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In vitro evaluation of MFS efflux pumps among multidrug resistant *Acinetobacter baumannii* isolated from patients hospitalized in ICU

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ABSTRACT

Multiple drug resistance (MDR) in Acinetobacter baumannii strains is a rapidly rising phenomenon worldwide. Different mechanisms are responsible for this resistance and efflux pumps are among them. In A. baumannii resistance resulted from efflux pumps are mainly in Major facilitator super family (MFS) and Resistance nodulation cell division (RND) families. Therefore, this study has been conducted to study in vitro evaluation of MFS efflux pumps among multidrug resistant A. baumannii isolated from patients hospitalized in intensive care unit (ICU). In this cross-sectional study, 100 MDR isolates were selected from a total of 200 clinical A. baumannii isolated from ICU; Multiplex- RT- PCR methods was used to in vitro evaluation of MFS efflux pumps among multidrug resistant A. baumannii isolated from patients hospitalized in ICU. According to the susceptibility test, among 200 A. baumannii isolates, the prevalence rate of MDR, non MDR and extremely drug resistance (XDR) were 56%, 34% and 10% respectively. Abaye 0369 gene expression was observed in MDR and non-MDR isolates in presence of tetracycline which is an indication of its role in antibiotic resistance against tetracycline. In confirming the role of MFS transporters such as abaye 0369 in tetracycline resistance, functional studies should be designed. For assessing the effect of the expression of other studied MFS transporters in tetracycline resistance, their expression rate should be determined using Real Time PCR.

Key words: *Acinetobacter baumannii*, MFS, *abaye* genes

INTRODUCTION

Acinetobacter baumannii genus is defined as a facultative anaerobe coccobacillus with gram-negative, non-fermenting, non-motile, catalase-positive, and oxidase-negative properties. This opportunistic microorganism could be a causing urinary tract infection, septicemia and meningitis. There are not many drugs that can fight the infections caused by this bacterium because involved high resistance mechanisms [1]. *Acinetobacter baumannii* strains that are resistant against at least three classes of well-known antibiotic classes are called multidrug-resistant *A. baumannii* strains (MDR) [2]. MDR-AB strains are considered formidable pathogens especially among patients diagnosed with pneumonia who are hospitalized in intensive care unit (ICU). Crude mortality rate of nosocomial pneumonia caused by *A. baumannii* is reported to be 30% to 70% [3]. According to the studies, MDR strains of *A. baumannii* in European countries as well as other regions is on the rise which could be a result of aerial travels from countries with high MDR strains to European regions. Developing countries such as Iran have serious problems in treating the infections resulted from MDR strains. Long hospitalization periods, Prolonged stay in the ICU, exposure to antimicrobial agents, mechanical ventilation, colonization pressure, invasive procedure, recent surgery and

underlying diseases are among risk factors involved in infection and colonization of MDR strains [4]. As the *Infectious Diseases Society* of *America* (IDSA) is considered *A. baumannii* as a Red alert pathogen. Before 1970s, *A. baumannii* could be treated with most of the well-known antibiotics; but nowadays, has found resistance against nearly all antibiotics [5]. Such resistance mechanisms could be observed in MDR strains of *A. baumannii* such as beta-lactamases, outer membrane proteins (OMP [porin]) changes; Aminoglycoside-modifying enzymes (AMEs), Multiple Efflux pump, and point mutations [6]. Protein carriers involved in efflux system are those which have role in reduction of drug concentration or toxic substances through transporting them from inner and outer membrane space to a place out of cell environment. MEPs are classified into 6 major families:

1. ATP-binding cassette (ABC)
2. Major facilitator super family (MFS)
3. Resistance nodulation cell division (RND)
4. Multidrug and toxic compound extrusion (MATE)
5. Small multidrug resistance (SMR) family
6. drug/metabolite transporter (DMT) superfamily [7]

Efflux transporters are expressed in every living cell and protect them from toxic effects of organic compounds as well actively export various classes of antibiotics out of cell. MDR is often a result of overexpression of these transporters. Increased rate of antibiotic effluxes could lead to reduced drug aggregation and increment in MIC [8]. Major facilitator superfamily (MFS) in its normal form is not multidrug transporters; but, nonetheless they usually act as exclusive exporters for various classes of antibiotic agents [7]. In *A. baumannii* resistance by efflux pumps is mainly caused by MFS and RND families [8]. The most important genes involved in antibiotic resistance by MFS efflux pumps are abaye genes. Therefore, due to lack of studies on MFS efflux pumps and their abaye genes in Iranian strains, this study has been conducted to in vitro evaluation of MFS efflux pumps among multidrug resistant *A. baumannii* isolated from patients hospitalized in ICU**.**

MATERIALS AND METHODS

Statistical population

In this cross-sectional study, 200 clinical isolates [urine (n=20), respiratory (n=80), blood (n=17), wound (n=51), and CSF (n=12)] were investigated using microbial methods and biochemical tests (n=16) and subsequently the presence of *Acinetobacter baumannii* were confirmed through bla_{OXA51} gene [9]. Samples were collected from ICU of Motahari hospital, Gonbad (n=50), Namazi hospital of Shiraz (n=50) and hospitals of Tehran (Children Medical Center and Imam Khomeini (n=100)) during March 21, 2011 to February 19, 2013, 50 MDR isolates and 50 non-MDR isolates were entered the final study to studding the effect of desired antibiotics on the expression of 5 selected MFS transporter genes.

Bacterial strains and antibiotic susceptibility testing

Bacterial strains were cultured in 37 degrees in Toy Soy Broth (TSB) and antibiotic susceptibility testing was carried out using disk diffusion method of Kirby Bauer (KB).

Determining MIC of MDR and non-MDR strains

Based on CLSI guidelines MDR strains were placed alongside selected antibiotics (tobramycin, levofloxacin, tetracycline, and erythromycin) and their MIC were determined using micro broth dilution [10]. Standard strains of *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were used as control bacteria.

DNA extraction

DNA extraction was done using the mentioned method already [11], briefly, in boiling DNA extraction, we dissolved 5 colonies of isolates in 200 µl high pure distilled water and incubated for overnight, then boiled for 10 minutes and coiling process was done in ice and centrifuged in 8000 rpm for 5 minutes. Supernatant was used as template in PCR reaction.

Determining selected MFS genes using multiplex PCR

First, MFS gene sequences (*abaye*) as well as *fum C* gene (fumarase) were selected from gene bank; then, aligning process was done using BioEdit software in which primerPlex and Gene Runner Software were applied to design desired primers for selected genes as well as *Fum C* gene (Fumarase) as the house keeping or positive control gene

and was ordered to Takapouzist company for synthesis (table 1). PCR reaction setup was initially prepared as uniplex and then as multiplex; the needed values for each reaction were as following: 10 µl of Master Mix (Amplicon, Denmark), 1.5 µl of each primer (Takapouzist, Iran), and 2µl of 100 pg of genomic DNA in 25 µl PCR reaction and 7 µl of distilled water; primers of *fum* and *abaye* genes were used in the concentration of 2.5 pmol. PCR cycle was as following: one cycle for initial denaturation at 94 º C for 3 minutes, 30 cycles with denaturation at 94 º C for 45 seconds, annealing stage in 47 º C for 30 seconds, extension temperature at 72 º C for 30 seconds and final extension temperature in 72 º C for 5 minutes; at last, electrophoresis was performed on 2% agarose gel and staining was prepared using safety dye.

Table 1 The necessary primers for *abaye* and *fumarase* genes

abaye genes	Primers sequences	Amplicons size (bp)	Tm (ºC)
0369	5'-GTGATAGCATCTACGGTAAGTA-3' 5'-CGGGTACTATACAAAATGATCG-3'	155	58.4/50.6
0685	5'-CGTATTTTACAAGCCTTAGGTG-3' 5'GGTAGCCACTTGATATAGAGAT-3'	300	58.4/51.1
3640	5'-GCGATGGATATTTATCTTCCAG-3' 5'-CCAGCATCGAACTGAAAAG-3'	361	58.4/51.1
3035	5'-CATGTCTATGTATTGTGCTGAC-3' 5'-CGTAAGGTGATAATCTAGTCCA-3'	422	58.4/50.27
0224	5'-GTCATCTTAGGTCTACAACAGT-3' 5'-GAGTCCTACTAATTGTTCAGGT-3'	504	58.4/49.7
fumC	5`-TTAAATGCTCATCCTGACTA-3` 3`- TGCCGTAACTAACATAAGAG-5`	528	48.1/47.1

Assessing the expression patterns of *abaye* genes

RNA extraction

Based on High pure kit manual, 5-10 ml of TSB medium were poured in 15 ml falcons and from desired bacterium (50 MDR and 50 Non-MDR strains) were once cultured in presence of antibiotic with lower than MIC concentration (sub MIC); they also were cultured without the presence of antibiotic and were kept into shaking incubator until reaching OD of 0.3 to 0.4 due to the highest growth rate of bacterium is in log phase; then, the falcon contents were centrifuged in 10000 rpms for a 5 minutes using a standard refrigerated centrifuge, so the bacterium would participate and bacterial plaque would be achieved. At the next phase, the topmost liquid was offloaded so that only sediment would remain; then, 750 ml of extract solution was added and after putting into vortex for 15 minutes, then was kept at room temperature for 5 minutes; in the next step, 200 ml of chloroform was added and intense vortex session was followed and after remaining 5 minutes in room temperature, was centrifuged in 4 º C temperature with 10000 rpm so two separately blue and chloroform phases would be formed; from the topmost liquid 400 ml was taken to a new 1.5 ml micro- tube accompanied by an equal amount of isopropanol; after enough shaking, the tube was kept in room temperature for 10 minutes and then for 10 minutes was centrifuged with 11800 rpms; again the topmost liquid was drop off and the sediment containing RNA would be remained. In the subsequent step, 1 ml of ethanol 70% was added for rinsing purpose and after a 5 minutes 10000 rpms centrifuging and offloading, RNA was appeared in the form of the remaining sediment. Since the alcohol should be completely extracted, RNA tube was kept in room temperature so it should be dried up and with low possible moisture. 25 ml of *DNase/RNase*-free distilled *water* was added and a few taps were applied on the tube for RNA's better dissolving; in order to RNA that were sticking to tube's walls to come down, the tube was kept in a 65 º C incubator for a 5 minutes. Finally, for determining the quality of extracted RNA, its OD was read through Nano Drop device in 260/280 and 230/260 wavelengths which showed an OD of 1.8-2 which was in line with the values specified by the standard kit.

Treatment of extracted RNA with DNase

Based on the kit's manual, 1.7 ml of extracted RNA was mixed with 2 ml of RNase free DNase and incubated for 30 minutes in 37 º C; then, 1 µl of DNase stop solution was added and the substance was kept in 65 º C temperature for 10 minutes. This process was done to ensure the elimination of genomic DNA as well as confirmation on the presence of RNA.

cDNA synthesis

According to the kit's direction, 2 ml of RNA which has been treated with DNase were mixed with 1 µl of random hexamer enzyme and added to sufficient amount of water to take the solution's volume up to 10 ml; then, it was incubated in 65 º C for 5 minutes and was cooled on ice; then added 10 ml of RT- PREMIX solution in order to take the total volume up to 20 ml. Micro tube was slowly shook upside down and was incubated in 25 º C for 10 minutes

and 50 º C for 60 minutes respectively, after that, in order to stop the reaction, it was kept in 70 º C condition for 10 minutes and was immediately cooled on ice and the cDNA synthesis process was completed. The rest of the RT-PCR procedures are nearly identical to PCR. Finally, the data were entered and analyzed using spss software (ver. 19.0.0; SPSS Inc., Chicago, IL) where significant differences in variables were analyzed by $\chi 2$ test.

RESULTS

According to the susceptibility test, among 200 *Acinetobacter baumannii* isolates, the prevalence rate of MDR, non MDR, and XDR were 56%, 34% and 10% respectively; based on CLSI guidelines, MDR strains were incubated with selected antibiotics (tobramycin, levofloxacin, tetracycline, and erythromycin) and their MIC were determined using micro broth dilution (table 2).

Table 2: Determining the MIC of selected antibiotics for 200 isolates of *Acinetobacter baumanii*

Resistant phenotype (number)	Antibiotics types	The number of sensitivity cases	MIC (ug/ml)								
			<0.5	1	2	4	8	16	32	64	128
	Tobramycin	2	0	1	1	0	3	6	8	1	1
XDR	Levofloxacin	7	0	0	7	0	1	5	3	3	2
(21)	Erythromycin	1	0	0	0	0	1	12	3	1	4
	Tetracycline	8	0	3	5	0	7	1	4	1	0
	Tobramycin	24	4	6	9	5	24	34	21	9	0
MDR	Levofloxacin	22	9	13	0	24	331	16	18	0	1
(112)	Erythromycin	12	0	12	0	21	19	26	21	13	0
	Tetracycline	31	20	8	4	14	33	26	21	1	0
	Tobramycin	11	0	0	9	2	23	16	17	0	0
Non MDR	Levofloxacin	14	1	10	3	7	21	10	0	10	0
(67)	Erythromycin	10	4	17	0	11	18	7	14	7	0
	Tetracycline	17	0	5	1	11	17	23	10	0	0

Tracing MFS genes using multiplex PCR

At first, PCR reaction was setup for uniplex and then multiplex method (Figure 1). As is shown in table 3, after assessing the clinical isolates regarding the presence of MFS transporters, it was revealed that 0685 and 3035 genes are present in all studied clinical isolates. Frequency of other desired genes in MDR and non-MDR isolates was varied.

Table 3: Determining the MIC50 and MIC90 of selected antibiotics in 200 studied isolates of *A. baumannii*

MIC (µg/mL)	Antimicrobial agents	phenotype		
MIC (µg/mL)		XDR	MDR	Non-MDR
	TOB	32	32	16
MIC_{50}	**LVX**	64	32	64
	CIP	128	128	32
	TET	32	32	16
MIC_{90}	**TOB**	16	16	8
	LVX	16	8	8
	CIP	16	16	8
	TET	8	8	8

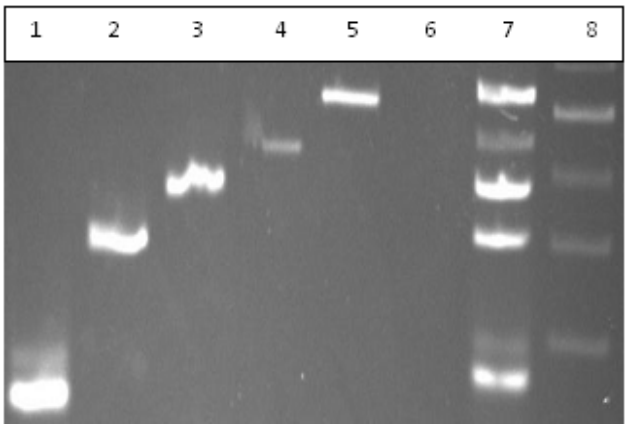

Figure 1. Multiplex PCR setup for tracing selected MFS genes Well 1-5. Uniplex PCR product of selected genes Well 7. Multiplex PCR product (5 Plex PCR) of selected genes Well 1. Multiplex PCR product of 0369 gene Well 2. Multiplex PCR product of 0685 gene Well 3. Multiplex PCR product of 3640 gene Well 4. Multiplex PCR product of 3035 gene Well 5. Multiplex PCR product of 0224 gene Well 8. 100 bp DNA Ladder

Evaluating the expression of MFS genes using RT- PCR method

After RNA extraction, evaluation of gene expression with RT- PCR revealed that none of the 100 MDR and non-MDR isolates have shown the expression of *abaye 0369* either with the presence of erythromycin, levofloxacin, tobramycin or without them. At the presence of tetracycline, *abaye 0369* gene expression was observed in 80% and 78% of MDR and non-MDR strains respectively; such phenomenon was not found without presence of tetracycline (table 4).

Table 4: Frequency of MDR and non-MDR isolates of *Acinetobacter baumannii* with expression of selected MSF transporters in the presence of sub-MIC concentration of selected antibiotics and without them

		Percentage of isolates expressing *abaye* genes									
		0224		3035		3640		0685		0369	
Resistance phenotype (No.)	**Antibiotic types**	**In lack of antibiotics**	**In presence of antibiotics**	**In lack of antibiotics**	**In presence of antibiotics**	**In lack of antibiotics**	**In presence of antibiotics**	**In lack of antibiotics**	**In presence of antibiotics**	**In lack of antibiotics**	**In presence of antibiotics**
Non MDR (50)	Erythromycin	14	14	100	100	4	4	46	46	0	0
	Levofloxacin	6	6	100	100	20	18	34	34	0	0
	Tobramycin	14	14	100	100	6	6	44	44	0	0
	Tetracycline	12	12	100	100	32	32	38	0	2	78
MDR (50)	Erythromycin	9	0	100	100	1	0	42	42	0	0
	Levofloxacin	11	0	100	100	11	0	34	34	0	0
	Tobramycin	8	0	100	100	6	0	40	40	0	0
	Tetracycline	5	0	100	100	13	0	52	0	0	80

As is evident from table 4, the expression of *abaye 0685* gene was observed in presence of erythromycin, levofloxacin, tobramycin, and without them while such was not the case in the presence of tetracycline. The expressions of *abaye 3640* and *abaye 0224* were observed in the presence of levofloxacin, tobramycin, and tetracycline in non-NDR isolates; without the presence of antibiotics, such expressions was observed in MDR isolates (table 4). *Abaye 3035* gene expression was observed in 100% MDR and non-MDR isolates with and without antibiotics. Based on statistical analysis between studied MFS gene expression and the presence of antibiotics and lack of them as well as the appearance of MDR phenotype no significant relationship was found ($P>0.05$). The only exception in these cases was the expression of *abaye 0369* in strains with MDR and non-MDR phenotypes in the

presence of tetracycline; statistically, a significant relationship was observed between the expression of this MFS transporter and the presence of tetracycline (P=0.001).

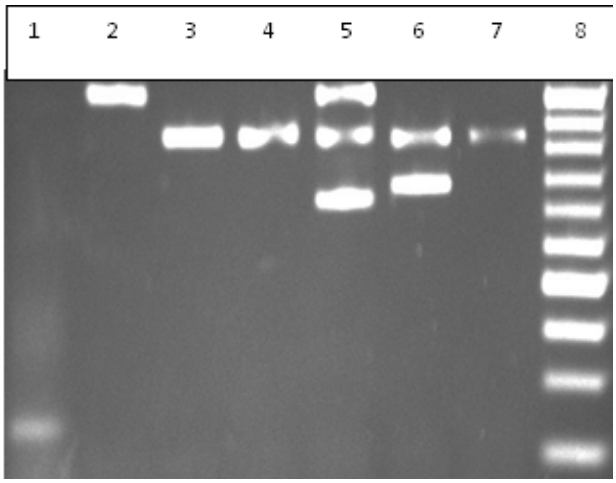

Figure 2: A sample of RT-Multiplex PCR product in evaluation of expression of selected genes Well 1 Multiplex PCR product on RNA adjoined with DNase Well 2 Multiplex PCR product of housekeeping gene Well 3 Multiplex PCR product sample without antibiotic Well 4 Multiplex PCR product sample with erythromycin antibiotic Well 5 Multiplex PCR product sample with levofloxacin antibiotic Well 6 Multiplex PCR product sample with tobramycin antibiotic Well 7 Multiplex PCR product sample with tetracycline antibiotic Well 8 Marker

DISCUSSION

MDR *A. baumannii* (MDR- AB) is considered as an emerging pathogen in health care settings specifically in ICU which has significantly reduced clinical active antibiotics that would have been used for fighting against this bacterium. Multi-drug resistance (MDR) in *Acinetobacter* strains is raising problem worldwide which have prompt a significant concern in public health [12]. In our study, MDR rate was 56% which was similar to many other local and international studies. In various studies conducted in Iran during 2008-2011, MDR-AB rate has had an increasing pattern in a way that it has been reported from 30% up to 94% [12]. In *Escherichia coli* from 39 efflux pumps involved in antibiotic resistance, most of them belong to MFS family and as was mentioned before, in *A. baumannii*, resistance toward antibiotics through efflux pumps are generally via efflux pumps of MFS and RND families [8]. Some gram-positive bacteria such as *Staphylococcus aureus*, *Bacillus subtilis*, and *Streptococcus pneumoniae* have efflux pumps of NorA, Bmr, and PmrA respectively from MFS family which have a notable role in resistance against fluoroquinolones and are generally similar to each other [13]. Efflux pumps of QacA/B in *Staphylococcus aureus* are parts of MFS family which would cause resistance toward biocides of ammonium quaternary compound [14]. In *Listeria monocytogenes* there is a MdrL efflux pump from MFS family which have a role in resistance against cephalosporins. Efflux pump of TetA,B which is a member of MFS family and has a notable effect on the resistance against tetracycline and minocycline, has a nucleotide sequence similarity in gram-negative and gram-positive bacteria [15]. In *A. baumannii*, efflux pumps of MFS family such as CraA and CmlA have significant role in resistance to chloramphenicol and efflux pump of SmvA affects the resistance towards erythromycin. Expressions of *bye 0369* gene was observed in Non-MDR and MDR isolates with presence of tetracycline which is an indication of this gene's role in antibiotic resistance against tetracycline, in fact, have a similar role with efflux pump Tet (A) that can consider it as one of MFS pumps [16]. Efflux pumps of MdfA and MefA are also affecting the resistance against tetracycline in *Salmonella typhimurium* and *Streptococcus pyogenes* of MFS family respectively. Assessing the presence of MFS genes in clinical isolates showed that *abaye 0685* gene as well as *abaye 3035* is present in all clinical isolates which make them a suitable marker for tracing *A. baumannii* isolates. Although unlike non-MDR isolates, *abaye 3640* and *abaye 0224* did not show any expression in MDR strains in presence of selected antibiotics; this difference was not significant enough that would make them a suitable marker for differentiating non-MDR from MDR isolates. Expression of *abaye 3035* gene was evaluated in presence of selected antibiotics as well as without them in all isolates and it was presented that its expression or lack of it does not have any effect on antibiotic resistance; it has a physiologic role in *A. baumannii*. *Abaye 3640* gene and *abaye 0224* showed expression in 15% and 11.5% of non-MDR isolates respectively, while in MDR strains and

in presence of antibiotics such expression was not observed; since this difference was not statistically enough significant (P = 0.11), the relationship between their expression or lack of theses and resistance phenotype cannot be confirmed. In regard to *abaye 3640* and *abaye 0224* genes showed expression in the presence of selected antibiotics as well as without them, it's possible that they could be the general transporters of this bacterium. As was mentioned before, due to the expression of several genes in the presence of selected antibiotics as well as without them, their expression or lack of these could not yet related to the antibiotic resistance; although, it's possible that the difference in their expression could be an indication of their role in creating the resistance, to investigate this hypothesis requires further studies be designed.

CONCLUSION

This study showed that MFS transporter of *abaye 0369* has an expression in the presence of tetracycline while such phenomenon did not occur without presence of this antibiotic. Thus, there is a possibility that this transporter might have a role in resistance against tetracycline. Expression of MFS transporter of *abaye 3035* was observed in all isolates and in the presence of all selected antibiotics as well as lack of them. Therefore, it was confirmed that the expression of this gene or lack of it does not have any affect in the resistance against selected antibiotics. Since MFS transporter genes of *abaye 0685* and *abaye 3035* were present in all studied clinical isolates, it's possible that they could be used as markers to trace *A. baumannii* isolates. In order to confirm the role *abaye 0369* gene of MFS transporter in resistance against tetracycline, functional studies should be designed. For evaluating the effect of expression of other studied MFS transporters in antibiotic resistance, their expression should be accessed through Real Time PCR

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