Rifampin resistance in carbapenem-resistant *Acinetobacter baumannii* in Siriraj Hospital, Thailand

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ABSTRACT

There is a growing evidence on emergence of carbapenem-resistant *Acinetobacter baumannii* (CRAB) in Thailand and recent treatment guidelines recommend a combination therapy using carbapenem and/or polymyxin with rifampin. Rifampin would be added in a combination therapy. The susceptibility of this pathogen to rifampin is not known, so we studied the rifampin susceptibility and possible mechanisms of resistance used by CRAB. The disk diffusion test was performed on 111 clinical isolates using 5 µg rifampin disk following CLSI guidelines. The inhibition zone was interpreted based upon the recommendation for *Staphylococcus aureus* (inhibition zone <20 mm = resistant). Polymerase chain reaction (PCR) using the primers specific for *arr-2* encoding rifampin ADP-ribosyltransferase was performed in all isolates. The *rpoB* DNA sequences from two isolates, with or without *arr-2*, were compared. All isolates under study were rifampin resistant. Inhibition zone was <14 mm for all isolates. The *arr-2* was positive for 35 isolates (31.5%) and these isolates correlated with high level of resistance (inhibition zone <10mm). The DNA sequences of *rpoB* genes in *arr-2* negative isolate showed mutations L904S, P906R, K909N and M1262K that might have roles in rifampin resistance. Mutations of *rpoB* genes in some isolates and possession of *arr-2* in class 1 integron element were mechanisms for rifampin resistance and these resistant determinants can disseminate through both vertical and horizontal gene transfer. Under this circumstance, it is not recommended to use rifampin in the treatment of carbapenem-resistant *A. baumannii* in Thailand.

Keywords: Carbapenem resistant-*Acinetobacter baumannii*, Rifampin resistance, *arr-2* gene, RpoB

INTRODUCTION

Acinetobacter baumannii is a leading cause of nosocomial infections for patients under invasive medical devices in intensive care units (ICU)[1] and mortality rate is unexpectedly high of 30.0-70.0%.[2] The â-lactam antibiotics, amino glycosides and tigecycline are the drugs of choice for this pathogen, however resistance to these drugs have already been demonstrated.[3,4] Recently, the largest genomic resistance island, called AbaR1, harboring 45 antibiotic and antiseptic resistance genes within the vicinity of insertion sequences and transposons has been reported in *A. baumannii* strain AYE.[5] The increasing incidence of this multidrug resistant *A. baumannii* nosocomial infection has motivated the reintroduction of polymyxin B and colistin (polymyxin E) alone or in combination with carbapenems, aminoglycosides or ampicillin-sulbactam as a treatment option[6] and the resistant phenotypes to these antibiotics alone or in combinations have already been emerged.[7] Finally, rifampin was introduced in combination with colistin or imipenem in the treatment of this superbug.[8,9]

Rifampin executes its function by binding to the â subunit of DNA dependent-RNA polymerase (RpoB) inhibiting the transition from transcription initiation to the protein elongation.[10] Rifampin resistance is governed either by missense mutation in rifampin resistance determining region (RRDR) of the *rpoB* gene or inactivation of rifampin by ADP-ribosyltransferase (Arr). RRDR of *rpoB* gene according to *Escherichia coli* protein coordinates, are the cluster I (amino acids 507-533) and II (amino acids 560 to 572), which harbor most of the missense mutations, while a single mutation at residue 687 defines cluster III.[11,12] These mutations have been described in *E. coli, Streptococcus pneumoniae* and *Mycobacterium tuberculosis.*[10,11,13] Apart from mutations, several other mechanisms like, rifampin efflux pump in *Pseudomonas fluorescens*,[14] inactivation of rifampin by ribosylation[15] and phosphorylation[16] are described.

In the present scenario, rifampin has been used in combination with other drugs to combat pan-drug resistant *A. baumannii* and has given promising results[8,9] and there is a growing concern regarding pan-drug resistant *A. baumannii* pathogen in Thailand, and use of this drug as a therapeutic option is questionable as susceptibility pattern of these isolates to rifampin is not known. Here, we investigated the rifampin susceptibility of carbapenem-resistant clinical isolates of *A. baumannii*

Table-1: Primers used in this studya

Primer name	Sequence 5'-3'	Amplicon (bp)	Gene and nucleotide position[b] rpoB
Abrp1F	TTTCTCCGTGTGTCGGGAAGC	565	-82 to -61
Abrp1R	TTGCGTGCGGGTGTGCCTTTAGCT		485-07
Abrp2F	CGTACGTGAAATGTCAGCCAA	608	512-33
Abrp2R	CAGATGAAGCAATCGCGCTGAGC		999-19
Abrp3F	CAGCCATCTTATCACCAGGCT	622	825-45
Abrp3R	TATCGGTGCTGAAGTTACTGCTGG		1424-47
Abrp4F	ACTGAGTTTCACCTTTAGGCGT	584	1380-01
Abrp4R	AGGTACAGGTATGGAAGCGAACGT		1940-63
Abrp5F	AATTACACCGCCACGGTTTGC	514	1891-11
Abrp5R	TCACTATGGTCGTGTTTGTCCA		2383-04
rpoBR2	GCTTTTGCGTAT	375	2321-32
rpoBF1	ACGTTCTGTTGGTGA		2788-02
rpoBInverse1	TTCTGCTTGGCTTAAACGCT	562	2614-33
Abrp6R	CGTGATGGTGAAGTAATTGCTGCA		3147-71
Abrp7F	GCTTAACACCGCCTTCTGCCAA	549	3093-14
Abrp7R	AATTACACCGTTCGCCAGGCGTA		3619-41
Abrp8F	CGGTAAGGAATGATACGTGCT	619	3545-65
Abrp8R	GGCCATATCGGCCTGCGTAATTCC		+50 to +74
			arr-2
Arr-2F	CATTTCGAGGACGGTCGTAT	417	159-79
Arr2-R	GCCTATTGCGCATAAAATGG		+66-85+

aAll primers were designed in this study, bNucleotide position corresponds to the A. baumannii strain AYE, GenBank accession number CU459141. F stands for forward primer and R stands for reverse primer otherwise indicated.

and report that all of our isolates are highly resistant to rifampin and its use in the patient management is not recommended at the present context.

MATERIALS AND METHODS

Bacterial strains and genomic DNA extraction

One hundred and eleven carbapenem resistant clinical isolates of A. baumannii, collected in the year 2003 and 2004 from patients in Siriraj Hospital, Thailand were studied. These organisms were identified by biochemical tests as suggested by Bovet and Gremont.[17] Chromosomal DNA was extracted using the Genomic DNA purification kit (Puregene, Minneapolis, Minnesota, USA) following the manufacturer's protocol. Extracted DNA was stored at -20°C and 4°C until used. The RpoB sequences of following A. baumannii strains were used for the comparison; SDF, AYE and ACICU (GenBank accession numbers CU468230, CU459141 and CP000863, respectively).

Antibiotic susceptibility test

Clinical isolates of *A. baumannii* were tested for carbapenem resistance by monitoring carbapenemase activity and disk diffusion test was carried using rifampin disk (5ìg) (BBL) on a Muller-Hinton agar plates following CLSI recommendation.[18] As there is no CLSI interpretive criteria for rifampin disk diffusion test for *A. baumannii* and related Gram-negative non-fermentative rods, interpretive criteria of susceptible phenotype (zone of >20 mm) for *S. aureus* has been used in this study.

Polymerase chain reactions (PCR), DNA sequencing and sequence analysis

All the oligonucleotide primers that have been used in this study are summarized in Table-1. PCR primers were designed from *A. baumannii* strain, AYE genome sequence obtained from the ncbi.nlm.nih.gov (GenBank accession number CU459141). PCR was carried out for amplification of *arr-2* and a whole open reading frame of *rpoB* gene. PCR amplification was done in 50 µl reaction volumes containing 25 pmol of each primer, 2mM dNTP (Finzymes), 1 µl of genomic DNA extract, 1 U of DNA polymerase (Finzymes) and 5 µl of supplied PCR buffer. PCR reactions were performed using Biorad thermocycler using the following profile: initial

denaturation at 95C for 5 minutes, 30 cycles of denaturation at 95C for 1 minute, annealing at 55C for 1 minute and extension at 72C for 1 minute and a final extension at 72C for 7 minutes. Amplified products were detected by agarose gel electrophoresis in 1.0% Tris-Acetate-EDTA (TAE) agarose (Research organics, inc. USA) stained with ethedium bromide. The *rpoB* amplicons were purified using PCR clean up kit (Nucleospin extract II, MN) and were sequenced on both strands (1st base sequencing, Malaysia). RpoB protein sequences were aligned using the clustalX (version 1.8).

RpoB sequence comparison

RpoB sequences of these isolates were also compared with *M. tuberculosis*, H37Rv (ATCC 27294), *P. aeruginosa* POA1, *Klebsiella pneumoniae* 342, *Salmonella* Typhimurium LT2 and *E. coli* (K12 MG1655).

Nucleotide sequence accession numbers. Nucleotide sequences for *rpoB* gene of isolates Ab03-128 and Ab03-168 have been deposited in GenBank under the following accession numbers FJ715473 and FJ715474, respectively.

RESULTS

Rifampin susceptibility test

Of 111 carbapenem resistant *A. baumannii* isolates under study, all isolates were resistant to rifampin. Rifampin susceptibility and diameter of zone of inhibition are summarized in Table-2. Inhibition zone ranged from no zone (26.1%) to 14 mm. 21.6% of isolates had inhibition zone between 6-8 mm and 49.8% of isolates had 9-11mm. Only one isolate had 14 mm inhibition zone.

Molecular basis of rifampin resistance

Amplification of the genomic DNA of all isolates using the primer pair Arr-2F and Arr-2R yielded an amplicon size of 417 bp in 35 isolates (31.5%) (Table-2). Seventy six of the isolates (68.5%) were *arr-2* negative. Whole open reading frame (ORF) of *rpoB* gene from *arr-2* positive (Ab03-168) and negative isolate (Ab03-128) was amplified and sequenced. DNA sequences were translated using translation tool at www.expasy.ch. The RRDR of RpoB sequences of these isolates were aligned with RpoB sequences of *A. baumannii;* SDF, AYE, ACICU, *E. coli* and *M. tuberculosis* (Fig. 1). There was no mutation in RRDR of *arr-2* negative isolate (Ab03-128) and sequence was similar to other *A. baumannii*, *M. tuberculosis* and *E. coli*. RRDR of RpoB of *M. tuberculosis* had T508 while others had S508. There were six amino acids difference in RpoB sequences outside the RRDR between *arr-2* positive and negative isolate. Ab03-128 had four mutations L904S, P906R, K909N and M1262K (Fig. 2 and 3), while Ab03-168 had E297D and R307L (data not shown).

Table-2: Rifampin susceptibility and arr-2 gene PCR in CRAB

ZI (mm)	Rifampin^r		*arr-2* positive	
	2003 (n=58)	2004 (n=53)	2003(n=19)	2004 (n=16)
No zone	11 (9.9)	18 (16.2)	7 (8.1)	12 (10.8)
6.0-8.0	13 (11.7)	11 (9.9)	9 (8.1)	4 (3.6)
9-11.0	30 (27.0)	24 (22.6)	3 (2.7)	
12-14.0	4 (2.7)			

ZI, Zone of Inhibition. Rrifampin resistant. All siolated were resistant to rifampin. 35/111 (31.5%) were arr-2 positive.

RpoB mutation located in a conserved region

The RpoB sequences inside and outside RRDR were also compared with other *A. baumannii*; AYE, SDF and ACICU, *E. coli* K12 MG1655, *K. pneumonia* 342, *S.* Typhimurium LT2 and *M. tuberculosis*. RRDR region was conserved in *arr-2* positive and negative isolates (Fig. 1). Amino acid sequences outside RRDR were also conserved in same regions. These novel mutations were located in these conserved regions.

	Cluster I	**Cluster II**
AYE	516 GSSQLSQFMDQNNPLSEITHKRRVSAL 543	569 PIETPEGPNIGLI 581
ACICU	516 GSSQLSQFMDQNNPLSEITHKRRVSAL 543	569 PIETPEGPNIGLI 581
SDF	516 GSSQLSQFMDQNNPLSEITHKRRVSAL 543	569 PIETPEGPNIGLI 581
Ab03-168	516 GSSQLSQFMDQNNPLSEITHKRRVSAL 543	569 PIETPEGPNIGLI 581
Ab03-128	516 GSSQLSQFMDQNNPLSEITHKRRVSAL 543	569 PIETPEGPNIGLI 581
E. coli K12	507 GSSQLSQFMDQNNPLSEITHKRRVSAL 533	560 PIETPEGPNIGLI 572
M. tuberculosis H37Rv	426 GTSQLSQFMDQNNPLSEITHKRRVSAL 453	479 PIETPEGPNIGLI 491
	*	

Fig. 1. ClustalX alignment of two major RRDR (cluster I and II) of RpoB from clinical isolates under study (Ab03-128 and Ab03-168), A. baumannii strains (AYE, ACICU, and SDF), E. coli and M. tuberculosis. Ab03-128 (arr-2 negative) and AYE, Ab03-168 (arr-2 positive) are rifampin resistant and others are rifampin susceptible. RRDR were conserved among A. baumannii and E.coli except M. tuberculosis had *T508 (E. coli numbering). Numbers indicate codon position in RpoB of respective species.

AYE	887 TAGDILVGKV TPKGETQLTP EEKLLRAIFG EKAADVKDSS LRVPSGTKGT 936
ACICU	887 TAGDILVGKV TPKGETQLTP EEKLLRAIFG EKAADVKDSS LRVPSGTKGT 936
SDF	887 TAGDILVGKV TPKGETQLTP EEKLLRAIFG EKAADVKDSS LRVPSGTKGT 936
Ab03-168	887 TAGDILVGKV TPKGETQLTP EEKLLRAIFG EKAADVKDSS LRVPSGTKGT 936
Ab03-128	887 TAGDILVGKV TPKGETQSTR EENLLRAIFG EKAADVKDSS LRVPSGTKGT 936
P. aeruginosa POA1	883 QAGDILVGKV TPKGETQLTP EEKLLRAIFG EKASDVKDSS LRVPTGTKGT 932
E. coli K12	878 TGGDILVGKV TPKGETQLTP EEKLLRAIFG EKASDVKDSS LRVPNGVSGT 927
K. pneumoniae 342	878 TGGDILVGKV TPKGETQLTP EEKLLRAIFG EKASDVKDSS LRVPNGVSGT 927
S. Typhimurium LT2	878 TGGDILVGKV TPKGETQLTP EEKLLRAIFG EKASDVKDSS LRVPNGVSGT 927
M. tuberculosis H37Rv	791 RDGDILVGKV TPKGETELTP EERLLRAIFG EKAREVRDTS LKVPHGESGK 841
	* * * * * * * * * * * * *

Fig. 2. ClustalX alignment of RpoB outside RRDR of A. baumannii under study (Ab03-128 and Ab03-168), AYE, ACICU and SDF, other Gram-negative bacteria and M. tuberculosis. Ab03-128 (arr-2 negative) had 904S, 906R, and 909N while arr-2 positive and resistant strains, AYE, and Ab03-168, arr-2 negative but susceptible strains, ACICU and SDF, and rest of the Gram-negative bacteria had L904, P906, and K909. This region was conserved among Gram-negative bacteria. *Mismatch amino acids.

DISCUSSION

Rifampin is a principal drug used to treat *M. tuberculosis* infection and is derived from rifamycin, a product of *Nocardia mediterranei*[19]. It is bactericidal drug which has been widely used for several other multidrug resistant pathogens like methicillin resistant *Staphylococcus aureus* (MRSA),[20] streptococci,[21] legionellae,[22] brucellae,[23] *P. aeruginosa,*[24] and *A. baumannii.*[8,9] This has been used in combination with carbapenems and/or polymyxin to treat CRAB. We have already documented increasing prevalence of CRAB in this hospital and rifampin would be added in the treatment. On determining the rifampin susceptibility of 111 clinical isolates, we found that all of CRABs studied were resistant to rifampin. Similar resistance to rifampin has already been demonstrated in *E. coli*,[10] *S. pneumonia*,[11] mycobacteria,[13] *Neisseria meningitides*,[25] *P. aeruginosa,*[26] and recently in *A. baumannii.*[27] There is no CLSI interpretive criteria for rifampin disk diffusion test for *A. baumannii*, the interpretive criteria of <20mm for *S. aureus* as resistant was used in this study.[18] This can be adopted as the future guideline for the interpretation of the rifampin disk diffusion test for *A. baumannii* and related organisms, but it needs validation with larger number of sample and with isolates from different geographical locations.

AYE	1241 DRPVTVGYMY MLKLNHLVDD KMHARSTGSY SL 1272
ACICU	1241 DRPVTVGYMY MLKLNHLVDD KMHARSTGSY SL 1272
SDF	1241 DRPVTVGYMY MLKLNHLVDD KMHARSTGSY SL 1272
Ab03-168	1241 DRPVTVGYMY MLKLNHLVDD KMHARSTGSY SL 1272
Ab03-128	1241 DRPVTVGYMY MLKLNHLVDD KKHARSTGSY SL 1272
P. aeruginosa POA1	1237 DRPVTVGYMY MLKLNHLVDD KMHARSTGSY SL 1253
E. coli K12	1237 DRPVTVGYMY MLKLNHLVDD KMHARSTGSY SL 1253
K. pneumoniae 342	1237 DRPVTVGYMY MLKLNHLVDD KMHARSTGSY SL 1253
S.Typhimurium LT2	1237 DRPVTVGYMY MLKLNHLVDD KMHARSTGSY SL 1268
M. tuberculosis H37Rv	
	1014 PYPVTVGYMY I MKLHHLVDD KI HARSTGPY SM 1045
	* * * * * * *

Fig. 3. ClustalX alignment RpoB outside RRDR of A. baumannii under study (Ab03-128 and Ab03-168), AYE, ACICU, and SDF, other Gram-negative bacteria and M. tuberculosis. Ab03-128 (arr-2 negative) had 1262K while arr-2 positive and resistant strains, AYE, and Ab03-168, arr-2 negative and susceptible strains, ACICU and SDF and rest of the Gram-negative bacteria had M1262, except for M. tuberculosis which had I1262. This region was conserved among Gram-negative bacteria and M. tuberculosis. *Mismatch amino acids.*Mismatch amino acids.

Molecular basis of resistance to rifampin in *A. baumannii* and other Gram-negative bacteria is classically due to the presence of *arr-2* gene which is carried on class 1 integron element.[26] Arr is naturally occurring enzyme in *M. smegmatis* and variants of *arr* gene, *arr-2*, *-3*, *-4* and *-5* have been described.[28] Amplification with the *arr-2* specific primers gave positive PCR for 35 isolates. These *arr-2* positive isolates in this study correlated with high level of resistance, no inhibition zone (54.2%) or inhibition zone between 6-10 mm (37.1%). This has also been reported by Elizabeth *et al*. where MIC was e"32 µg/ml.[27] Surprisingly, *arr-2* negative isolates were also rifampin resistant, albeit at low level, and this highlights some other mechanisms of resistance in those isolates.

Missense mutations in and out of clusters I, II, and III of *rpoB* is a common mechanism of resistance to rifampin.[29] Amino acids 507-511, 513, 522, 526 and 531 of RRDR forms a part of rifampin binding site and mutation at these residues are associated with no rifampin binding and a high level of resistance.[10] All these residues were not mutated in sequenced RpoB of both isolates. *M. tuberculosis* has residue T508 (*E. coli* numbering) and mutation to 508S has been reported in 2.0% isolates, however, the resistance pattern due to this mutation was not confirmed.[10] Both of these isolates also had S517 (*M. tuberculosis*, 427) which might go along with this report but presence of S508 (*M. tuberculosis*, 427) in rifampin susceptible *E. coli* and 517S in rifampin susceptible *A. baumannii;* SDF and ACICU *rpoB* sequences suggest this doesn't have any role in rifampin resistance.

On comparison of amino acid sequences outside these clusters with *A. baumannii* (SDF, AYE and ACICU) and other species, mutations L904S, P906R, K909N and M1262K were identified in *arr-2* negative strain Ab03-128. These mutations were located in a region which was conserved in different species and these residues might play critical role for rifampin resistance in isolates without *arr-2.* These mutations have never been described in *A. baumannii* and other species.[10-13] More sequencing of rifampin resistant (*arr-2* negative) strains and mutagenesis studies are warranted to support this data.

In summary, 100% of CRAB isolates were rifampin resistant and molecular mechanisms to this resistance were based on the possession of class 1 integron borne *arr-2* and mutations outside RRDR of RpoB in those isolates which were devoid of *arr-2,* understanding that there exists several mechanisms for rifampin resistance which have not been explored here. We conclude on our findings that rifampin use in the treatment of CRAB is not recommended and urge for the search of novel drug targets and discovery of newer drugs to overcome the problem with this pathogen.

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