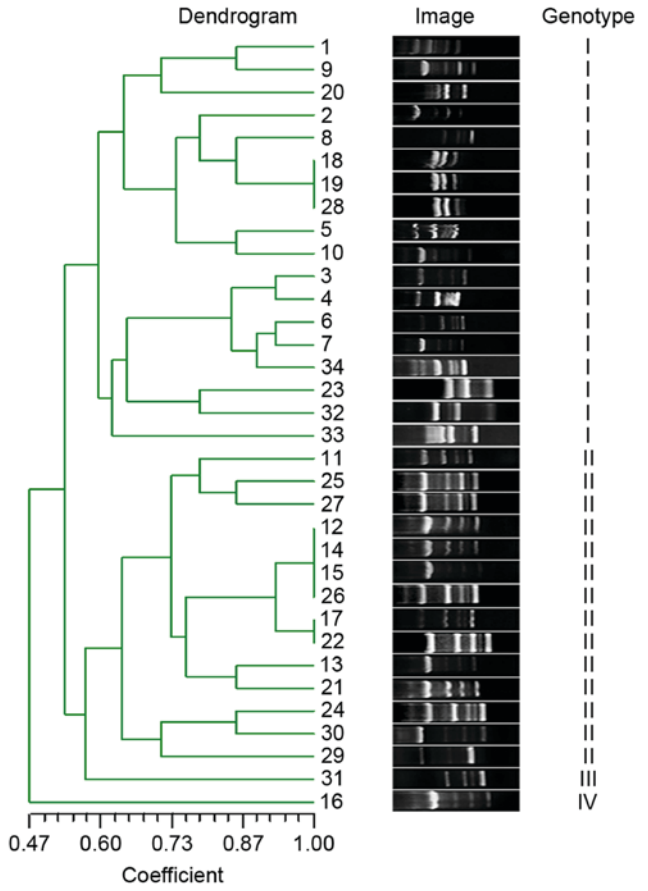

Figure 1. RAPD fingerprinting of MDRAB strains. The gel image displayed diversity RAPD genotyping pattern of each MDRAB isolates representing various phenotypes from different wards. The genotypic similarities were calculated by NTsys 2.10e using dice similarity index and UPGMA, presented in the left of the glue image with coefficients and lines. Four genotypes (I-IV) were formed at a 60% similarity level. RAPD, randomly amplified polymorphic DNA; MDRAB, multidrug resistant *Acinetobacter baumannii*; UPGMA, unweighted pair group average.

Discussion

Antibiotic resistance patterns observed in *A. baumannii* exhibits the capacity to cause an epidemic globally. MDRAB has been demonstrated to lead to serious hospital-acquired infection, as limited therapeutic options are available (20). In the present study, the features of 34 MDRAB strains isolated from the Second Xiangya Hospital were investigated, including antimicrobial susceptibility, genotypes, screening of antibiotic resistance genes, MIC assay and antibiotic interactions.

In the antimicrobial susceptibility study, eight phenotypes (A-H) were classified using the K-B test. The results of the drug susceptibility were in accordance with previous studies (21,22) that highlighted the efficiency of cefoperazone/sulbactam against MDRAB. However, the expression of characteristic bacterial phenotypes may be easily affected by various environmental factors. Therefore, only determining the phenotype was not sufficient for the complete epidemiological typing of various strains. Organism identification based on the genotype is considered to be more reliable, as the genotype of each organism is unique and invariable. In the present study, a *AP2* primer known to be efficient in RAPD genotyping was used to amplify the genomic DNA of MDRAB strains (23). In total, four genotypes (I-IV) were formed at a 60% similarity level. The genotypic distribution was polyclonal, which was opposite to a previous study (24). In general, the 'classic' outbreaks of MDRAB may be more frequently induced by a single clone spread among people, whereas for the prevalence of polyclones in the Second Xiangya Hospital, it may be associated the existence of mobile genetic elements, for example integrons. The variety of patient wards and isolate origin was hypothesized to be responsible for the transmission of different subtypes. When comparing the phenotypes with diverse fingerprinting profiles, it is noteworthy to select isolates of the same phenotype with different genotypes, as it

Table IV. Distribution of positivity of various resistance genes.

No.	*aac(3)-I*	*aac(6')-I*	*ant(3")-I*	*OXA-23*	*OXA-24*	*TEM-1*	*IMP-5*	*AmpC*	*ArmA*	*RmtA*	*RmtB*	*Aph(3)*	*AdeRS*	*AdeB*	*Int1*	*ParC*
1	N	N	P	P	P	P	N	P	P	P	N	P	N	P	P	P
2	N	N	P	P	P	P	P	P	N	P	P	P	N	P	P	P
3	N	N	P	P	P	P	P	P	P	P	N	P	N	P	P	P
4	N	N	P	P	P	P	P	P	P	P	N	P	N	P	P	P
5	N	N	P	P	P	P	P	P	P	P	N	P	N	P	P	P
6	N	N	P	P	P	P	P	P	P	N	N	P	N	P	P	P
7	N	N	P	P	P	P	P	P	N	P	N	P	N	P	P	P
8	N	N	P	P	P	P	P	P	P	P	P	P	N	P	P	P
9	N	N	P	P	P	P	P	P	P	P	P	P	N	P	P	P
10	N	N	P	P	P	P	N	P	P	P	N	N	N	P	P	P
11	N	N	P	P	P	P	P	P	P	P	N	P	N	P	P	P
12	N	N	P	P	P	P	P	P	P	P	N	P	N	P	P	P
13	P	N	P	P	P	P	P	P	P	P	N	P	N	P	P	P
14	N	N	P	P	P	P	P	P	P	P	P	P	N	P	P	P
15	N	N	P	P	P	P	P	P	P	P	N	P	N	P	P	P
16	N	N	P	P	P	P	P	P	P	P	P	P	N	P	P	P
17	N	N	P	P	P	P	P	P	P	P	N	P	N	P	P	P
18	N	N	P	P	P	P	P	P	P	P	N	P	N	P	P	P
19	N	N	P	P	N	P	P	P	P	P	N	P	N	P	P	P
20	N	P	N	P	P	P	P	P	P	P	P	P	N	P	P	P
21	N	N	P	P	P	P	P	P	P	P	N	P	N	P	P	P
22	N	N	P	P	P	P	P	P	P	P	P	P	N	P	P	P
23	P	P	N	P	N	P	N	P	P	P	N	P	N	P	P	P
24	P	P	N	P	N	P	N	P	P	P	N	P	N	P	P	P
25	N	N	N	N	N	P	N	P	N	P	P	P	N	P	P	P
26	P	N	N	P	P	P	N	N	P	P	P	P	N	P	N	P
27	P	P	P	P	P	P	N	P	P	P	N	P	N	P	P	P
28	P	P	N	P	P	P	N	P	P	P	P	P	N	P	N	P
29	P	P	P	P	P	P	N	P	P	P	P	P	N	P	P	P
30	N	P	N	P	N	P	N	N	P	P	P	N	N	P	P	P
31	P	P	P	P	P	P	P	P	P	P	P	P	N	P	P	P
32	P	P	P	P	P	P	P	P	P	P	P	P	N	P	P	P
33	N	P	N	P	P	P	N	P	P	P	N	P	N	P	P	P
34	P	P	P	P	P	P	N	P	P	P	N	N	N	N	P	P
P (%)	29.41	32.35	76.47	97.06	85.29	100.00	64.71	94.12	91.18	97.06	41.18	91.18	0.00	97.06	94.12	100.00

P (%), percentage of strains positive for each gene; P, positive; N, negative.

Table V. MIC parameters of drugs alone use or in combination.

Antibiotics usage	MIC_{50} (μg/ml)	MIC_{90} (μg/ml)	MIC_G (μg/ml)
AK alone	>256.00	>256.00	>222.24
SCF I alone	64.00	>256.00	>90.83
SCF II alone	128.00	>256.00	>130.35
MEM alone	33.60	>64.00	>46.39
MINO alone	3.50	>25.00	>5.50
CIP alone	>16.00	>16.00	>14.13
AK/SCF I			
AK	8	8	8
SCF I	32	256	29.88
AK/SCF II			
AK	8	>256	>24.24
SCF II	64	>256	>57.41
MEM/SCF I			
MEM	2	32	2.82
SCF I	8	128	17.18
MEM/SCF II			
MEM	2	>64	>6.14
SCF II	8	>256	>26.12
CIP/SCF I			
CIP	0.5	>16	>0.96
SCF I	32	>256	>48.24
CIP/SCF II			
CIP	0.5	>16	>1.85
SCF II	64	>256	>87.76
MINO/SCF I			
MINO	2	2	2
SCF I	8	8	8
MINO/SCF II			
MINO	2	4	3.24
SCF II	8	8	8
AK/MEM			
AK	8	8	>37.18
MEM	16	>64	>89.82
AK/CIP			
AK	>256	>256	>176.99
CIP	>16	>16	>12.25
CIP/MEM			
CIP	>16	>16	>14.40
MEM	16	>64	>24.65
CIP/MINO			
CIP	0.5	1	0.53
MINO	2	4	2.47
MEM/MINO			
MEM	2	2	2
MINO	2	2	2

MIC, minimum inhibitory concentration; MIC_{50}, the concentration that inhibits the growing of 50% of strains; MIC_{90}, the concentration that inhibits the growing of 90% of strains; MIC_G, the average MIC; AK, amikacin; SCF, SCFI 1:1 and SCFII 2:1, cefoperazone/sulbactam; MEM, meropenem; MINO, minocycline; CIP, ciprofloxacin; FIC, fractional inhibitory concentration index.

manifested that the environment may affect the discrepancy between genotype and phenotype.

To additionally investigate the resistant mechanisms of MDRAB, the expression of the genes associated with drug resistance was detected among the 34 isolates. Differences were observed in the genetic characteristics of β-lactam, aminoglycoside and quinolones resistance. Previously, the carbapenem resistance associated with class D β-lactamase genes had been suggested to cause serious therapeutic problems in clinical practice (25,26). The high positive rate of *OXA-23* (33/34) in the present study was consistent with a previous study (27), while the proportion of *OXA-24*-positive strains (29/34) was increased compared with a previous study (28). The production of *OXA-23* and *OXA-24* β-lactamase may be the major cause for the selected MDRAB representing 100% resistance to imipenem and meropenem. Other β-lactamase genes, including *TEM-1* (class A), *IMP-5* (class B) and *AmpC* (class C) were also identified in the present study, which may be associated with the resistance of MDRAB to various types of β-lactams including aztreonam, ceftazidime, cefepime, and cefoperazone/sulbactam. At present, aminoglycoside-modifying enzymes and 16S rRNA methylases have been suggested to be the most important mechanism for bacterial resistance against aminoglycosides (14). In the present study, the co-existence of *aph(3)* (91.18%), *ant(3'')-I* (76.47%), *aac(3)-I* (29.41%), *aac(6')-I* (32.35%), *armA* (91.18%), *rmtA* (94.12%) and *rmtB* (41.18%) resistance genes confers the high resistance to amikacin and gentamicin in MDRAB. Mutations in *ParC* were observed in all isolates, which may assist in explaining the 100% resistance to ciprofloxacin. The result was similar to a previous study (29). Among the isolates, the positive rates of *Int1* and *adeB* were 94.1 and 97.1%, respectively, while the *adeRS* was completely negative. Integron and efflux pump genes are non-specific resistance genes. Concurrently, integrons are widely present in MDRAB, particularly *Int-1,* which provides *A. baumannii* with a gene capture system adapted to circumvent the challenges of multiple-antibiotic treatment regimens (30). The results of the present study demonstrated that *Int1* serves a crucial role in multiple drug resistance. In addition, the identification of the co-existence of *Int-1* and the majority of the β-lactamase and aminoglycoside genes was notable, which is in accordance with a previous study (31). This highlighted the importance of the roles of *Int-1* in the horizontal spreading of antibiotic resistance genes, which may finally result in the polyclonal prevalence of MDRAB in the Second Xiangya Hospital. *AdeB* is a vital component of the AdeABC efflux pump, and the expression of adeABC is regulated by the *adeRS* two-component regulatory system (32). The sophisticated feedback between them may account for the high positive ratio of *adeB* and absolute absence of *adeRS* observed in the present study.

The results of the present study confirmed that several genes were associated with MDR. Abuse of antibiotic chemotherapeutics affords major challenges in treating MDRAB infections. At present, traditional therapy regimens are not efficient to manage these life-threatening infections. Tigecycline and polymyxins have been considered as the last resort for treating MDRAB infections (4,5). However, they are still widely used across mainland China due to the lack of any other qualified commercial products. On this basis, the present study aimed to identify an effective regimen to manage MDRAB infections through a combination of antibiotics that are frequently used in clinical practice. In the present study, 6 drugs (iAK, SCF I, SCF II, MEM, MINO and CIP) were selected to study the *in vitro* activity of drug combinations against MDRAB. The lower MIC of SCFI compared with

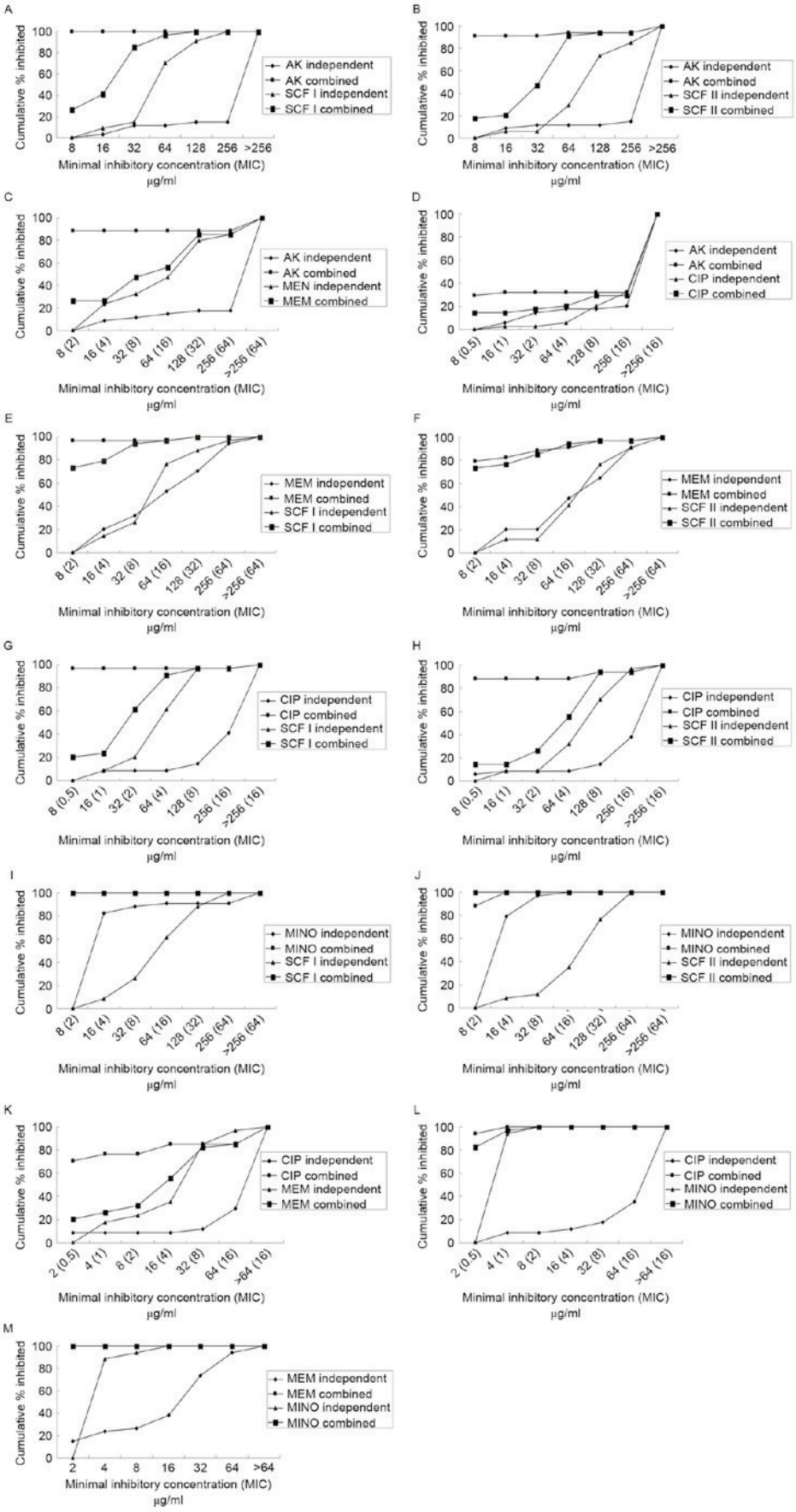

Figure 2. Cumulative percentages of MDRAB strains that were inhibited by increasing concentrations of various types of drugs used alone or combination. (A) AK+SCFI; (B) AK+SCFII; (C) AK+MEM; (D) AK+CIP; (E) MEM+SCFI; (F) MEM+SCFII; (G) CIP+SCFI; (H) CIP+SCFII; (I) MINO+SCFI; (J) MINO+SCFII; (K) CIP+MEM; (L) CIP+MINO; and (M) MEM+MINO. There were two cumulative percentage lines overlapped (to 100% bacteriostasis) in parts I and M of MINO combining with SCFI and MEM. AK, amikacin; SCF, SCFI 1:1 and SCFII 2:1, cefoperazone/sulbactam; MEM, meropenem; MINO, minocycline; CIP, ciprofloxacin.

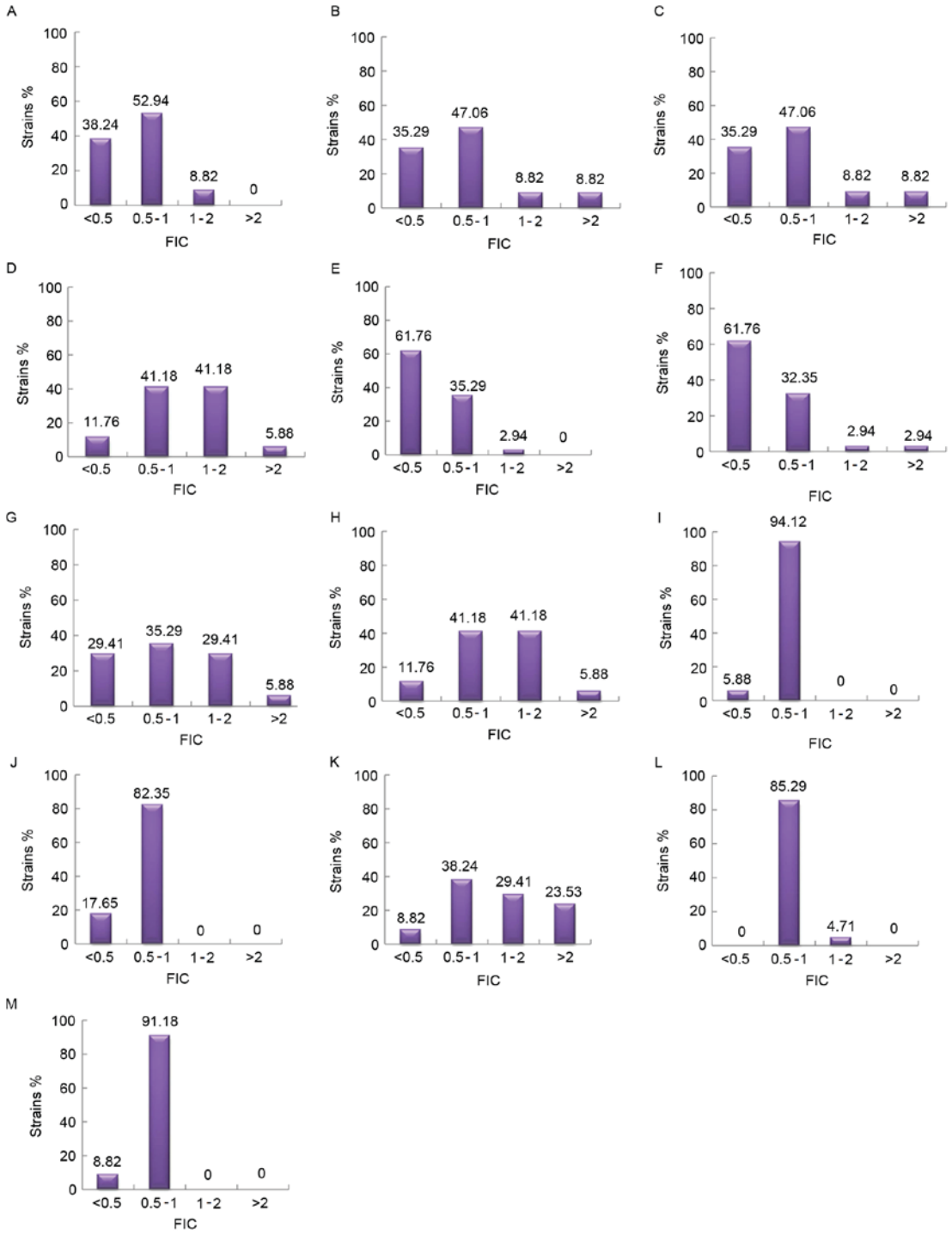

Figure 3. The distribution of FIC of various combinations of antimicrobial drugs. (A) AK+SCFI; (B) AK+SCFII; (C) AK+MEM; (D) AK+CIP; (E) MEM+SCFI; (F) MEM+SCFII; (G) CIP+SCFI; (H) CIP+SCFII; (I) MINO+SCFI; (J) MINO+SCFII; (K) CIP+MEM; (L) CIP+MINO; and (M) MEM+MINO. Synergy, FIC ≤0.5; addition, 0.5< FIC ≤1; indifference, 1< FIC ≤2; antagonism, FIC >2. AK, amikacin; SCF, SCFI 1:1 and SCFII 2:1, cefoperazone/sulbactam; MEM, meropenem; MINO, minocycline; CIP, ciprofloxacin; FIC, fractional inhibitory concentration index.

SCF II demonstrated a comparatively increased rate of *in vitro* activity of SCF I against MDRAB, which was incompatible with previous studies (33,34). This discrepancy may arise from the geographical and biological evolutional differences. In addition, a high percentage (76.47%) of MDRAB was susceptible to minocycline, indicating the potential of this drug for the treatment of this fatal infection (35). The chequerboard assay indicated synergism for all the tested combinations, particularly the combination of MEM+SCF I, and MEM+SCF II, with the exception of CIP+MINO. Conversely, the combinations of MINO+SCF I, MINO+SCF II, CIP+MINO and MEM+MINO demonstrated additive effects. In summary, the combination of cefoperazone-sulbactam or meropenem-minocycline has been indicated to be more active compared with ciprofloxacin-amikacin, which is similar to the recent surveillance data (16,21). The present study also demonstrated that meropenem and cefoperazone-sulbactam were generally active in MDRAB. In a previous study, the combined

utilization of meropenem and sulbactam was considered a therapeutic option for *A. baumannii* infection (36). However, only a small number of studies have been focused on the study of the efficiency of a MEM+SCF combination. The present study attempted to investigate the *in vitro* activity of two types of SCF (SCF I 1:1; SCF II 2:1) combined with MEM against 34 strains of MDRAB. The results indicated a marked synergistic interaction in the majority tested isolates. Although no significant differences were observed in the activity of cefoperazone-sulbactam combined with meropenem, it revealed a novel potential option for clinical combination therapy. Meropenem belongs to the family of β-lactam antibiotics, while cefoperazone-sulbactam is a type of the third-generation cephalosporin and β-lactamase inhibitor. When meropenem is combined with cefoperazone-sulbactam, they may bind to different types of penicillin bonding proteins, executing their bactericidal effects. Concurrently, sulbactam may irreversibly inhibit β-lactamase activity. This may be the most probable explanation for the synergism observed. MINO was active against MDRAB whenever it is used alone or combination. Although it is only a second-line antibiotic for the majority of clinical bacterial infections, its potential antibacterial activity against MDRAB should not be neglected. In the present study, the combination of AK+CIP produced an antagonism of 70.59%. Therefore, the combined use of these drugs should be avoided in clinical practice.

In conclusion, the identification of fingerprinting diversity highlights the issues with the polyclonal and horizontal spread of MDRAB in the Second Xiangya Hospital. Although the co-occurrence of numerous resistance-encoding genes presented a completely threaten for the active therapy, the determination of efficacious combinations among minocycline, meropenem and cefoperazone-sulbactam, particularly MEM+SCFI and MEM+SCFII, provides improved choices for the rational clinical combination therapy for MDRAB infections. Additionally, MINO may be the alternative choice to overcome the critical resistance of *A. baumannii*. The present study failed to depict the pharmacokinetics and pharmacodynamics of these drug combinations. Future studies should focus on updating these data and proceed to additionally identify the clinical effects of combination therapy.

Acknowledgements

The present study was supported by the China National Natural Scientific Foundation (grant no. 81470133), and the Science and Technology Planning Project of Hunan Province of China (grant no. 2015JC3035).

References

1. Visca P, Seifert H and Towner KJ: Acinetobacter infection - an emerging threat to human health. IUBMB Life 63: 1048-1054, 2011.
2. Munoz-Price LS and Weinstein RA: Acinetobacter infection. N Engl J Med 358: 1271-1281, 2008.
3. Dent LL, Marshall DR, Pratap S and Hulette RB: Multidrug resistant Acinetobacter baumannii: A descriptive study in a city hospital. BMC Infect Dis 10: 196, 2010.
4. Principe L, D'Arezzo S, Capone A, Petrosillo N and Visca P: In vitro activity of tigecycline in combination with various antimicrobials against multidrug resistant *Acinetobacter baumannii*. Ann Clin Microbiol Antimicrob 8: 18, 2009.
5. Lim TP, Tan TY, Lee W, Sasikala S, Tan TT, Hsu LY and Kwa AL: In-vitro activity of polymyxin B, rifampicin, tigecycline alone and in combination against carbapenem-resistant *Acinetobacter baumannii* in Singapore. PLoS One 6: e18485, 2011.
6. Weng XB and Mi ZH: Phylogenetic analysis on pandrug-resistant *Acinetobacter baumannii* strains. Zhonghua Liu Xing Bing Xue Za Zhi 32: 948-949, 2011 (In Chinese).
7. Zhao WS, Liu GY, Mi ZH and Zhang F: Coexistence of blaOXA-23 with armA and novel gyrA mutation in a pandrug-resistant *Acinetobacter baumannii* isolate from the blood of a patient with haematological disease in China. J Hosp Infect 77: 278-279, 2011.
8. Wen Y, Ouyang Z, Yu Y, Zhou X, Pei Y, Devreese B, Higgins PG and Zheng F: Mechanistic insight into how multidrug resistant *Acinetobacter baumannii* response regulator AdeR recognizes an intercistronic region. Nucleic Acids Res 45: 9773-9787, 2017.
9. Yang H, Huang L, Barnie PA, Su Z, Mi Z, Chen J, Aparna V, Kumar D and Xu H: Characterization and distribution of drug resistance associated β-lactamase, membrane porin and efflux pump genes in MDR A. Baumannii isolated from Zhenjiang, China. Int J Clin Exp Med 8: 15393-15402, 2015.
10. Jiang W, Liu H, Zhong M, Yang YC, Xiao DW and Huang WF: Study on the resistant genes to carbapenems and epidemiological characterization of multidrug-resistant *Acinetobacter baumannii* isolates. Microb Drug Resist 19: 117-123, 2013.
11. Ramirez MS and Tolmasky ME: Aminoglycoside modifying enzymes. Drug Resist Updat 13: 151-171, 2010.
12. Petersen A, Guardabassi L, Dalsgaard A and Olsen JE: Class I integrons containing a dhfrl trimethoprim resistance gene cassette in aquatic Acinetobacter spp. FEMS Microbiol Lett 182: 73-76, 2000.
13. Azizi O, Shakibaie MR, Badmasti F, Modarresi F, Ramazanzadeh R, Mansouri S and Shahcheraghi F: Class 1 integrons in non-clonal multidrug-resistant *Acinetobacter baumannii* from Iran, description of the new blaIMP-55 allele in In1243. J Med Microbiol 65: 928-936, 2016.
14. Tada T, Miyoshi-Akiyama T, Kato Y, Ohmagari N, Takeshita N, Hung NV, Phuong DM, Thu TA, Binh NG, Anh NQ, *et al*: Emergence of 16S rRNA methylase-producing *Acinetobacter baumannii* and *Pseudomonas aeruginosa* isolates in hospitals in Vietnam. BMC Infect Dis 13: 251, 2013.
15. Lee JK, Lee YS, Park YK and Kim BS: Mutations in the gyrA and parC genes in ciprofloxacin-resistant clinical isolates of Acinetobacter baumannii in Korea. Microbiol Immunol 49: 647-653, 2005.
16. American Thoracic Society; Infectious Diseases Society of America: Guidelines for the management of adults with hospital-acquired, ventilator-associated, and healthcare-associated pneumonia. Am J Respir Crit Care Med 171: 388-416, 2005.
17. Clinical and Laboratory Standards Institute: Performance standards for antimicrobial susceptibility testing: Twenty-second informational supplement M100-S21. CLSI 31: 64-65, 2011.
18. Zhang T, Wang M, Xie Y, Li X, Dong Z, Liu Y, Wang L, Yang M, Song H, Cao H and Cao W: Active efflux pump adeB is involved in multidrug resistance of *Acinetobacter baumannii* induced by antibacterial agents. Exp Ther Med 13: 1538-1546, 2017.
19. Benson DA, Karsch-Mizrachi I, Lipman DJ, Ostell J and Wheeler DL: GenBank. Nucleic Acids Res 33 (Database Issue): D34-D38, 2005.
20. Daoud Z, Mansour N and Masri K: Synergistic combination of carbapenems and colistin against *P. aeruginosa* and *A. baumannii*. Open J Med Microbiol 3: 253-258, 2013.
21. Huang J, Chen EZ, Qu HP, Mao EQ, Zhu ZG, Ni YX, Han LZ and Tang YQ: Sources of multidrug-resistant Acinetobacter baumannii and its role in respiratory tract colonization and nosocomial pneumonia in intensive care unit patients. Chin Med J (Engl) 126: 1826-1831, 2013.
22. Xu T, Xia W, Rong G, Pan S, Huang P and Gu B: A 4-year surveillance of antimicrobial resistance patterns of *Acinetobacter baumanni* in a university-affiliated hospital in China. J Thorac Dis 5: 506-512, 2013.
23. Koeleman JG, Stoof J, Biesmans DJ, Savelkoul PH and Vandenbroucke-Grauls CM: Comparison of amplified ribosomal DNA restriction analysis, random amplified polymorphic DNA analysis, and amplified fragment length polymorphism fingerprinting for identification of *Acinetobacter* genomic species and typing of *Acinetobacter baumannii*. J Clin Microbiol 36: 2522-2529, 1998.

24. Fontana C, Favaro M, Minelli S, Bossa MC, Testore GP, Leonardis F, Natoli S and Favalli C: *Acinetobacter baumannii* in intensive care unit: A novel system to study clonal relationship among the isolates. BMC Infect Dis 8: 79, 2008.
25. Niranjan DK, Singh NP, Manchanda V, Rai S and Kaur IR: Multiple carbapenem hydrolyzing genes in clinical isolates of *Acinetobacter baumannii*. Indian J Med Microbiol 31: 237-241, 2013.
26. Feizabadi MM, Fathollahzadeh B, Taherikalani M, Rasoolinejad M, Sadeghifard N, Aligholi M, Soroush S and Mohammadi-Yegane S: Antimicrobial susceptibility patterns and distribution of blaOXA genes among *Acinetobacter* spp. Isolated from patients at Tehran hospitals. Jpn J Infect Dis 61: 274-278, 2008.
27. Thapa B, Tribuddharat C, Srifuengfung S and Dhiraputra C: High prevalence of bla(OXA)-23 in oligoclonal carbapenem-resistant *Acinetobacter baumannii* from Siriraj Hospital, Mahidol University, Bangkok, Thailand. Southeast Asian J Trop Med Public Health 41: 625-635, 2010.
28. Chen Z, Liu W, Zhang Y, Li Y, Jian Z, Deng H, Zou M and Liu Y: Molecular epidemiology of carbapenem-resistant *Acinetobacter* spp. from XiangYa Hospital, in Hunan Province, China. J Basic Microbiol 53: 121-127, 2013.
29. Sung JY, Kwon KC, Cho HH and Koo SH: Antimicrobial resistance determinants in imipenem-nonsusceptible *Acinetobacter calcoaceticus-baumannii* complex isolated in Daejeon, Korea. Korean J Lab Med 31: 265-270, 2011.
30. Peymani A, Farajnia S, Nahaei MR, Sohrabi N, Abbasi L, Ansarin K and Azhari F: Prevalence of class 1 integron among multidrug-resistant Acinetobacter baumannii in Tabriz, northwest of Iran. Pol J Microbiol 61: 57-60, 2012.
31. Farajnia S, Azhari F, Alikhani MY, Hosseini MK, Peymani A and Sohrabi N: Prevalence of PER and VEB type extended spectrum betalactamases among multidrug resistant acinetobacter baumannii Isolates in North-West of Iran. Iran J Basic Med Sci 16: 751-755, 2013.
32. Yoon EJ, Courvalin P and Grillot-Courvalin C: RND-type efflux pumps in multidrug-resistant clinical isolates of *Acinetobacter baumannii*: Major role for AdeABC overexpression and AdeRS mutations. Antimicrob Agents Chemother 57: 2989-2995, 2013.
33. Jones RN, Barry AL, Packer RR, Gregory WW and Thornsberry C: In vitro antimicrobial spectrum, occurrence of synergy, and recommendations for dilution susceptibility testing concentrations of the cefoperazone-sulbactam combination. J Clin Microbiol 25: 1725-1729, 1987.
34. Barry AL and Jones RN: Criteria for disk susceptibility tests and quality control guidelines for the cefoperazone-sulbactam combination. J Clin Microbiol 26: 13-17, 1988.
35. Pei G, Mao Y and Sun Y: In vitro activity of minocycline alone and in combination with cefoperazone-sulbactam against carbapenem-resistant Acinetobacter baumannii. Microb Drug Resist 18: 574-577, 2012.
36. Deveci A, Coban AY, Acicbe O, Tanyel E, Yaman G, and Durupinar B: In vitro effects of sulbactam combinations with different antibiotic groups against clinical *Acinetobacter baumannii* isolates. J Chemother 24: 247-252, 2012.