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Original Article

Comprehensive study to investigate the role of various aminoglycoside resistance mechanisms in clinical isolates of *Acinetobacter baumannii*

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ABSTRACT

Therapeutic resistance towards most of the current treatment regime by *Acinetobacter baumannii* has reduced the prescribing antibiotic pattern and option is being re-shifted towards more toxic agents including aminoglycosides. The present investigation aimed at to study various mechanisms towards aminoglycoside non-susceptibility in clinical isolates of *A. baumannii*. The bacteria were subjected to genetic basis assessment for the presence of aminoglycoside modifying enzymes (AME), 16S rRNA methylase encoding genes and relative expression of AdeABC and AbeM efflux pumps in relation to their susceptibility to five aminoglycosides. When isolates were subjected to typing by repetitive extragenic palindromic (REP) PCR, isolates could be separated into thirteen definite clones. The majority of isolates (94%) were positive for AME encoding genes. Possession of *ant(2′)-Ia* correlated with non-susceptibility towards gentamicin, amikacin, kanamycin, tobramycin; while, presence of *aph(3′)-VIa* attributed to resistance towards amikacin, kanamycin; possession of *aac(3′)-Ia* allied with non-susceptibility to amikacin, tobramycin and presence of *aac(3′)IIa* correlated with kanamycin non-susceptibility. Presence of *armA* was detected in 34.4%, 34.2%, 29.2%, 40.3%, and 64.2% of isolates showing non-susceptibility to gentamicin, amikacin, kanamycin, tobramycin and netilmicin, respectively. No isolates were found to carry *rmtB* or *rmtC*. Amikacin non-susceptibility in comparison to other aminoglycosides correlated with over production of *adeB*.

Overall, the results represented a definitive correlation between presence of AME encoding genes as well as *armA* and resistance of *A. baumannii* towards aminoglycosides. On the other hand, the up-regulation of AdeABC and AbeM systems was found to have only the partial role in development of aminoglycoside resistance.

1. Introduction

Acinetobacter baumannii is an opportunistic pathogen notorious for causing diverse nosocomial infections, principally in immunocompromised patients besides those subjected to invasive procedures [1]. During last few decades clinicians are facing challenges as the organism switched from multi-drug (MDR) to extensive (XDR) drug resistant [2]. The impressive number of antimicrobial resistance mechanisms makes selection of an appropriate empirical

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treatment exceedingly difficult [3]. Despite the development of new antimicrobial agents, aminoglycosides continue to play an important role in the treatment of severe illnesses caused by *A. baumannii*, usually in combination with β-Lactam agents. Resistance to aminoglycosides has primarily been the consequence of aminoglycoside inactivation, through *N*-acetylation, *O*-nucleotidylation, and/or *O*-phosphorylation, with varying effects depending upon the particular agent [4]. However, other mechanisms such as 16S rRNA methylation and over expression of the RND (Resistance-Nodulation-Division) -type efflux pump (AdeABC) and the MATE (Multi-antimicrobial extrusion protein) -type efflux pump (AbeM) have also been suggested [5–7]. Published reports highlights more versatility and complexity in aminoglycoside resistance mechanisms in *A. baumannii* conferring more than one resistant mechanism at one time and geographical areas [8,9]. These studies are scarce and though have dealt with differences in the phenotype of aminoglycoside resistance, however, have not been designed to focus on all potential mechanisms accounted for resistance of *A. baumannii* toward aminoglycosides including, efflux pumps, AMEs and 16S rRNA methylases together. Moreover, emerging resistance towards these antibiotics have challenged the therapeutic approach including, Iran. Thus, this study intended to investigate the genetic basis of aminoglycoside resistance, clarify the role of each resistance mechanisms comprising expression of efflux pumps in *A. baumannii* clinical isolates originated from different hospitals in North West of Iran.

2. Materials and methods

2.1. Bacteria

A total of 87 consecutive non-duplicate *A. baumannii* were isolated from various clinical specimens of patients admitted to four referral University Teaching hospitals in Tabriz (n = 76) and Uremia (n = 11), the two main cities in North West of Iran, during January to April, 2014. Source of these isolates was as follows: wound (n = 40), tracheal aspirates (n = 20), blood (n = 11), urine (n = 8), catheter (n = 2), CSF (n = 2) and other body fluids (n = 4). All isolates were initially identified using conventional microbiology methods [10]. Later, species identification was confirmed by PCR amplification of the intrinsic $bla_{\text{oxa-51-like}}$ allele [11] followed by PCR for partial sequencing of RNA polymerase beta-subunit encoding gene *rpoB* gene as described previously [12].

2.2. Susceptibility testing

Antimicrobial susceptibility testing was performed by minimum inhibitory concentration (MICs) assay towards Gentamicin, Amikacin, Kanamycin, Netilmicin and Tobramycin using E-test (Liofilchem, Italy) on cation-adjusted Mueller-Hinton II Agar (Himedia, India) and results were interpreted according to the guidelines of the Clinical and Laboratory Standards Institute [13].

2.3. Rep-PCR typing

The putative REP-like elements in the bacterial chromosomes were amplified using REP1 and REP2 primers and condition as described elsewhere [14]. Amplification reactions were carried out in a Master gradient thermal cycler (Eppendorf), with an initial denaturation (3 min at 95 °C) followed by 30 cycles of denaturation (30 s at 90 °C), annealing (1 min at 45 °C), and extension (8 min at 65 °C), with a single final extension (16 min at 65 °C). Amplified products were subjected to electrophoresis through a 1.5% agarose gel at 70 V for 2 h and stained with DNA SafeStain (SinaClon, Iran). Strains belonging to the same type showed identical profiles or highly similar profiles (up to two bands difference).

2.4. Polymerase chain reaction (PCR) amplification of the aminoglycoside resistance

Amplification reactions were carried out in final volume of 25 μL containing 12.5 μl Taq PCR Master Mix (Ampliqon, Denmark), 0.4 μM each primer and 1 μL template DNA for the genes encoding the following aminoglycoside-modifying enzymes: APH(3′)-Ia, APH(3′)-VIa, AAC(3′)-Ia, AAC(3′)-IIa, AAC(6′)-Ib, AAC(6′)-Ih, ANT(2′)-Ia, and ANT(3′)-Ia, using specific primers and PCR conditions as described previously [15,16]. In addition all isolates were subjected to a separate multiplex-PCR for amplification of the *armA*, *rmtB* and *rmtC* genes using the primers and PCR conditions as described [5].

2.5. RNA extraction and complementary DNA (cDNA) synthesis

Total RNA was extracted from clinical isolates of *A. baumannii* using RNX-Plus solution and RNase-free DNase set (Sina Clon, Iran) according to the manufacturer's instructions. RNA concentration and purity was determined by Nano Drop spectrophotometer (ND-1000, Wilmington, USA). A total of 1 μg DNA-free RNA was used to synthesis cDNA using the 2-steps RT-PCR kit (Vivantis, Malaysia) following the manufacturer's protocol.

2.6. qRT-PCR (real-time PCR)

Quantification of *adeB* and *abeM* transcripts was performed using SYBR Premix Ex Taq II (Tli RNaseH Plus) (Takara Bio, Inc. Japan) with 200 ng cDNA and 0.2 μM each primers specific to *adeB* [17] and *abe*M [18] in a final volume of 20 μl on the Rotor Gene 6000 Real-Time PCR system (Corbett). The reaction conditions were 95 °C for 2 min followed by 40 cycles of 95 °C for 10 s and 60 °C for 1 min. Following PCR cycling, melting point data were collected and a dissociation curve was examined for each well. The 16S rRNA gene was used as a housekeeping gene to normalize the expression of target genes [17]. Results were given as the relative expression of the mRNA compared with that of *A. baumannii* ATCC 19606.

2.7. Statistical analysis

Categorical variables were compared by the chi-square test and differences in means were assessed by independent t-test using SPSS 19.0 statistical software (SPSS Inc., Chicago, IL). A statistically significant difference was considered if P value was <0.05.

3. Results

3.1. Antibiotic susceptibility to aminoglycosides

Among tested aminoglycosides, kanamycin showed the least activity with MICs ranging from 1 to ≥256 μg/ml (MIC_{50} = ≥256 μg/ml) whereas, netilmicin sustained it's activity against the most of the isolates, with susceptibility rate of 67.8% (MIC_{50} = 8 μg/ml). Regarding gentamicin, amikacin and tobramycin, MICs ranged from 0.5 to ≥256 μg/ml, 2- ≥256 μg/ml and 0.25- ≥256 μg/ml, respectively (MIC_{50} for gentamicin = ≥256 μg/ml; for amikacin and tobramycin = 32 μg/ml).

In order to study the diversity in various resistance mechanisms against each aminoglycoside, isolates were divided into 12 phenotypic groups based on the results of aminoglycoside susceptibility testing.

3.2. Genotypic diversity among A. baumannii clinical isolates

Table 2 depicts variable resistance phenotypes obtained in this study. REP analysis could recognize 13 (A–M) distinct patterns among tested isolates (Fig. 1). The majority of isolates clustered in Type F (31%) and J (28.7%). Sixteen isolates (59.2%) in type F showed high level of resistance (MIC $\geq$ 256 μg/ml) to gentamicin, amikacin, kanamycin and tobramycin while maintained full susceptibility (MIC = 8 μg/ml) to netilmicin. Regarding type I, all isolates were highly resistant to all tested aminoglycosides, including netilmicin.

3.3. AMEs and 16S rRNA methylase

Distribution of tested AMEs and 16S rRNA methylases in different phenotypic groups are presented in Table 1. The most common AME gene was *aph(3′)-VIa* (62.1%) followed by *aac(3′)-Ia* (46%), *ant(2′)-Ia* (44.8%), *aac(3′)-Ih* (29.9%), *ant(3′)-Ia* (27.6%) and *aac(6′)-Ib* (26.4%). When different phenotypic groups were compared for possessing different aminoglycoside resistance genes, there was significant association between harboring of *ant(2′)-Ia* and non-susceptibility to gentamicin (p = 0.00), amikacin (p = 0.04), kanamycin (p = 0.03) and tobramycin (p = 0.00), presence of *aph(3′)-VIa* and non-susceptibility to amikacin (p = 0.04) and kanamycin (p = 0.03). In addition, harboring of *aac(3′)-Ia* gene was associated with non-susceptibility to amikacin (p = 0.00) and tobramycin (p = 0.02). Other significant correlation was the presence of *aac(3′)-IIa* genes and non-susceptibility to kanamycin (p = 0.00). No significant correlation was observed between the presence of tested AME genes and non-susceptibility to netilmicin.

When isolates were analyzed for the presence of *armA* gene by PCR, the molecular test could detect presence of this gene in 34.4%, 34.2%, 29.2%, 40.3%, 11.4% and 64.2% of groups showing non-susceptibility to gentamicin, amikacin, kanamycin, tobramycin and netilmicin, respectively. On the other hand, 3 isolates with MIC of gentamicin and tobramycin in susceptible range (MIC = 2–4 μg/ml and MIC = 1–2 μg/ml respectively) and 6 netilmicin susceptible isolates (MIC = 4–8 μg/ml) were positive for the presence of *armA*. All isolates were negative for the presence of *rmtB* and *rmtC*.

3.4. Efflux pumps expression

The expression analysis of *adeB* and *abeM* in different phenotypic groups are summarized in Table 2. Real-time PCR experiments showed that expression level of the *ade*B in 39 (44.8%) isolates was higher (1.5–711.46 fold) than that of *A. baumannii* ATCC 19606. Among isolates with increased mRNA level of *adeB*, 28 (71.7%), 35 (89.7%), 38 (97.4%), 25 (64.1%) and 14 (35.8%) isolates were non-susceptible to gentamicin, amikacin, kanamycin, tobramycin and netilmicin, respectively. Regarding *abeM* expression by the isolates, 20 (22.9%) of them exhibited increased expression level (1.5–98.01 fold). Of these, 13 (65%), 18 (90%), 19 (95%), 11 (55%) and 7 (35%) isolates were non-susceptible to gentamicin, amikacin, kanamycin, tobramycin and netilmicin, respectively. Statistical analysis revealed significant association between increased level of *adeB* and non-susceptibility to amikacin (p = 0.04). However, no statistically significant association was found between over expression of *adeB* and resistance to other aminoglycosides. In addition, *abeM* over expression was not statistically associated with non-susceptibility to tested aminoglycosides.

4. Discussion

A. baumannii, possess numerous antibiotic resistance mechanism and is almost approaching finale in the pharmacopeia.

Table 1
The primer sequences, amplicon sizes and annealing temperatures for amplification.

Genes	Primer Sequence	Amplicon Size (bp)	Annealing Temperature (°C)	Ref.
bla$_{OXA-51}$-like	F: 5′-TAA TGC TTT GAT CGG CCT TG-3′ R: 5′-TGG ATT GCA CTT CAT CTT GG-3′	353	57	[11]
rpoB	F: 5′-TAYCGYAAAGAYTTGAAAGAAG-3′ R: 5′-CMACACCYTTGTTMCCRTGA-3′	350	60	[12]
Rep-elements	REP1: 5′-IIIICGICGICATCIGGC-3′ REP2: 5′-ICGICTTATCIGGCCTAC-3′	variable	45	[14]
aph(3′)-Ia	F: 5′- CGAGCATCAAATGAAACTGC-3′ R: 5′- GCGTTGCCAATGATGTTACAG-3′	623	54	[16]
aph(3′)-VIa	F: 5′- CGGAAACAGCGTTTTAGA-3′ R: 5′- TTCCTTTTGTCAGGTC-3′	716	49	[16]
aac(3′)-Ia	F: 5′- GACATAAGCCTGTTCGGTT-3′ R: 5′- CCCGCTTTCTCGTAGCA-3′	372	49	[16]
aac(3′)-IIa	F: 5′- ATGCATACGCGGAAGGC-3′ R: 5′- TGCTGGCACGATCGGAG-3′	822	49	[16]
aac(6′)-Ib	F: 5′- TATGAGTGGCTAAATCGAT-3′ R: 5′- CCCGCTTTCTCGTAGCA-3′	395	54	[16]
aac(6′)-Ih	F: 5′- TGCCGATATCTGAATC-3′ R: 5′- ACACCACACGTTCAG-3′	407	49	[16]
ant(2′)-Ia	F: 5′- ATCTGCCGCTCTGGAT-3′ R: 5′- CGAGCCTGTAGGACT-3′	404	49	[16]
ant(3′)-Ia	F: 5′- ATGAGGGAAGCGGTGATCG-3′ R: 5′- TTATTTGCCGACTACCTTGGT-3′	624	62	[15]
armA	F: 5′- ATT CTG CCT ATC CTA ATT GG-3′ R: 5′- ACC TAT ACT TTA TCG TCG TC-3′	315	55	[5]
rmtB	F: 5′- GCT TTC TGC GGG CGA TGT AA -3′ R: 5′- ATG CAA TGC CGC GCT CGT AT-3′	173	55	[5]
rmtC	F: 5′- CGA AGA AGT AAC AGC CAA AG -3′ R: 5′- ATC CCA ACA TCT CTC CCA CT-3′	711	55	[5]
AdeB	F: 5′- ACGGACGACCATCTTTGAGTATT-3′ R: 5′- CAGTTGTTCCATTTCACGCATT-3′	83	60	[17]
AbeM	F: 5′- GGTAGGTGTAGGCTTATGGA-3′ R: 5′- CTTCGGCAACTAATGGTGT-3′	80	60	[18]
16S rRNA	F: 5′- CAGCTCGTGTCGTGAGATGT-3′ R: 5′- CGTAAGGGCCATGATGACTT-3′	150	55	[17]

Table 2
The contribution of tested aminoglycoside resistance determinants among different phenotypic groups.

Groups	MIC range (μg/ml)					No. of isolates	Rep profile	Detected genes									Expression Level of	
								aac(3′)-Ia	*aac(3)-IIa*	*aac(6′)-Ib*	*aac(6)-Ih*	*ant(2′)-Ia*	*ant(3′)-Ia*	*aph(3′)-Ia*	*aph(3′)-VIa*	*armA*	*adeB*	*abeM*
Group 1 Gentamicin-non susceptible (n = 61)	8–64					11	A,F,G,H,I,J,L	3	1	2	3	6	7	3	7	–	<1: 5 1.09–7.63: 2 11.16–83.36: 4	<1: 8 1.05–7.81: 3
	≥256					50	B,D,E,F,G,I,M	28	2	13	15	31	9	9	33	21	<1: 23 1.09–9.12: 22 11.63–711.48: 5	<1: 38 1.23–7.51: 10 12.03–16: 2
Group 2 Gentamicin- susceptible (n = 26)	≤4					26	A,C,F,G,,I,J,K	9	4	8	8	2	8	2	14	3	<1: 14 1.32–10.26: 9 30.6–98.6: 3	<1: 19 1.56–8.63: 7
Group 3 Amikacin-non susceptible (n = 70)	32–128					23	A,C,D,F,G,I,J,M	8	3	9	8	7	7	5	18	3	<1: 11 1.09–10.26: 6 11.16–83.36: 6	<1: 16 1.05–8.63: 5 35.65–98.01: 2
	≥256					47	A,B,C,E,F,I,M	29	2	12	13	28	12	7	30	21	<1: 19 1.09–13.41: 25 44.95–711.48: 3	<1: 34 1.54–7.51: 11 12.03–16: 2
Group 4 Amikacin-susceptible (n = 17)	≤16					17	A,F,G,H,I,J,K	3	2	2	5	4	5	2	6	–	<1: 10 3.96–10.26: 4 36.16–98.68: 3	<1: 15 3.05–7.81: 2
Group 5 Kanamycin-non susceptible (n = 82)	32–128					6	A,H,I,L	–	1	2	3	1	3	–	1	–	<1: 5 36.16	<1: 5 7.81
	≥256					76	A-F,G,I,J,L,M	37	4	21	22	38	21	14	53	24	<1: 34 1.09–10.26: 31 11.16–711.48: 11	<1: 56 1.05–12.03: 20
Group 6 Kanamycin- susceptible (n = 5)	≤16					5	A,E,I,K	3	2	–	1	–	–	–	–	–	<1: 3 1.81–2.12: 2	<1: 4 7.51
Group 7 Tobramycin-non susceptible (n = 52)	8–128					11	A-H,I	4	1	4	5	9	3	4	9	2	<1: 4 1.7–11.16: 5 12.53, 36.16	<1: 8 1.23–7.81: 3
	≥256					41	F,I	24	–	9	9	25	9	5	25	19	<1: 20 1.09–11.63: 19 44.95, 711.48	<1: 33 1.54–7.32: 8
Group 8 Tobramycin- susceptible (n = 35)	≤4					35	A,C,F,G,I-M	12	6	10	12	5	12	5	20	3	<1: 18 1.09–10.26: 11 19.98–98.6: 6	<1: 24 1.05–12.05: 9 35.65–98.01: 2
Group 9 Netilmycin- nonsusceptible (n = 28)	16–128					9	A,E,F,I	6	2	1	1	7	3	1	7	–	<1: 6 1.85, 11.63, 13.41	<1: 7 3.98–7.51: 2
	≥256					19	I	7	–	6	5	2	3	4	7	18	<1: 5 1.09–6.25: 12 44.95, 711.48	<1: 13 1.54: 3 12.03–16: 3
Group 10 Netilmycin- susceptible (n = 59)	≤8					59	A-D,F-L	27	5	16	20	30	18	9	40	6	<1: 31 1.09–10.26: 20 11.16–98.6: 8	<1: 45 1.05–8.63: 12 35.65–98.01: 2
Group 11 G,A,K,T,N- non susceptible (n = 23)	G	A	K	T	N													
Sub group 1	≥256	≥256	≥256	≥256	≥256	18	I	6	–	3	4	2	3	4	6	17	<1: 5 1.09–6.25: 11 44.95, 711.48	<1: 15 1.54: 3
Sub group 2	≥256	≥256	≥256	≥256	16	5	F	5	–	1	1	5	2	–	5	–	<1: 3 11.63–13.41: 2	<1: 5 –
Group 12 G,A,K,T,N- susceptible	G	A	K	T	N													
AS1	4	8	1	2	8	1	I	1	–	–	–	-	-	-	-	–	0.33	0.01
AS2	2	8	2	1	1	1	A	1	–	–	1	-	-	-	-	–	0.66	0.85
AS3	2	4	8	1	4	1	K	1	–	–	–	-	-	-	-	–	0.5	0.1
Total			-			87	A -M	40	7	23	26	39	24	14	54	24	<1: 42 >1: 45	<1: 65 >1: 22

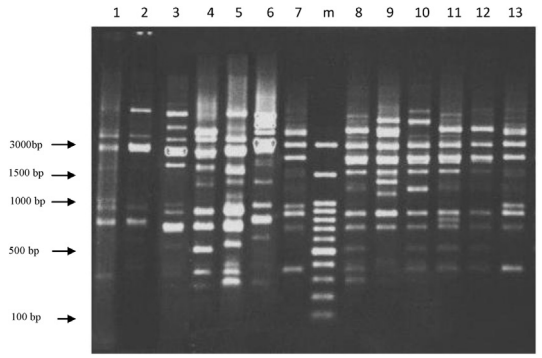

Fig. 1. Repetitive extragenic palindromic (REP)-PCR patterns of genomic DNA from tested *A. baumannii*. Lane 1: rep pattern A; Lane 2: rep pattern B; Lane 3: rep pattern C; Lane 4: rep pattern D; Lane 5: rep pattern E; Lane 6: rep pattern F; Lane 7: rep pattern G; Lane m: DNA Marker 100 bp plus (100–3000 bp); Lane 8: rep pattern H; Lane 9: rep pattern I; Lane 10: rep pattern J; Lane 11: rep pattern K; Lane 12: rep pattern L; Lane 13: rep pattern M.

Present investigation evaluated different aminoglycoside resistance mechanisms in clinical isolates of *A. baumannii* obtained from hospitals of North West Iran. Our finding demonstrated higher rate of resistance to clinically used aminoglycosides in our region, as 96.5% of all isolates were resistant to at least one of the tested aminoglycosides. Remarkably, non-susceptibility to amikacin was considerably higher (80%) than that published till now from Iran [19], Germany, Italy and France [20]. This higher rate of amikacin resistance could be attributed to the extensive use of this antibiotic in our hospitals. A positive correlation between increased amikacin consumption and the emergence of amikacin resistance mediated by modifying enzymes has been described earlier [21].

Based on the prior investigations, aminoglycoside resistance has been a consequence of (i) enzymatic inactivation by aminoglycoside modifying enzymes (AMEs), (ii) modification of target site and (iii) limited drug uptake due to loss of permeability or over expression of efflux pumps. In an attempt to clarify the significance of each resistance mechanism against aminoglycosides, *A. baumannii* isolates were divided into 12 groups based on their aminoglycosides susceptibility pattern and were assessed for the occurrence of various AME genes, 16S rRNA methylase (*armA*, *rmtB* and *rmtC*), as well as mRNA level of Ade ABC and AbeM efflux pumps. We detected the presence of AME encoding genes in 79 (94%) of aminoglycoside non-susceptible isolates (84 isolates), underscoring the major role of AMEs in the dissemination of aminoglycoside resistant strains of *A. baumannii.* Among AME encoding genes studied in the current investigation, Aph(3′)-VI encoding gene was the most common with distribution rate of 62%. The dominance of *aph(3′)-VI* (90.6%) has earlier been documented from Iran in *A. baumannii* strains [22]. When occurrence of aminoglycoside resistance genes was compared among different phenotypic groups in the present investigation, prevalence of *aph(3′)-VI* was found significantly higher in amikacin non-susceptible (48 of 70) group versus susceptible (6 of 17) and similar finding was a characteristic feature for kanamycin non-susceptibile (54 of 82) versus susceptible (none of 5) groups, demonstrating effective role of Aph(3′)-VI in amikacin and kanamycin non-susceptibility. The finding is in agreement to the fact that kanamycin, amikacin and gentamicin are the usual substrates for APH(3′)-VI [23]. However, we could not find a significant correlation between harboring of *aph(3′)-VI* and gentamicin resistance. Instead, non-susceptibility to gentamicin among our isolates allied with possession of *ant(2′)-Ia* (p = 000). Presence of ANT(2′)-Ia encoding gene correlated with non-susceptibility to tobramicin and kanamycin, as expected substrates as well as amikacin as unexpected substrate. Similarly other genotypic results did not match with their corresponding resistance pattern, for example, *aac(3′)-IIa* genotype did not correlate with it's counterpart phenotype. The presence of *aac(3′)-IIa* was associated with non-susceptibility to kanamycin, instead of gentamicin or tobramycin non-susceptibility. Similar observation has been reported in a study conducted in South Africa on SAK strain of *A. baumannii*, whereby a marked increase in the level of AAC(3′)-II activity was observed when kanamycin was added to the culture medium of SAK [24].

Since 2003, 16S rRNA methylation via methyltransferase enzymes has emerged as a new mechanism of high level resistance to aminoglycosides in gram negative bacteria including *A. baumannii* [5]. In our study, *armA* was detected in 94.4% of isolates with high level of resistance to all of the assessed aminoglycosides (17 out of 18). Research studies from China and Vietnam have reported similar prevalence of *armA* (98% and 87.1%, respectively) among aminoglycoside high resistant *A. baumannii* strains [25,26]. Surprisingly, *armA* was found in *A. baumanii* isolates showing susceptibility towards gentamicin and tobramycin (3 isolates each) and netilmicin (6 isolates) in our study. Similar observation was also documented previously by Dally et al. who reported two *A. baumannii* isolates with *armA* gene and phenotype of susceptibility to amikacin [27], and assumed modulation of ArmA activity at transcribtional, translational, or post-translational level—options.

Efflux mediated resistance to aminoglycosides in Acinetobacter was primary reported in the study conducted by Magnet et al. [6], whereby RND-type efflux pumps of AdeABC was shown to be involved in low level resistance to aminoglycoside in BM4454, a multi drug resistant strain of *A. baumannii.* The importance of AdeABC efflux pumps in development of resistance to multiple classes of antibiotics is highlighted in several reports [28,29]. However, very few studies have dealt with differences in the phenotype of aminoglycoside resistance and AdeABC expression among Acinetobacter strains. In the present study, we evaluated the role of AdeABC efflux pump in development of aminoglycosides resistance by comparing clinical isolates with various susceptibility patterns against aminoglycosides. According to the obtained results, 44.8% (39 of 87) of isolates had enhanced expression of *adeB* in comparison to reference strain ATCC 19606, and among them 28 (71.7%), 38 (97.4%) and 25 (64.7%) were non-susceptibile to gentamicin, kanamycin and tobramycin, respectively. However, elevated level of *adeB* was obvious among 50%, 40% and 49.1% of isolates susceptible to gentamicin, kanamycin and tobramycin, respectively, demonstrating non-significant association between the enhanced expression of *adeB* and resistance to mentioned aminoglycosides. However, we could find significant correlation between over expression of *adeB* and non-susceptibility to amikacin (p = 0.04). This finding is interesting and contrary to the prior observation of Magnet et al. [6], according to whom kanamycin and amikacin are less effectively transported in comparison to other aminoglycosides by AdeABC, because of possessing hydrophilic structure. Moreover, study conducted by Nemec et al. [30], suggests that association exists between decreased susceptibility to netilmicin and up regulation of AdeABC efflux pump. They constructed this association on the basis of observation that presence of the genes was essential for the activity of the AdeABC and reduced netilmicin susceptibility. However, expression of Ade ABC pump was not assayed in their study. In our investigation, although a mean 4-fold higher level of *adeB* expression was observed in netilmicin non-susceptible isolates (MICs of 16-≥256 μg/ml) compared with susceptible isolates (MIC≤4 μg/ml), but *adeB* expression did not significantly correlate with netilmicin resistance, suggesting

AdeABC efflux pump is not solely responsible for resistance to netilmicin. Notably, in the study conducted by Morita et al. [31] on PA7 strain of *Pseudomonas aeruginosa,* interplay between the MexXY efflux pump, a member of RND family and the AAC modifying enzyme has been found to provide high-level resistance toward aminoglycoside in *P. aeruginosa*. However, we could not find similar correlation between the co-existence of tested AMEs and over expression of AdeABC efflux pump and development of high level resistance to aminoglycosides in *A. baumannii*.

Augmented efflux as a result of *abeM* over expression in *Escherichia coli*, has been indicated in development of resistance to kanamycin and gentamicin [7]. Our results showed that *abeM* expression is not an important contributor to overt aminoglycoside resistance in 76.1% (64 of 84 aminoglycoside resistant) of isolates. Similar finding has also been reported among multidrug resistant strains of *A. baumannii* from Taiwan [32]. Thus, it seems that *abeM* expression did not guarantee aminoglycoside resistance phenotypes. Nevertheless, we could not exclude the possibility that AbeM efflux pumps might play a role in resistance against aminoglycosides, as evidenced in 20 of 84 isolates showing non-susceptibility to tested aminoglycosides in the present study.

In conclusion, our results mark a definitive role of aminoglycoside modifying enzymes and ArmA in development of resistance in *A. baumannii* towards aminoglycosides. On the other hand, up-regulation of AdeABC and AbeM had restrictive role in reducing the susceptibility of aminoglycosides in *A. baumannii*.

Disclosure statement

No competing financial interest exist.

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