Disruption of *adeB* gene has a greater effect on resistance to meropenems than *adeA* gene in *Acinetobacter* spp. isolated from University Malaya Medical Centre

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ABSTRACT

Introduction: The AdeABC pump of *Acinetobacter* spp. confers resistance to various antibiotic classes. This pump is composed of the AdeA, AdeB, and AdeC proteins where AdeB is a member of the resistance-nodulation-division efflux pump superfamily. The *adeA*, *adeB*, and *adeC* genes are contiguous and adjacent to *adeS* and *adeR*, which are transcribed in the opposite direction and which specify proteins homologous to sensors and regulators of two-component systems, respectively. In this study, an attempt is made to elucidate the role of the AdeABC efflux pump in carbapenem resistance in *Acinetobacter* spp.

Methods: 39 carbapenem-resistant clinical isolates of *Acinetobacter* spp. were used. Minimum inhibitory concentrations were evaluated using the agar dilution method according to Clinical and Laboratory Standards Institute standards. The presence of carbapenem hydrolysing oxacillinases and AdeABC efflux pump genes were determined by PCR amplification. Subsequently, each gene was inactivated by plasmid insertion in order to study the contribution of these genes in developing antibiotic resistance and the resulting mutants were tested for their antimicrobial susceptibilities.

Results: Among the multidrug-resistant strains, 36 strains had all the three (A, B, C) genes detected, while the remaining three strains had one or two of the genes detected. Inactivation of these individual genes showed decreased antimicrobial susceptibility indicating its contribution towards the development of antimicrobial resistance.

Conclusion: The presence of AdeABC multidrug efflux pump plays a major role in the development of antimicrobial resistance in *Acinetobacter* spp. The presence of either one or an interplay between these genes may have an effect on antimicrobial resistance in *Acinetobacter* spp.

Keywords: *Acinetobacter* spp., AdeABC efflux pump, antimicrobial resistance, carbapenam resistance, multidrug resistance

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INTRODUCTION

Acinetobacter spp. are aerobic Gram-negative bacteria that are non-fermentative and are ubiquitous in the environment. This opportunistic pathogen can be involved in a range of nosocomial infections, including blood stream infections, meningitis, ventilator-associated pneumonia, wound and urinary tract infections. The emergence of antibiotic resistance among acinetobacters in hospitalised patients is a serious and recurrent problem for the treatment of infections.[1] Although carbapenems have been the most successful β-lactam antibiotics for the treatment of *Acinetobacter* spp. infections, there are increasing reports of carbapenem resistance among these pathogens.[2] There are several factors leading to carbapenem resistance in *Acinetobacter* spp. which include the ability of β-lactamases to hydrolyse carbapenems, the presence of metallo-β-lactamases, oxacillinases, mobile genetic elements, the reduced expression of outer membrane proteins (OMP) and penicillin-binding proteins.[3-5] AdeABC efflux pump-mediated antibiotic resistance includes resistance to aminoglycosides, β-lactams, chloramphenicol, erythromycin, tetracyclines, and the dye ethidium bromide in *Acinetobacter baumannii*, and has been reported previously by Magnet et al.[6]

The efflux system is a mechanism of antibiotic resistance involving the extrusion of toxic substrates from

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Table I. Primer sequences of the *adeABC*, and *adeRS* genes.

Resistance genes	Forward sequence	Reverse sequence	Amplicon size (bp)
AdeA	AAAATTTT*CTCTAGCCGATGTCGCTCAA	AAAATTTT*ATACCTGAGGCTCGCCACTG	510
AdeB	AAAATTTT*ACCGCGCTAACTTAGGTATGC	AAAATTTT*TCTGAACTTGCACCTGAGGCA	931
AdeC	AAAATTTT*TGCGGCAGGTTAGCCCATCG	AAAATTTT*GCGGAACAGGATGACCTGCT	435
AdeR	AAAATTTT*GACTACGATATTGGCGACAT	AAAATTTT*ACGGTTCGCTCTAGTGCATC	560
AdeS	AAAATTTT*GATTGGCATGCGCCTCGCAA	AAAATTTT*GCCTGCATGTGAATAGCGTA	580

* CCCGGG (*Sma*I restriction site added to the primer sequences)

within cells into the external environment with the help of transport proteins. In the bacteria kingdom, multidrug transporters can be divided into five major families; the major facilitator, the multidrug and toxic efflux, the small multidrug resistance, the ATP-binding cassette, and the resistance-nodulation-division (RND) superfamily.[7] The RND-type superfamily is the most commonly found efflux system in Gram-negative bacteria including *Acinetobacter* spp. The RND efflux transporters act as part of a tripartite system which includes a membrane fusion protein, and an OMP.[6] The AdeABC efflux pump belongs to the RND-type superfamily which consists of *adeA* (membrane fusion), *adeB* (multidrug transporter), and *adeC* (outer membrane) genes. These three genes are contiguous, and adjacent to them are two-component regulatory systems, *adeR* and *adeS*, which are transcribed in the opposite direction.[8] The main aim of our study was to determine the role of AdeABC efflux pump genes in carbapenem resistance among the *Acinetobacter* spp. isolated from University Malaya Medical Centre, Kuala Lumpur, Malaysia.

METHODS

39 isolates of carbapenem-resistant *Acinetobacter* spp. were obtained from patients who were hospitalised in University Malaya Medical Centre, Kuala Lumpur, Malaysia, from August 2003 to March 2004. These species were identified by the conventional method as described in the Manual of Clinical Microbiology[9] and the API20NE System (bioMérieux SA, Marcy-1'Etoile, France). Growth at 44°C was performed to differentiate between *Acinetobacter baumannii* and *Acinetobacter calcoaceticus*.[10] Further species level identification was done by amplified ribosomal DNA restriction analysis (ARDRA) as described by Koeleman et al.[11] Imipenem, meropenem, ceftazidime, cefotaxime, gentamicin, amikacin, ciprofloxacin and ampicillin (Oxoid, UK) susceptibility was tested by a disk diffusion method.[12] Minimum inhibitory concentrations (MICs) for the *Acinetobacter* spp. isolates were determined by an agar dilution method[13] and interpreted according to the guidelines of the Clinical Laboratory Standards Institute.[14] The total genomic DNA was prepared using a standard boiling method by heating 2–5 colonies of bacteria suspension to 95°C for five minutes in a final volume of 50 µL of distilled sterile water. After centrifugation at 13,000 g, the suspensions were used as DNA templates. Plasmid DNA was extracted using the HiYield™ Plasmid Mini Kit (Yeastern Biotech Co Ltd, Taiwan).

Primers specific for $bla_{\text{OXA-23}}$ were used to detect the presence of the $bla_{\text{OXA-23}}$ gene encoding Class D β-lactamases in *Acinetobacter* spp. as described by Alfaz-Shah et al.[15] This amplification was performed using primer pairs 5'-GATGTGTCATAGTATTCGTCG-3' (forward) and 5'-TCACAACAACTAAAAGCACTG-3' (reverse). This set of primers gave an amplicon size of 1058 bp. The PCR reaction mixture in a final volume of 50 µL containing 20 pmol (each) primer, 200 µM (each) deoxynucleotide triphosphate, 1 × reaction buffer, 1.5 mM $MgCl_2$, 2.5 U *Taq* DNA polymerase (Fermentas, Lithuania, USA) and approximately 1 µL of template DNA was prepared. The master mix was then dispensed equally into each tube and vortexed. The tubes were then subjected to polymerase chain reaction (PCR) amplification using Thermal Cycler (BioRad, Hercules, USA). The thermocycling conditions used were: an initial denaturation step at 94°C for five minutes, followed by 25 cycles of denaturation at 94°C for 25 s, annealing at 52°C for 40 s, elongation at 72°C for 50 s, followed by a final extension step at 72°C for six minutes.

Primers of the *adeA*, *adeB*, *adeC*, *adeR*, and *adeS* were designed, and the forward and reverse primer sequences of all these genes are listed in Table I. *Sma*1 restriction sites were added to each forward and reverse primer sequences of each gene for ligation purposes. The presence of each gene was detected using *Pfu* DNA polymerase according to the manufacturers' recommendations (Fermentas Inc, Lithuania). PCR mixes contained 1 µL of heat-extracted template DNA, 1 µL (20 pmol) of each primer and PCR premix containing 2.5 U of *Taq* DNA polymerase in a final volume of 50 µL. The thermocycle protocol used was: an initial denaturation step at 94°C for five minutes, followed by 25 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 30 s, elongation at 72°C for 45 s, followed by a

Table II. Minimum inhibitory concentrations of β-lactams for three *Acinetobacter baumannii* strains and its mutants.

Strain*	IMP (mg/ml)	MEM (mg/ml)	CAZ (mg/ml)	CTX (mg/ml)	CN(mg/ml)	AK (mg/ml)	CIP (mg/ml)	AMP (mg/ml)
1 (wild type)	0.032	0.064	0.008	0.064	0.016	0.004	0.008	> 0.512
1*adeA*⁻	0.032	0.032	0.004	0.032	0.008	0.002	0.004	0.512
1*adeB*⁻	0.032	0.016	0.004	0.004	0.002	0.000125	0.00025	0.512
1*adeR*⁻	0.032	0.016	0.004	0.002	0.002	0.00025	0.000125	0.512
1*adeS*⁻	0.032	0.016	0.004	0.004	0.001	0.00025	0.000125	0.512
15 (wild ype)	0.032	0.064	> 0.512	0.032	0.128	0.008	0.064	> 0.512
15*adeA*⁻	0.032	0.032	0.256	0.016	0.064	0.004	0.032	0.256
15*adeB*⁻	0.032	0.016	0.256	0.002	0.016	0.0005	0.004	0.256
15*adeC*⁻	0.032	0.064	0.256	0.064	> 0.512	0.008	0.064	0.256
15*adeR*⁻	0.032	0.016	0.256	0.002	0.032	0.001	0.002	0.256
15*adeS*⁻	0.032	0.016	0.256	0.002	0.032	0.001	0.002	0.256
63 (wild type)	0.016	0.032	> 0.512	0.064	0.128	0.004	0.032	> 0.512
63*adeA*⁻	0.016	0.016	0.256	0.032	0.064	0.002	0.016	0.256
63*adeB*⁻	0.016	0.008	0.256	0.002	0.008	0.00025	0.002	0.256
63*adeC*⁻	0.016	0.032	0.256	0.064	0.512	0.008	0.064	0.256
63*adeR*⁻	0.016	0.008	0.256	0.002	0.032	0.001	0.002	0.256
63*adeS*⁻	0.016	0.008	0.256	0.002	0.032	0.001	0.002	0.256

*The minus (−) sign shows created mutants lacking the respective genes.
IMP: imipenem; MEM: meropenem; CAZ: ceftazidime; CTX: cefotaxime; CN: gentamicin; AK: amikacin; CIP: ciprofloxacin; AMP: ampicillin.
CLSI breakpoints: IMP, MEM and CN (S ≤ 4; I = 8; R ≥ 16); CAZ and AMP (S ≤ 8; I = 16; R ≥32); CTX (S ≤ 8; I = 16-32; R ≥64); AK (S ≤ 16; I = 32; R ≥64); CIP (S ≤ 1; I = 2; R ≥4).

final extension step at 72°C for seven minutes.

The involvement of *adeA*, *adeB*, *adeC*, *adeR* and *adeS* genes in carbapenem resistance in *Acinetobacter* spp. was performed according to the modified method of Héritier et al.[16] Briefly, plasmid pAT801, which confers resistance to ampicillin, was used as a shuttle vector. This vector is able to replicate in *Acinetobacter baumannii* and *Escherichia coli*, and it consists of part of the pWH1266 and pUC18 vectors.[17] Inactivation of bla_{TEM-1} resistance gene of pAT801 was performed as described previously by Héritier et al,[16] giving rise to plasmid pAT801-RA, conferring resistance to rifampin. An internal fragment to the *adeA*, *adeB*, *adeC*, *adeR*, and *adeS* genes was amplified using primer pairs as listed in Table I. Each PCR product was cloned into the *Sma*1 digested pAT801-RA vector individually and electrotransformed into another strain of *Acinetobacter* spp. harbouring the respective *ade* genes in order to disrupt the *ade* gene sequence and hence to study its function. The transformants were selected on ticarcillin (50 μg/ml) and rifampin (25 μg/ml) containing plates. The successful disruption of *adeA*, *adeB*, *adeC*, *adeR* and *adeS* genes individually was screened by M13 primers and primers specific to the inserts. The derivative mutants were then subjected to antimicrobial susceptibility testing using various antibiotics, including imipenem, meropenem, ceftazidime, cefotaxime, gentamicin, amikacin, ciprofloxacin and amoxicillin.

RESULTS

Using the API20NE system for species identification, all the strains were detected as *Acinetobacter baumannii-calcoaceticus* complex. Growing the strains at 44°C showed that among the 39 imipenem-resistant strains, 36 were *Acinetobacter baumannii* and only three were *Acinetobacter calcoaceticus*.[10] Further species confirmation was done by ARDRA (results not shown). All the strains were multiresistant to all the antimicrobials tested including imipenem and meropenem. The positive strains for bla_{OXA-23} gene were detected by PCR which resulted in the amplicon of size 1058 bp. Among the 39 carbapenem-resistant strains, 37 were detected positive for the presence of this gene. This PCR product of 1058 bp was sequenced and the data showed that this gene has 96% amino acid sequence homology to bla_{OXA-23}.

PCR amplification of AdeABC and AdeRS showed that 36 were positive for the presence of *adeA*, *adeB*, *adeR* and *adeS* genes and 34 strains carried *adeC* gene. In this study, the three representative *Acinetobacter baumannii* strains, strain 1, strain 15 and strain 63, were chosen and further analysed on the gene association with resistance in these strains. Strain 15 and strain 63 harboured all the five *ade* genes whereas strain 1 harboured all the genes except the *adeC* gene. Upon disruption of each *ade* gene by the insertion-inactivation method, the resulting mutants were determined for antimicrobial susceptibility testing using various antibiotics. Comparing the MIC values of the wild-type strains of strain 1, strain 15 and strain 63, the derivative mutants showed lower MIC values than its parent strains. The MIC values for all the mutants ranged from one- to six-fold lower for ceftazidime, cefotaxime, gentamicin, amikacin, ciprofloxacin, amoxicillin and meropenem, as shown in Table II. The gene knockout

studies showed that the decrease in the MIC_{AK}, MIC_{CN}, MIC_{CIP}, and MIC_{CTX} is greater in the mutant knockouts of the *adeB* gene as compared to *adeA* gene. Mutants for *adeB* gene showed a four- to six-fold decrease in MICs as compared to *adeA* gene, which only showed a one-fold decrease in MICs compared to that of the wild strain. Similar results were observed in gene knockouts of *adeR* gene and *adeS* gene. The MIC_{MEM} in each mutant knockout of the *adeB* gene, *adeR* gene and *adeS* gene, respectively, showed a two-fold decrease in MICs and only one-fold decrease in MIC_{CAZ}, and MIC_{AMX}. However, all the mutants showed no differences in the MIC values for imipenem as compared to the wild-type strains.

DISCUSSION

Carbapenem resistance is an emerging problem in *Acinetobacter* spp. worldwide, and is said to be attributed to various causes, such as the presence of metallo-β-lactamases, oxacillinases, and reduced expression of OMPs.[4,5] In our study, we report that besides the involvement of *bla*$_{OXA-23}$ oxacillinases gene, the RND-type efflux pump also contributes to carbapenem resistance in *Acinetobacter* spp. Generally, the presence of β-lactamases confer resistance to β-lactam antibiotics as they are capable of hydrolysing the four members of β-lactam antibiotics including penicillins, cephalosporins, monobactam and carbapenems. β-lactamases can be divided into four classes (A, B, C and D) according to their sequence similarities.[18] Based on their different catalytic mechanisms, two groups have been established, where the Class B enzymes are metallo-β-lactamases that require zinc for their activity, while the Class A, C, and D β-lactamases contain serine groups in their active site.[19] Oxacillinases are Ambler class D β-lactamases with hydrolytic activity against penicillins, extended-spectrum cephalosporins, methicillin, and aztreonam.[20] However, most of the new OXA-type carbapenemases lack hydrolytic activity against oxacillin, cloxacillin and methicillin, but they display resistance to carbapenems.[15,20]

In our samples, 37 of the 39 carbapenem-resistant strains were positive for *bla*$_{OXA-23}$ gene. This gene was first demonstrated by Brown and Amyes from a clinical isolate of *Acinetobacter baumannii* from Scotland.[21] Since then, the enzyme has been discovered in England, Brazil, Polynesia, Singapore, Korea and China.[21,22] Besides that, other types of oxacillinases with carbapenemase activity have been also identified in different parts of the world, such as OXA-24 to -27, OXA-40, and OXA-58 genes.[5,23-26] In addition to these genes, the naturally-occurring oxacillinases such as OXA-51/-69 have also been studied in detail to elucidate their carbapenemase activities in *Acinetobacter baumannii*.[16,25]

The main aim of this study was to characterise the AdeABC efflux pump genes together with the two component-regulatory genes, AdeRS, and to study their contribution to carbapenem resistance in three selected *Acinetobacter baumannii* isolates. All strains had the *adeA*, *adeB*, *adeR*, and *adeS* genes, but only 34 had the *adeC* gene. Disruption of the *ade* gene resulted in lower MIC values than the parent strains for ceftazidime, cefotaxime, gentamicin, amikacin, ciprofloxacin, amoxicillin and meropenem (Table II). Knocking the gene out showed that the decrease in the MICs was greater in the mutants lacking *adeB* gene, *adeR* gene and *adeS* gene, while all the mutants showed no differences in the MIC values for imipenem as compared to the wild-type strains. This shows that the efflux pump has an effect on the resistance to meropenem compared to imipenem, and the resistance may be due to meropenem upregulating the efflux pump, as suggested by Livermore.[27] Besides that, among the mutants derived from gene knockout of *adeA*, *adeB*, *adeC*, *adeR* and *adeS*, only mutants derived from *adeC* resulted in a similar resistance to antimicrobial agents tested to that of wild-type strains. This indicates that the *adeC* gene was not responsible for the antimicrobial resistance to these antibiotics. Therefore, this finding suggests that the AdeAB pump may also be utilising other proteins, such as the OMP, as suggested by Marchand et al.[8]

Reports from other studies had also shown that the efflux pumps were responsible for a wide range of antibiotic resistance in Gram-negative bacteria.[6,8,15,16] A report by Magnet et al has shown that the AdeABC efflux pump contributes to multidrug resistance in *Acinetobacter baumannii* including fluoroquinolones, tetracyclines, chlorampenicol, erythromycin, trimethoprim and ethidium bromide.[6] However, there is still a scarcity of information on efflux-mediated carbapenem resistance in the *Acinetobacter* spp. Recently, Sinha and Srinivasa reported the efflux pump as a possible cause for carbapenem resistance.[28] In this study, only nine isolates in total were screened, and a comparison of MIC assays were demonstrated using reserpine as an efflux pump mechanism. It is recommended that screening of more isolates are required in order to conclude that the efflux pump has a role in carbapenem resistance. To date, the study conducted by Sinha and Srinivasa is the only most recent publication that has discussed the role of efflux pumps in the development of carbapenem resistance in *Acinetobacters* spp[28]

In conclusion, our study has shown that besides the involvement of *bla*$_{OXA-23}$ gene, the three component system, AdeABC, and the two-component regulatory system AdeRS, have also contributed to carbapenem resistance

in *Acinetobacter* spp. Therefore, it might be crucial to understand the carbapenem-resistant mechanisms for the development of novel therapeutic strategies, since there are multiple mechanisms which affect carbapenem resistance in *Acinetobacter* spp. A novel approach would be to attempt to develop efflux inhibitors as a possible development of new agents to control antimicrobial resistance in nosocomial pathogens, such as *Acinetobacter* spp.

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