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HIGH DISTRIBUTION OF 16S rRNA METHYLASE GENES *rmtB* AND *armA* AMONG *ENTEROBACTER CLOACAE* STRAINS ISOLATED FROM AN AHVAZ TEACHING HOSPITAL, IRAN

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The emergence of 16S rRNA methylase genes encoded on plasmids confers high-level aminoglycoside resistance (HLAR). This study aimed to investigate the prevalence of 16S rRNA methylases among *Enterobacter cloacae* strains isolated from an Ahvaz teaching hospital, Iran. A total of 68 *E. cloacae* clinical strains were collected between November 2017 and September 2018. The MICs of aminoglycosides were assessed using the agar dilution method. The presence of 16S rRNA methylase genes, including *armA, rmtA* to *rmtH*, and *nmpA* was evaluated by PCR. The transferability of 16S rRNA methylase-harboring plasmids was evaluated by conjugation assay. The genetic diversity of all isolates was evaluated by ERIC-PCR. The *armA* and *rmtB* genes were the only 16S rRNA methylase genes detected in this study (29 out of 68 isolates; 42.64%). The transferability by conjugation was observed in 23 *rmtB* or/and *armA* positive donors. HLAR phenotype was in 33 of 68 strains. Ten clonal types were obtained by ERIC-PCR and significant associations ($p < 0.05$) were between the clone types and aminoglycoside susceptibility, as well as with profile of the 16S rRNA methylase genes. In conclusion, both horizontal transfer and clonal spread are responsible for dissemination of the *rmtB* and *armA* genes among *E. cloacae* strains.

Keywords: 16S rRNA methylase, *Enterobacter cloacae*, ERIC-PCR

Introduction

Enterobacter spp. as important opportunistic pathogens have usually emerged in nosocomial infections. The extensive use of antibiotics in the hospital

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settings has increased the emergence of multidrug-resistant *Enterobacter* spp. isolates in hospitals. The most frequent human infections caused by *Enterobacter* spp. are bacteremia, endocarditis, septic arthritis, osteomyelitis, lower respiratory tract, urinary tract, and intra-abdominal infections [1].

Aminoglycosides are considered as a group of broad-spectrum antibiotics that have been used empirically for the treatment of life-threatening Gram-negative infections. They can inhibit the protein synthesis of bacteria by binding to the 16S rRNA. However, the widespread use of aminoglycosides has caused some selective pressures for the emergence of resistant organisms [2].

Resistance to aminoglycoside agents occurs primarily through three different mechanisms: (a) the modification of the 16S rRNA and ribosomal protein targets, (b) the reduction of uptake and increment of efflux, and (c) aminoglycoside-modifying enzymes (AMEs). Among them, only aminoglycoside phosphotransferases can produce a high level of resistance [3].

The high-level aminoglycoside resistance (HLAR) is established by 16S rRNA methylases, which are usually encoded on plasmids. Moreover, these genes can be simply transferred among Gram-negative bacilli populations especially in hospital setting through the horizontal gene-transfer mechanisms [4]. In contrast to AMEs that have a range of substrates, 16S rRNA methylases confer HLAR phenotype to almost all common aminoglycosides, such as amikacin, kanamycin, tobramycin, and gentamicin [5].

In 2002, the first 16S rRNA methylase gene, later known as *armA*, was identified as the part of a plasmid sequence of a clinical isolate of *Citrobacter freundii* in Poland [5]. Since then, ten 16S rRNA methylase plasmid-mediated genes, *rmtA* to *rmtH*, *armA*, and *nmpA*, have been identified in clinical or veterinary bacterial isolates [6–8]. Previous studies in European and East Asia countries indicated a relativity high prevalence of *armA, rmtB* or both genes in the *Enterobacteriaceae* family [9–13]. In this article, we first investigated the prevalence of 16S rRNA methylases in *Enterobacter cloacae* strains isolated from various clinical samples in Ahvaz, Iran. The molecular typing of these isolates was performed using enterobacterial repetitive intergenic consensus–polymerase chain reaction (ERIC-PCR) method.

Materials and Methods

A total of 86 non-duplicate clinical *E. cloacae* isolates were collected between November 2017 and September 2018 from clinical samples of hospitalized patients in ICU of the Golestan teaching Hospital in Ahvaz, Iran. All *E. cloacae* isolates from the different clinical samples were obtained from the

hospital laboratory. The study design was approved by the Research Ethics Committee of Ahvaz Jundishapur University of Medical Sciences, Iran (IR.AJUMS.REC.1396.409). All strains were identified by colony morphology, biochemical tests, and sequencing 16S rRNA gene [14].

Determination of antimicrobial susceptibility of aminoglycosides

The minimum inhibitory concentrations (MICs) of aminoglycoside agents, including amikacin, kanamycin, and gentamycin, were assessed using agar dilution method and their results were interpreted according to CLSI guidelines [15]. The presence of HLAR phenotype is defined as MICs ≥256 μg/ml of gentamicin, amikacin, or kanamycin.

DNA extraction

The whole genome was extracted using boiling method as described previously [16]. In addition, the extraction of plasmid DNA was performed using a Plasmid Purification Kit (CinnaGene, Iran) according to manufacturer's procedure.

ERIC-PCR typing and analysis

The genetic diversity of *E. cloaceae* isolates was evaluated using the ERIC-PCR [17]. The primer sequences used were ERIC-F (5′-ATGTAA GCTCCTGGGGATTCAC-3′) and ERIC-R (5′-AAGTAAGTGACTGGGGTGA GCG-3′). The PCR reaction was performed in the final volume of 20 μl. The amplification mixture consisted of 1U Taq DNA polymerase, 1.5 mM $MgCl_2$, 200 μM dNTPs, 0.25 μM of each primer, 10× PCR buffer, 5 μl of template DNA, and distilled water up to a final volume of 20 μl. The amplification process was performed in Mastercycler Nexus Thermal Cycler Gradient (Eppendorf, Hamburg, Germany) with one cycle of initial denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 60 s, annealing at 55 °C for 60 s, extension at 72 °C for 90 s, with a cycle of final extension at 72 °C for 10 min. The amplified products were resolved on agarose gel 1.5%, stained with 0.5 μg/ml ethidium bromide. The data analysis was performed using the Gel Compare II software version 6.6 (Applied Maths, Sint-Martens-Latem, Belgium). The similarity pattern was calculated using the Unweighted-Pair Group Method (UPGMA)/the Dice similarity coefficient with a position tolerance of 1.5%. Isolates with more than 80% similarity were considered an as clonal type.

Molecular identification of 16S rRNA methylases

The amplification of genes encoding 16S rRNA methylases, including *rmtA* to *rmtH*, *armA*, and *nmpA* on both DNA extracted from plasmid and whole genome was performed by PCR, as described previously [13]. The primer sequences used for these genes are presented in Table I. The single PCR reactions were established in the final volume of 20 µl. The amplification mixture consisted of 1U Taq DNA polymerase, 1.5 mM $MgCl_2$, 200 µM dNTPs, 0.35 µM of each primer, 10× PCR buffer, 3 µl of template DNA, and distilled water up to a final volume of 20 µl. The amplification conditions were as follows: one cycle of initial denaturation at 94 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 60 s, annealing temperatures (mentioned in Table I) for 30 s, extension at 72 °C for 30 s, with a cycle of final extension at 72 °C for 10 min. The PCR products were visualized on a 1% agarose gel stained with safe stain. DNA sequencing of PCR products of four randomly selected isolates was recommended for both the DNA strands (Bioneer, South Korea).

Conjugation experiment

The ability of the 16S rRNA methylase gene-positive isolates for the plasmid transfer into *E. coli* J53 Azide® as the recipient was evaluated using

Table I. Primer used for the amplification of the 16S rRNA methylase genes

Gene	Primer sequence (5′–3′)	bp	Annealing temperature (°C)
armA	F – AAAGTACAATCAGGGGCAGTT	269	54
	R – TCGTCGTCTTTAACTTCCCAA		
rmtA	F – CTAGCGTCCATCCTTTCCTC	634	55
	R – TTGCTTCCATGCCCTTGCC		
rmtB	F – GCTTTCTGCGGGCGATGTAA	173	54
	R – ATGCAATGCCGCGCTCGTAT		
rmtC	F – CGAAGAAGTAACAGCCAAAG	711	60
	R – A TCCCAACATCTCTCCCACT		
rmtD	F – CGGCACGCGATTGGGAAGC	401	62
	R – CGGAAACGATGCGACGAT		
rmtE	F – TGGTTGCAGAGGTTCTGTCGAGC	518	57
	R – CGGCGTAACAGACACGGCATCA		
rmtF	F – ATTCATCTGGGCTGCGTGCGAC	338	54
	R – ACCAGCTCGTCCGACACCGTAA		
rmtG	F – CGTGTATGCGCGTCTGTTGGGT	420	56
	R – ACGGTGCGTTCGATTCGCCATT		
rmtH	F – ACAAAAAGCCCAAGCAGGCGGT	259	58
	R – CGGTGCAGCATCAGCGGGTTTA		
nmpA	F – GGTCAGTTTGATCGTGTGCA	195	59
	R – AGCTGCAATAACAAACACCACA		

conjugation experiment [11]. The donor and recipient cells were mixed to each other in ratio 10:1 in lysogeny broth (LB) and incubated for an overnight at 37 °C. Transconjugants were selected on LB plates supplemented with sodium azide (100 μg/ml) and amikacin (128 μg/ml). The plasmid transfer of 16S rRNA methylase genes into transconjugants was confirmed by the amplification of these genes using primers used in the previous section.

Statistical analysis

The descriptive statistics and χ^2 tests were performed using SPSS version 16.0 (Chicago, IL, USA). Moreover, χ^2 test was used for finding the association between the resistance to aminoglycosides in various clonal types and a p value <0.05 was considered statistically significant.

Results

In this study, 68 *E. cloacae* isolates were collected from different clinical samples, including urine (47), wound (11), and blood (10). All isolates were confirmed as *E. cloacae* by standard biochemical tests and sequencing 16S rRNA gene. The majority of these strains was resistant to gentamicin ($n = 43$; 63.23%), followed by amikacin ($n = 38$; 55.88%) and kanamycin ($n = 35$; 51.47%). Overall, the 16S rRNA methylase genes were identified in 29 (42.64%) isolates. Moreover, 16 (23.52%) strains harbored only *armA*, 7 (10.29%) harbored only *rmtB*, and 6 (8.82%) harbored both *armA* and *rmtB* genes. According to the results obtained from sequencing, we observed that the *armA* and *rmtB* genes had 100% identity with *armA* in *K. pneumoniae* BM4536, (GenBank AY220558) and *rmtB* in *K. pneumonia* NCCHD 1261-1 (GenBank LC424160.1). However, none of the strains harbored *rmtA, rmtC, rmtD, rmtE, rmtF, rmtG*, and *nmpA* genes. All isolates harboring 16S rRNA methylase genes were highly resistant to gentamicin, amikacin, and kanamycin (MICs ≥ 256 μg/ml). The clinical samples of the 16S rRNA methylase gene-positive isolates were as follows: urine ($n = 20$), wound ($n = 3$), and blood ($n = 6$).

In this study, the HLAR phenotype was observed in 33 isolates. However, 4 out of these 33 isolates had neither *armA* nor *rmtB* gene.

Conjugation experiments showed the horizontal transfer of the *armA* and *rmtB* genes in 23 out of 29 strains. Susceptibility testing of these transconjugants to aminoglycosides approved the conjugation assay.

In this study, 68 *E. cloacae* strains were clustered into 10 clone types and 28 single type of ERIC-PCR. Figure 1 shows the dendrogram of ERIC-PCR of these isolates. In addition, Table II shows the distribution of MICs of amikacin,

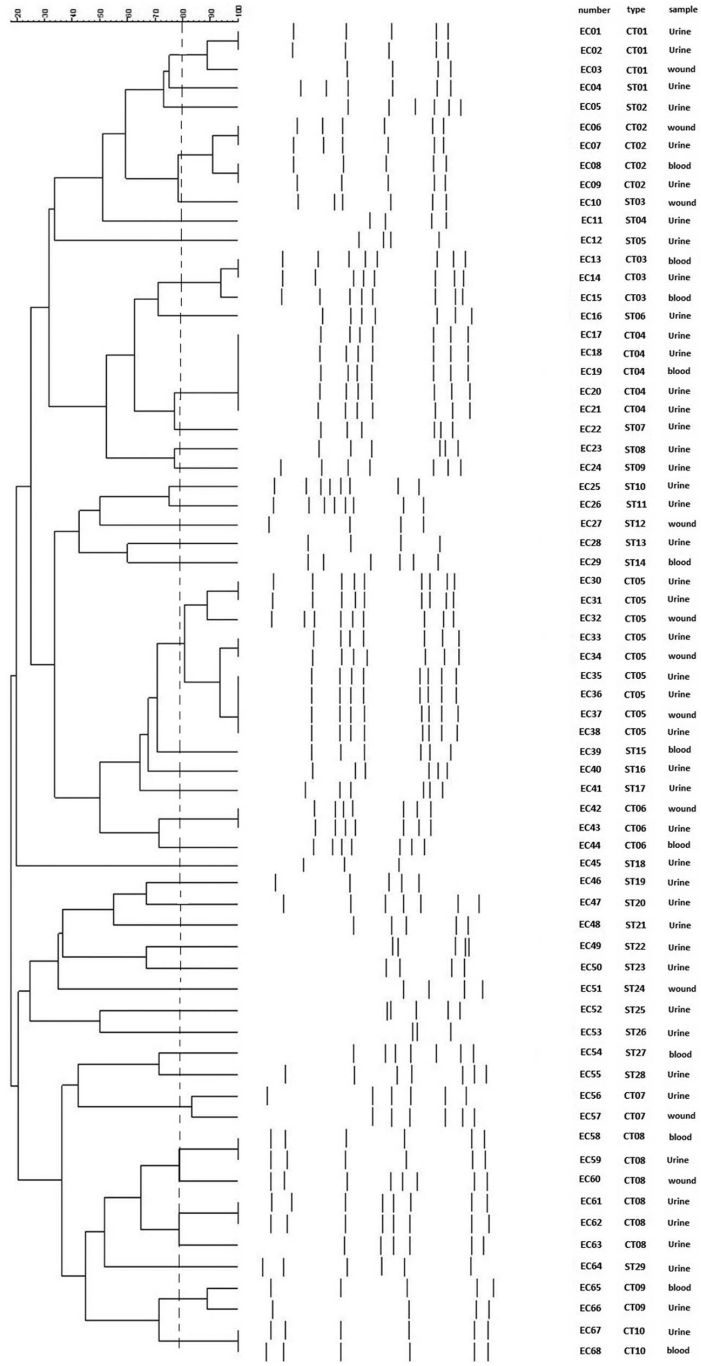

Figure 1. Dendrogram of 68 *E. cloacae* clinical strains based on ERIC-PCR profiles

Table II. The distribution of MICs of amikacin, gentamicin, and kanamycin, as well as the *armA* and *rmtB* genes

Strain	Type	AMI	GEN	KAN	*armA*	*rmtB*	Strain	Type	AMI	GEN	KAN	*armA*	*rmtB*
EC01	CT01	512	>512	512	+	+	EC35	CT05	8	2	16	−	−
EC02	CT01	512	>512	512	+	+	EC36	CT05	8	2	32	−	−
EC03	CT01	512	>512	>512	+	+	EC37	CT05	16	4	8	−	−
EC04	ST01	32	128	16	−	−	EC38	CT05	8	2	16	−	−
EC05	ST02	64	32	8	−	−	EC39	ST15	512	256	>512	+	+
EC08	CT02	512	512	512	−	+	EC42	CT06	512	>512	512	+	−
EC09	CT02	512	512	512	−	+	EC43	CT06	256	256	>512	+	−
EC10	ST03	8	16	16	−	−	EC44	CT06	512	>512	512	+	−
EC11	ST04	64	16	16	−	−	EC45	ST18	64	128	4	−	−
EC12	ST05	>512	>512	512	+	−	EC46	ST19	128	32	8	+	−
EC13	CT03	16	4	16	−	−	EC47	ST20	512	>512	512	−	+
EC14	CT03	16	2	16	−	−	EC48	ST21	256	512	>512	−	+
EC15	CT03	8	4	16	−	−	EC49	ST22	512	256	>512	−	+
EC16	ST06	>512	512	>512	+	+	EC50	ST23	64	32	128	−	−
EC17	CT04	256	512	512	+	−	EC51	ST24	512	256	512	−	−
EC18	CT04	512	512	>512	+	−	EC52	ST25	8	4	16	−	−
EC19	CT04	>512	512	512	+	−	EC53	ST26	2	4	8	−	−
EC20	CT04	512	256	>512	+	−	EC54	ST27	512	256	512	−	+
EC21	CT04	512	256	512	+	−	EC55	ST28	16	64	128	−	−
EC22	ST07	8	128	16	−	−	EC56	CT07	512	256	>512	−	−
EC23	ST08	256	512	>512	+	−	EC57	CT07	512	256	>512	−	−
EC24	ST09	16	8	4	−	−	EC58	CT08	512	512	512	+	−
EC25	ST10	>512	512	512	−	+	EC59	CT08	512	512	512	+	−
EC26	ST11	16	128	4	−	−	EC60	CT08	256	512	>512	+	−
EC27	ST12	256	256	512	−	−	EC61	CT08	256	>512	512	+	−
EC28	ST13	16	2	8	−	−	EC62	CT08	512	512	512	+	−
EC29	ST14	256	256	>512	+	+	EC63	CT08	512	>512	>512	+	−
EC30	CT05	16	4	32	−	−	EC64	ST29	16	4	32	−	−
EC31	CT05	8	4	16	−	−	EC65	CT09	16	2	32	−	−
EC32	CT05	8	4	16	−	−	EC66	CT09	8	4	16	−	−
EC33	CT05	16	2	32	−	−	EC67	CT10	8	2	32	−	−
EC34	CT05	16	2	16	−	−	EC68	CT10	16	0.5	4	−	−

Note: MIC: minimum inhibitory concentration; EC: *Enterobacter cloacae*; CT: clonal type; ST: single type; AMI: amikacin; GEN: gentamicin; KAN: kanamycin.

gentamicin, and kanamycin, as well as the *armA* and *rmtB* genes among 68 strains of *E. cloacae* based on ERIC-PCR patterns.

We found that all strains in a same clone type had similar 16S rRNA gene profile. There was a significant association ($p < 0.05$) between the clone types and antibiotic susceptibility to aminoglycoside agents, and the profile of 16S rRNA genes (e.g., all *armA*- or *rmtB*-harboring isolates) was clonally related.

Discussion

The emergence of 16S rRNA methylases genes among the *Enterobactericeae* family is raising serious concerns for the future of treatment with aminoglycosides. These genes are considered as one of the main determinants in the growing spread of HLAR phenotype [18]. Hence, the epidemiologic studies and analysis of the acquisition mechanisms of these determinants by clinical isolates are paramount for the prevention of their spread in healthcare settings.

In this study, the overall prevalence of the 16S rRNA methylase genes (*armA* and *rmtB*) among *E. cloacae* strains isolated from the various clinical samples was 42.64%. The frequency rates of the 16S rRNA methylase genes among *Enterobacteriaceae* family in other studies ranged from 0.66% to 46.34% [11, 18–22].

Our data indicated that the *armA* gene was more prevalent than the *rmtB* gene in *E. cloacae* strains. This result is consistent with other reports indicating that *armA* had a higher prevalence relative to *rmtB* among the *Enterobacteriaceae* family [11, 19–21]. Although the presence of *rmtA*, *rmtD*, and *rmtC* genes has been confirmed by PCR among the *Enterobacteriaceae* family in India [22] and Saudi Arabia [12], these genes were not detected in this study and some other countries [11, 19, 20, 23]. On the whole, the frequency rates of 16S rRNA methylase genes are highly dependent on the distribution pattern of plasmids harboring these genes.

As mentioned above, the 16S rRNA methylase genes are often encoded on self-transferable plasmids, which can be easily transferred to other species through the horizontal gene-transfer mechanisms, such as transconjugation and transformation. In this study, we confirmed the amplification of 16S rRNA methylase genes on both DNA extracted from plasmid and whole genome for all strains harboring these genes. In addition, we showed that the aminoglycoside resistance was transferred to the recipient *E. coli* J53 by conjugation in 23 out of 29 isolates producing *armA* and/or *rmtB* gene. This finding indicates that the 16S rRNA methylase genes are often located on self-transferable plasmids. However, the plasmid transfer in the six remaining strains failed via conjugation, which indicates

that these plasmids were not transferable to the recipient *E. coli* J53. In consistence with our results, some previous studies also indicated that most strains producing 16S rRNA methylase had self-transferable plasmids by conjugation [9, 11, 19]. However, Yu et al. [18] found that most *armA*- or *rmtB*-positive donors transferred their plasmids into the recipient strains through transformation. This finding highlights two main mechanisms of the horizontal gene transfer in the dissemination of HLAR determinants.

In this study, we observed that 4 out of 33 strains with HLAR phenotype were lacking the 16S rRNA methylase genes. Similar finding was also reported in a study conducted in China by Wang et al. [24] on *Acinetobacter baumannii* strains. Moreover, they showed that some strains with HLAR phenotype had genes encoding AMEs instead of the 16S rRNA methylase genes.

Molecular typing methods are considered as the important tools to identify the clonal relationship and the spread of nosocomial and geographical of an infectious agent. ERIC-PCR is a rapid and low-cost method that can differentiate the genetic variations of bacterial isolates. We showed that these 68 isolates were successfully differentiated into 10 clone types by ERIC-PCR. In addition, we indicated the clonal dissemination of *armA* and/or *rmtB* gene-positive *E. cloacae* strains.

On the other hand, one of the serious concerns of the dissemination of 16S rRNA methylase genes is the development of multidrug resistance through co-transfer of plasmids harboring these genes with other resistance determinants, including enzymes OXA types, MBLs, ESBLs, and PMQRs by the horizontal gene transfer. Moreover, the acquisition of resistance determinants to carbapenem and fluoroquinolones is a serious threat for the antibiotic therapy of infectious diseases caused by Gram-negative bacteria [25].

Conclusions

In this study, we showed the high distribution of *rmtB* and *armA* genes among *E. cloacae* strains. In addition, ERIC-PCR and conjugation indicated that both the horizontal gene transfer and clonal dissemination were responsible for the spread of the *rmtB* and *armA* in *E. cloacae* strains. Hence, the regional epidemiologic studies for finding these genes in the clinical isolates are critical for the prevention from the dissemination of HLAR phenotype organisms.

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Conflict of Interest

The authors declare no conflict of interest.

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