

Genetic Characterization of a Novel Composite Transposon Carrying *armA* and *aac(6)-Ib* Genes in an *Escherichia coli* Isolate from Egypt

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Abstract

Aminoglycosides are used in treating a wide range of infections caused by Gram-positive and Gram-negative bacteria; however, aminoglycoside resistance is common and occurs by several mechanisms. Among these mechanisms is bacterial rRNA methylation by the 16S rRNA methyl transferase (16S-RMTase) enzymes; but data about the spread of this mechanism in Egypt are scarce. Cephalosporins are the most commonly used antimicrobial agents in Egypt; therefore, this study was conducted to determine the frequency of 16S-RMTase among third generation cephalosporin-resistant clinical isolates in Egypt. One hundred and twenty three cephalosporin resistant Gram-negative clinical isolates were screened for aminoglycosides resistance by the Kirby Bauer disk diffusion method and tested for possible production of 16S-RMTase. PCR testing and sequencing were used to confirm the presence of 16S-RMTase and the associated antimicrobial resistance determinants, as well as the genetic region surrounding the *armA* gene. Out of 123 isolates, 66 (53.66%) were resistant to at least one aminoglycoside antibiotic. Only one *Escherichia coli* isolate (E9ECMO) which was totally resistant to all tested aminoglycosides, was confirmed to have the *armA* gene in association with *bla*_{TEM-1}, *bla*_{CTX-M-15}, *bla*_{CTX-M-14} and *aac(6)-Ib* genes. The *armA* gene was found to be carried on a large A/C plasmid. Genetic mapping of the *armA* surrounding region revealed, for the first time, the association of *armA* with *aac(6)-Ib* on the same transposon. In conclusion, the isolation frequency of 16S-RMTase was low among the tested aminoglycoside-resistant clinical samples. However, a novel composite transposon has been detected conferring high-level aminoglycosides resistance.

Key words: 16S rRNA methyl transferases, aminoglycosides, *armA*, β -lactamase

Introduction

Aminoglycosides are used in treating a wide range of infections caused by both Gram-negative and Gram-positive bacteria. They have been classified by the World Health Organization as critically important antimicrobial drugs in human medicine (WHO, 2011). They bind specifically to the aminoacyl site (A-site) of 16S rRNA within the prokaryotic 30S ribosomal subunits and interfere with protein synthesis. The most commonly encountered resistance mechanism to aminoglycosides is enzymatic inactivation. Other known mechanisms of aminoglycoside resistance include defect of cellular permeability, active efflux and rarely, nucleotide substitution of the target molecule (Magnet and Blanchard, 2005). Since 2003, a new mechanism of aminoglycoside resistance has emerged (Galimand *et al.*, 2003; Yokoyama *et al.*, 2003) which is mediated by a newly recognized group of 16S rRNA methyl transferases (16S-RMTase), with modest similarity to those produced by aminoglycoside-producing actinomycetes. Their presence confers an extraordinarily

level of resistance to clinically useful aminoglycosides, such as amikacin, tobramycin and gentamicin (Doi and Arakawa, 2007).

Seven types of plasmid-mediated 16S-RMTase (*armA*, *rmtA*, *rmtB*, *rmtC*, *rmtD*, *rmtE*, *rmtF* and *npmA*) have so far been identified (Davis *et al.*, 2010; Doi *et al.*, 2004; 2007; Doi and Arakawa, 2007; Galimand *et al.*, 2003; 2012; Wachino *et al.*, 2006; Yokoyama *et al.*, 2003). Published data concerning this new group of enzymes indicate that 16S-RMTase genes have already disseminated globally among pathogenic Gram-negative bacilli, although their overall prevalence appears to remain low (Doi and Arakawa, 2007).

Genes encoding 16S-RMTases are often carried by mobile genetic elements like transposons. They are frequently associated with other antimicrobial resistance genes such as *bla*_{CTX-M}, *bla*_{DHA}, *bla*_{NDM-1}, *bla*_{OXA-10}, *bla*_{TEM-1}, *bla*_{VIM-1} as well as plasmid-mediated quinolone resistance determinants, thus conferring multidrug resistance phenotypes. In addition, some pathogenic microbes such as *Salmonella* species have already acquired these genes. Therefore, this genetic apparatus

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may contribute to the rapid worldwide dissemination of this resistance mechanism among different pathogenic microbes (Yamane *et al.*, 2008; Yang *et al.*, 2011; Galani *et al.*, 2012; Poirel *et al.*, 2012; Wachino and Arakawa, 2012).

Egypt has a high population density (15th world) and a central location at the crossroad of major transportation routes, thus it is important to monitor the status of antibiotic resistance in this country. However, little data about the incidence of 16S-RMTase producing bacteria in Egypt are available. Since cephalosporins are the most commonly used antibiotics in Egypt (Hassan *et al.*, 2011), this investigation was conducted to determine the isolation frequency of 16S-RMTase producers among third-generation cephalosporin-resistant Gram-negative pathogenic bacteria in Egyptian medical facilities. In addition, this study also determined the plasmid origin of *armA*, the replicon type of plasmid, and the 16S-RMTase genetic neighborhood. Other associated antimicrobial resistance determinants have been studied.

Experimental

Materials and Methods

Bacterial strains. One hundred and twenty three isolates, previously characterized as being resistant to at least one of the third-generation cephalosporins, were included in the study. All isolates were collected between 2009 and 2010 from outpatients with suspected infections, in Abu El-Rish Children's Hospital, Cairo. Isolates were identified by API 20E identification system (Kashif *et al.*, 2012). *Escherichia coli* TOP10 was used as a recipient strain for transformation experiments.

Antimicrobial susceptibility testing. Antibiotic susceptibility to gentamicin (10 µg), tobramycin (10 µg) and amikacin (30 µg) was determined by the Kirby-Bauer disk diffusion method as proposed by Doi and Arakawa (2007).

Isolates with 16S-RMTase genes were tested by the Kirby-Bauer disc diffusion method for susceptibility to other antimicrobials, such as: ciprofloxacin (5 µg), cefoxitin (30 µg), cefotaxime (30 µg), piperacillin (100 µg), piperacillin/tazobactam (100/10 µg), trimethoprim/sulfamethoxazole (1.25/23.75 µg), imipenem (10 µg). The results were interpreted following the guidelines of the Clinical and Laboratory Standards Institute (2012a).

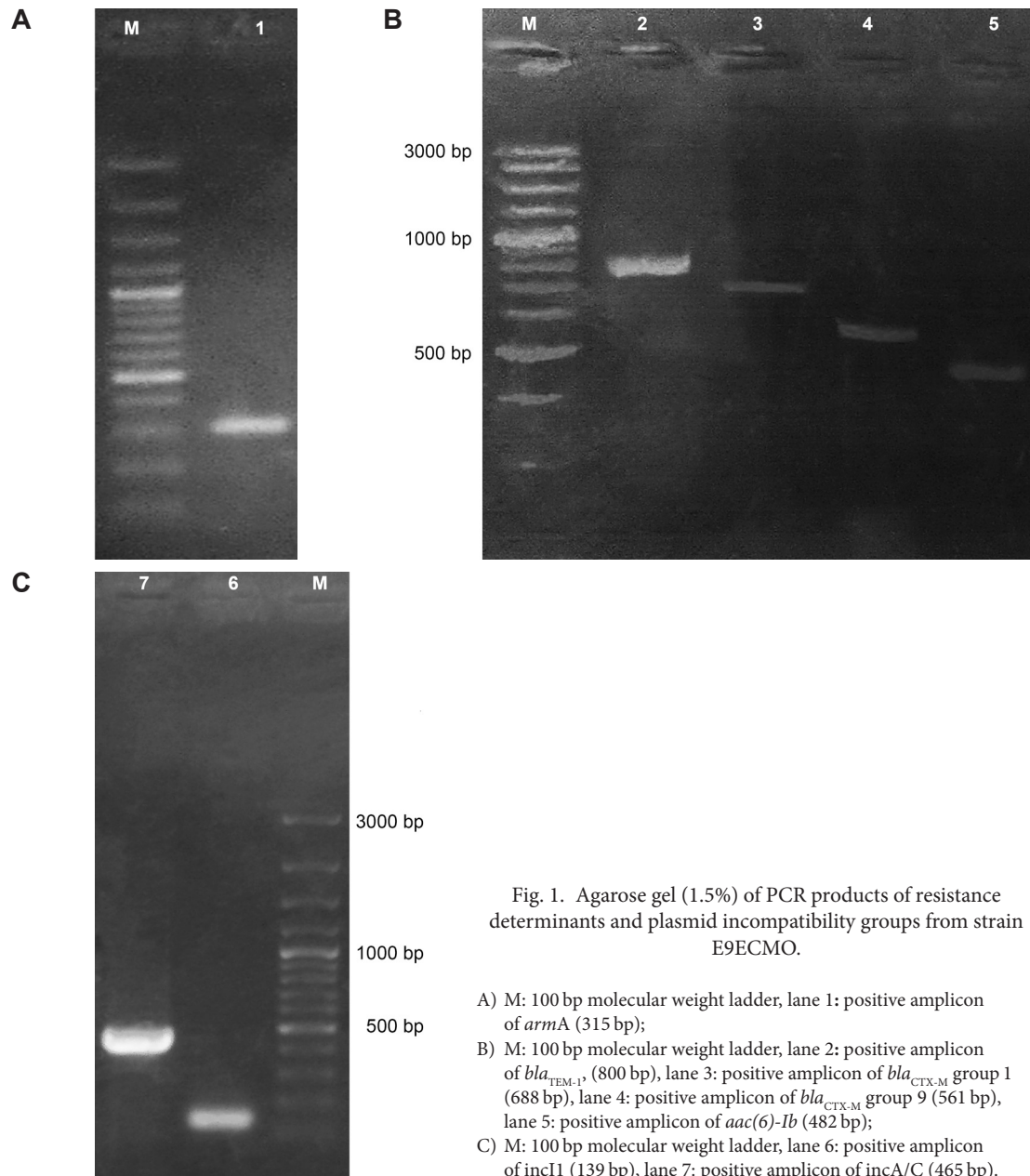
Characterization of 16S-RMTase genes and the associated antimicrobial resistance genes. Genomic DNA was extracted from isolates suspected to contain 16S-RMTase genes by the boiling method. The isolates were tested for *armA*, *rmtA*, *rmtB*, *rmtC*, *rmtD* and *npmA* genes by PCR amplification, which was per-

formed as previously described (Doi and Arakawa, 2007; Fritsche *et al.*, 2008). PCR identification of other resistance genes (*bla*_{TEM}, *bla*_{SHV}, *bla*_{CTX-M} group 1 and group 9 and quinolone resistance genes: *aac(6)-Ib-cr*, *qepA*, *qnrA*, *qnrB* and *qnrS*) was also performed as previously described (Park *et al.*, 2005; Cattoir *et al.*, 2007; Yamane *et al.*, 2008; Dallenne *et al.*, 2010). PCR products were purified with GeneJET PCR Purification Kit (Thermo Fisher Scientific, Lithuania) and sequenced by ABI 3730 xl DNA sequencer (Applied Biosystems, Foster City, CA). Similarity searches for the nucleotide sequences were performed with BLASTN program (<http://www.ncbi.nlm.nih.gov/blast>) with default settings.

Transformation of 16S-RMTase genes. Plasmid DNA was extracted with GeneJET Plasmid Miniprep Kit (Thermo Scientific, Lithuania) from 16S-RMTase positive strain (E9ECMO). Two microliters of plasmid extract from E9ECMO was electroporated into 50 µl of electrocompetent *E. coli* TOP10 cells using a Gene Pulser electroporator (Bio-Rad, Hemel Hempstead, UK), according to the manufacturer instructions. Transformants were selected on LB agar medium supplemented with either 100 µg/ml cefotaxime, or 100 µg/ml gentamicin. The Kirby-Bauer disc diffusion method was used to determine the antibiogram of the obtained transformants. The MICs of cefotaxime, ciprofloxacin, gentamicin and amikacin for isolate E9ECMO, the obtained transformants and *E. coli* TOP10 were determined by broth microdilution method (CLSI, 2012b).

Plasmid analysis. Plasmid DNA was extracted from E9ECMO and the obtained transformants, as previously described. The size of *armA* carrying plasmid was determined by restriction digestion using *BsaBI* (Thermo Scientific, Lithuania). Electrophoresis of the extracted and digested plasmid was performed on 0.5% agarose gels in 1 × TAE buffer and visualized by staining with ethidium bromide. Fragment size was determined by comparison with Gene-Ruler 1 kb DNA ladder (Thermo Scientific, Lithuania). Plasmid replicon typing was performed using plasmid DNA extracted from E9ECMO as well as from the transformants. This was done using PCR-based replicon typing as previously described by Johnson *et al.* (2007).

Determination of *armA* genetic context. Tn1548 was identified as the genetic platform that mobilizes *armA* between plasmids in most isolates. PCR mapping experiments were performed to investigate the genetic context of the *armA* using the method of Granier *et al.* (2011) with slight modifications. Some primer pairs used didn't produce any amplicons (Tn1-F/Tn1-R, Tn2-F/Tn2-R, Tn3-F/Tn3-R and Tn5-F/Tn5-R), so modification of the used primer pairs combinations was done (Tn1-F/Tn2-R, Tn2-F/Tn3-R and Tn4-F/Tn5-R). All PCR products were purified with GeneJET PCR Purification Kit (Thermo Fisher Scientific,



Lithuania) and sequenced using ABI 3730 xl DNA sequencer (Applied Biosystems, Foster City, CA). Similarity searches for the nucleotide sequences were performed with the BLASTN program (<http://www.ncbi.nlm.nih.gov>) using default settings.

Results and Discussion

As stated above, the aim of this study was to assess the spread of 16S-RMTase resistance genes among cephalosporin-resistant bacterial isolates. For this purpose, one hundred and twenty three isolates, obtained from Abu El-Rish Children Hospital in Cairo and previously determined as being resistant to third-generation cephalosporins, were screened for aminoglycoside resistance by the Kirby Bauer disk diffusion method.

Detection of 16S-RMTase producing isolates. Production of 16S-RMTase was suspected when no or little inhibitory zone was observed with any of the aminoglycoside disks (Doi and Arakawa, 2007). Out of 123 isolates tested, 66 were resistant to aminoglycosides and were thus candidates for production of 16S-RMTase. However, only one out of those 66 isolates was confirmed to possess *armA* gene by PCR (Fig. 1A) followed by sequencing (GenBank accession number KM357401 and KM357402). The positive isolate (E9ECMO) was identified as *E. coli*. Higher rates were recorded in areas such as Korea (32–45%) and China (90%) (Lee *et al.*, 2006; Xia *et al.*, 2011; Yang *et al.*, 2011). On the other hand, less than 1% prevalence of *armA* elements was recorded in some countries such as Taiwan (0.7%), Belgium (0.11%) and Japan (0.03%) (Yamane *et al.*, 2007; Bogaerts *et al.*, 2010). The low rate, recorded

Table I
Minimum Inhibitory Concentration (MIC) of selected antimicrobials against E9ECMO, *E. coli* TOP 10, transformant T1 and transformant T2.

Anti-microbials	MIC ($\mu\text{g/ml}$)			
	E9ECMO	<i>E. coli</i> TOP 10	T1	T2
Amikacin	< 256	2	< 256	> 256
Gentamicin	> 256	0.5	> 256	> 256
Ciprofloxacin	> 256	> 0.25	256	4
Cefotaxime	> 256	0.5	> 256	> 256

in this study, may be because most isolates collected in this study were from low socioeconomic children (2–16 years old) attending outpatient clinics in a governmental hospital in Cairo. Those Children are usually not in close contact with animals where 16S-RMTase were detected in several instances and thus can spread by horizontal dissemination of plasmids (Xia *et al.*, 2011).

E9ECMO is multidrug resistant. E9ECMO was also resistant to ciprofloxacin, cefoxitin, cefotaxime, piperacillin, piperacillin/ tazobactam and trimethoprim/sulfamethoxazole. The resistance genes *bla*_{TEM-1}, *bla*_{CTX-M} group 1 (*bla*_{CTX-M-15}), *bla*_{CTX-M} group 9 (*bla*_{CTX-M-14}) and *aac(6)-Ib* were also detected by PCR and sequencing in E9ECMO (Fig. 1B). This is the same resistance tendency as that reported by Bogaerts *et al.* (2010). Confirmed sequences of the resistance genes observed in the present study have been deposited in GenBank under accession numbers KM357388, KM357389, KM357390, KM357391, KM357403, KM357404, KM357405 and KM357406.

Transformation of 16S-RMTase genes. Analysis of transformants revealed the presence of two types of transformants with two corresponding phenotypes: i) Transformant, T1, was resistant to gentamicin, tobramycin, amikacin, ciprofloxacin, cefoxitin, cefotaxime, piperacillin, piperacillin/tazobactam and trimethoprim/sulfamethoxazole. ii) Transformant, T2, showing only high-level resistance to cefotaxime and the tested aminoglycosides (gentamicin, amikacin and tobramycin) as shown in Table I. PCR testing confirmed the presence of *armA*, *bla*_{TEM-1}, *bla*_{CTX-M} group 1 and group 9 and *aac(6)-Ib* in transformant T1 while only *armA*, *bla*_{CTX-M} group 9 and *aac(6)-Ib* were present in transformant T2.

Plasmid analysis. Plasmid DNA extracted from E9ECMO and each of the obtained transformants, was separated using gel electrophoresis. The plasmid profile of E9ECMO and transformants T1 showed the same number and pattern of plasmids. However, the plasmid profile of transformant T2 confirmed the presence of only one large plasmid in T2 (Fig. 2). Thus, *armA* is carried on a plasmid pMO2; conferring a multidrug resistance phenotype.

Only one linear fragment was obtained after restriction of pMO2 with *BsaBI* of approximate size 13500 bp. Thus, the size of pMO2 was approximately 13500 bp. PCR-based replicon typing of plasmids showed that plasmids extracted from both E9ECMO and transformant T1 belonged to incompatibility groups A/C and I1 while pMO2 belonged to incompatibility group A/C (Fig. 1C). This indicates that *armA*, *bla*_{CTX-M} group 9 and *aac(6)-Ib* are borne on A/C type plasmid which is one

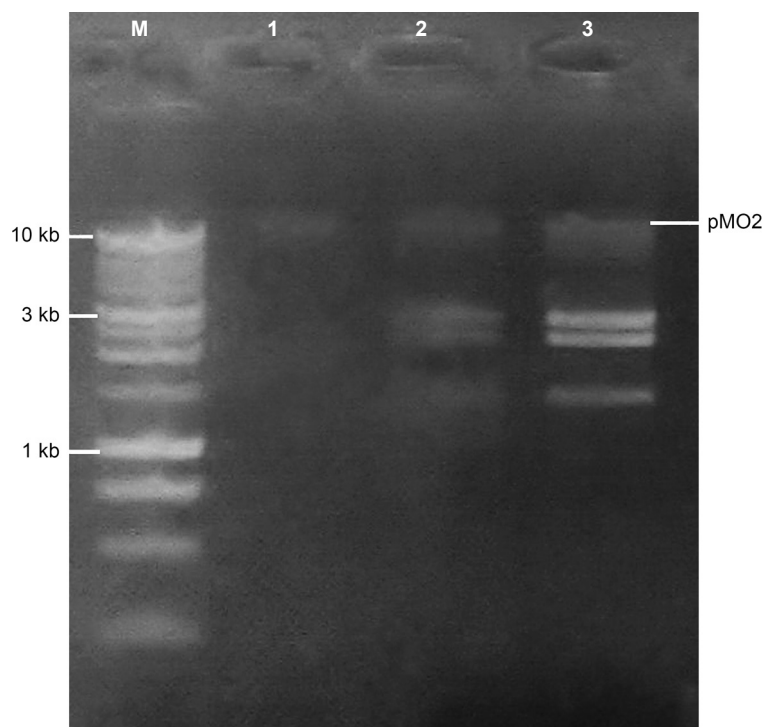


Fig. 2. Agarose gel (0.5%) showing the plasmid profile of lane M: 1 kb molecular weight ladder, 1: transformants T2, 2: transformants T1, 3: Isolate E9ECMO.

of the prevalent plasmid families known of carrying *armA* (Carattoli, 2009). The antimicrobial resistance pattern of E9ECMO was consistent with the phenotype suggested by Kang *et al.* (2008) for isolates having the *armA* gene carried on A/C type plasmid. This confirmed the presence of linkage between *armA* to other associated antimicrobial resistance genes as well as to the specific plasmid backbone.

Genetic context of *armA* in pMO2. Some primer pairs used didn't produce any amplicons, so modification of the used primer pairs combinations was done. PCR mapping and sequencing experiments revealed variation in the sequences upstream of the *armA* gene in transformant T2 from that of the genetic context of *armA* genes reported in *E. coli* AY522431 and *Citrobacter freundii* AF550415 (Gołebiewski *et al.*, 2007; González-Zorn *et al.*, 2005a). This is the first time to record a deletion of *ant3*⁹ (aminoglycoside resistance gene) and *qacEΔ1* (quaternary ammonium compounds resistance gene), which were commonly recorded upstream from *armA* in Tn1548. They were replaced by another aminoglycoside resistance gene, *aac(6)-Ib*, and this explains the failure to obtain amplicons from

some previously used primer pair combinations (Galimand *et al.*, 2005; González-Zorn *et al.*, 2005b; Johnson *et al.*, 2007; Zhang *et al.*, 2008). This coincides with González-Zorn *et al.* (2005b) conclusion about the spread of *armA* within a unique transposon composite. We reveal, for the first time, the presence of *aac-6-lb* gene together with *armA* on the same transposon. The association of *armA* together with other aminoglycosides resistance gene (*aac-6-lb*) accounts for the high level aminoglycosides resistance conferred by this transposon. This genetic combination is worrisome; mutation in *aac(6)-lb* gene can produce its *cr* allele which confers resistance to fluoroquinolones limiting the available choices for treatment of *armA* associated infections (Park *et al.*, 2006).

In addition, other antimicrobial resistance genes were detected on the same transposon. The gene *sul1* was detected upstream from *armA* and confer resistance to sulphonamides. The genes coding for the macrolide resistance efflux-pump *mefE/mel*, and a macrolide phosphotransferase gene, *mph*, were also identified downstream of *armA* gene (Fig. 3A, B). This association necessitates reinforcing the control measures

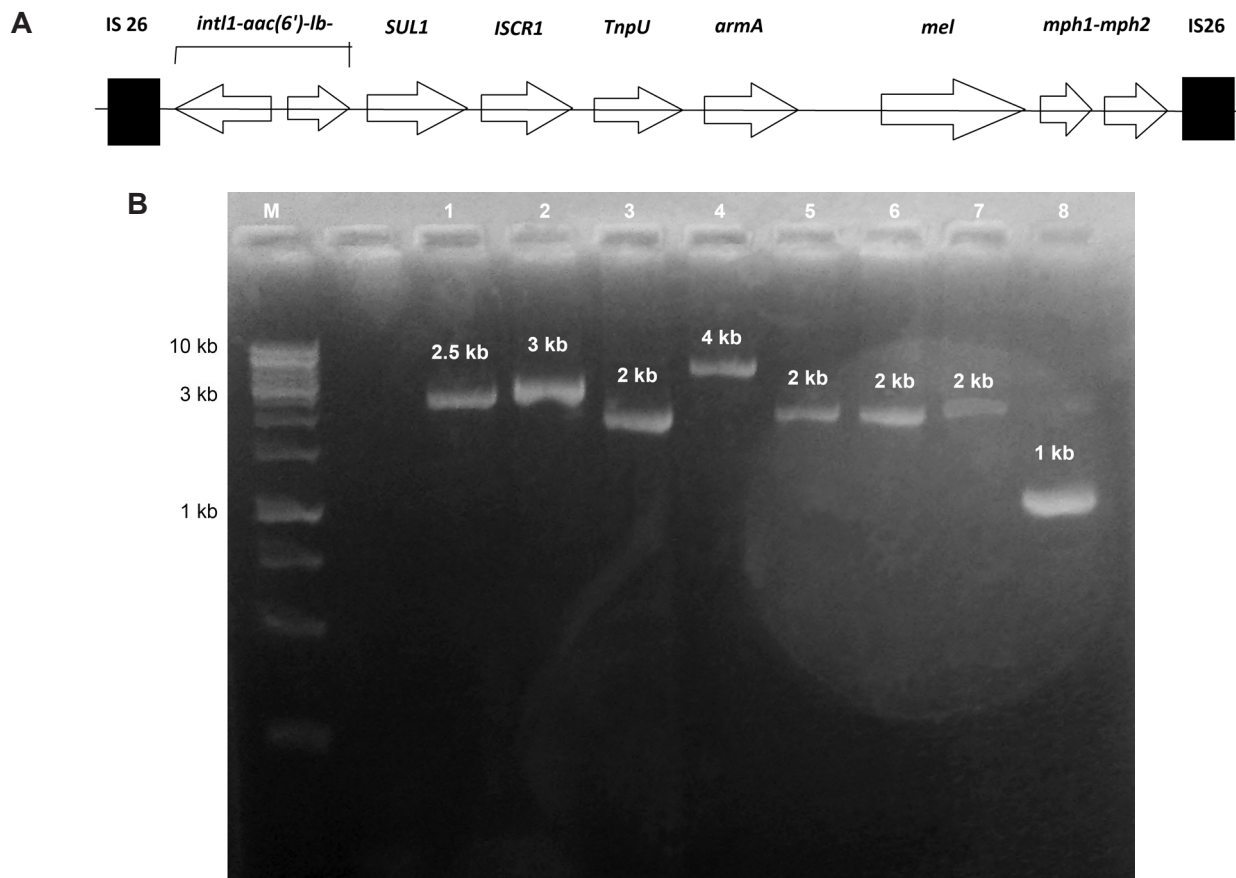


Fig. 3.

- A) Genetic structure of transposon 1548 in plasmid pMO2 from strain E9ECMO;
 B) Agarose gel (1%) of PCR amplicons obtained from transposon 1548 mapping in plasmid pMO2 from strain E9ECMO. M: 1 kb molecular weight ladder; lanes 1 to 8: represent amplicons from the following primer pairs combination: (Tn1-F & Tn2-R, Tn 2-F & Tn3-R, Tn4-F & Tn4-R, Tn4-F & Tn5R, Tn6-F & Tn6-R, Tn7-F & Tn7-R, Tn8-F & Tn8-R, Tn9-F & Tn9-R).

in both human and animal environment to avoid the spread of this aminoglycosides resistance mechanism that could be selected by sulphonamides and macro-lides consumption.

To the best of our knowledge this is the first article discussing the genetic context of 16S-RMTase genes in a clinical isolate from Egypt. A partial sequence Tn1548 variant observed in the present study has been deposited in GenBank under accession numbers KM357386, KM357387, KM357392-KM357400 and KM357407.

In conclusion, a novel mobile genetic element carried on a plasmid has been detected in this study, combining *armA* and *aac-6-lb*. The pattern and sequence of the genetic context suggests a composite transposon. The dissemination of this transposon may lead to pan aminoglycosides resistance with an impact on morbidity, mortality, and costs of healthcare in both clinical and livestock-breeding environments. Organisms possessing 16S-RMTase genes are usually more likely to develop multidrug resistance, because of associated β -lactamase genes. Therefore, despite the low rate of prevalence of *armA* in Abu El-Rish children hospital, strict surveillance should be implemented to limit its local spread.

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