

## ORIGINAL ARTICLE

## Prevalence of 16S rRNA methylase genes among $\beta$ -lactamase-producing Enterobacteriaceae clinical isolates in Saudi Arabia

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**Background:** Co production of 16S rRNA methylases gene and  $\beta$ -Lactamase gene among Enterobacteriaceae isolates conferring resistance to both therapeutic options has serious implications for clinicians worldwide.

**Methods:** To study co existence of 16S rRNA methylases (*armA*, *rmtA*, *rmtB*, *rmtC*, *rmtD*, and *npmA*) and  $\beta$ -Lactamase (*bla*<sub>TEM-1</sub>, *bla*<sub>SHV-12</sub>, *bla*<sub>CTX-M-14</sub>) genes, we screened all phenotypic positive  $\beta$ -Lactamase producing enterobacteriaceae by polymerase chain reaction (PCR) targeting above genes. A total of 330 enterobacteriaceae strains were collected during study period out of that 218 isolates were identified phenotypically as  $\beta$ -Lactamase producers, which include 50 (22.9%) *Escherichia coli*; 92 (42.2%) *Klebsiella pneumoniae*, 44 (20.2%), *Citrobacter freundii* and 32 (14.7%) *Enterobacter* spp.

**Results:** Among this 218, only 188 isolates harbored the resistant gene for  $\beta$ -Lactamase production. Major  $\beta$ -Lactamase producing isolates were *bla*<sub>TEM-1</sub> type. 122 (56 %) isolates were found to produce any one of the 16S rRNA methylase genes. A total of 116 isolates co produced  $\beta$ -Lactamase and at least one 16S rRNA methylases gene Co production of *armA* gene was found in 26 isolates with *rmtB* and in 4 isolates with *rmtC*. The *rmtA* and *rmtD* genes were not detected in any of the tested isolates. Six isolates were positive for a 16S rRNA methylase gene alone.

**Conclusion:**  $\beta$ -Lactamase producing isolates appears to coexist with 16S rRNA methylase predominantly *armA* and *rmtB* genes in the same isolate. We conclude the major  $\beta$ -Lactamase and 16S rRNA methylases co-producer was *K. pneumoniae* followed by *E. coli*. We suggest further work on evaluating other  $\beta$ -lactamases types and novel antibiotic resistance mechanisms among Enterobacteriaceae.

Keywords:  *$\beta$ -lactamase; Enterobacteriaceae; 16S rRNA methylase gene*

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The widespread use of antibacterial drugs has resulted in the emergence of multidrug and pan-drug resistant Gram negative bacterial strains, which pose challenges in an era when new antibiotic choices are limited (1, 2). Multidrug resistant strains are intermediate or resistant to at least three drugs in the classes of  $\beta$ -lactams, carbapenems, aminoglycosides, and fluoroquinolones, whereas pan-drug resistant strains are treatable only with a colistin (3). Pan-drug resistant pathogens with acquired resistance to almost all available antimicrobial

agents have emerged in recent years and challenged the treatment of infections (2, 3). Strains in the Enterobacteriaceae family have been well documented for their resistance to clinically used antibiotics (4, 5). Extended spectrum  $\beta$ -lactamase (ESBL) and metallo  $\beta$ -lactamase (MBL) production are the commonly recognized resistance mechanisms in Enterobacteriaceae strains worldwide (6).

Usually, aminoglycosides such as amikacin and tobramycin are used to treat infections by Gram negative pathogens, in combination with  $\beta$ -lactam agents.

<sup>5</sup>Equally contributed.

But increasing resistance to aminoglycosides by novel mechanisms is being reported (7, 8). 16S rRNA methylases have emerged recently as a mechanism of high level aminoglycoside resistance to most of the clinically important aminoglycosides, including arbekacin, amikacin, tobramycin, and gentamicin (4, 9). In 2003, the first 16S rRNA methylase gene, *armA*, was identified. Since then, eight plasmid-mediated 16S rRNA methylases (*armA*, *rmtA*, *rmtB*, *rmtC*, *rmtD*, *rmtE*, *rmtF*, and *npmA*) have been found in clinical isolates of Gram negative bacilli (10–12). Recently, *rmtD* (which was first identified in a pan-resistant *Pseudomonas aeruginosa* strain isolated in Brazil and hitherto only found in Latin America), was also detected in a *Klebsiella pneumoniae* isolate in Brazil (13–15). *armA* and *rmtB* have been found in many species of Gram negative bacilli in Asia (4, 16).

Dissemination of  $\beta$ -lactamase producing clinical isolates with acquired 16S rRNA methylase genes has become a global concern. Unreasonable use of  $\beta$ -lactams, including cephalosporins, carbapenams, and aminoglycosides for the treatment of Gram-negative nosocomial infections has facilitated the emergence of pan-resistant strains associated with therapeutic failures (3, 4). Coexistence of 16S rRNA methylase genes and  $\beta$ -lactamase genes among Enterobacteriaceae isolates confers resistance to both therapeutic options. This study was undertaken to determine the prevalent mechanisms of resistance among  $\beta$ -lactamase positive Enterobacteriaceae strains and also to investigate by polymerase chain reaction (PCR) the coexistence of  $\beta$ -lactamase and 16S rRNA methylase antibiotic resistance genes in these strains.

## Materials and methods

### Bacterial strains

Between January 2011 and December 2011, 330 non-duplicate Enterobacteriaceae isolates were consecutively collected from patients at El Iman Hospital, Riyadh: *E. coli*, *K. pneumoniae*, *C. freundii*, and *Enterobacter* spp (*E. aerogenes* and *E. cloacae*). The strains were obtained mostly from urine (72%) and blood (11%), but also from wounds, sputum, and other body fluids. Identification of the clinical isolates was confirmed by using GNI cards on the VITEK-60 system (bioMerieux, Marcy l'Etoile, France) and conventional methods, namely, Gram staining, morphology on differential media (MacConkey agar), and biochemical tests (manitol motility, Mannite, citrate utilization, indole production, triple sugar ion, and methyl red and Voges Proskauer tests).

### Antimicrobial susceptibility testing

Antimicrobial susceptibilities were determined by using GNS cards on the VITEK-60 system (bioMerieux, France). Antimicrobial susceptibility to amikacin, tobramycin,

and gentamicin was confirmed by the disk diffusion test using commercial disks for cefotaxime, ceftazidime, ciprofloxacin, levofloxacin, ceftiofloxacin, imipenem, tetracycline, and trimethoprim/sulfamethoxazole (Himedia, Mumbai, India) according to the criteria recommended by the Clinical and Laboratory Standards Institute (CLSI) (17). Minimum inhibitory concentrations (MICs) of amikacin, tobramycin, and gentamicin were determined by agar dilution according to CLSI guidelines and interpretative criteria. *E. coli* ATCC 25922, *Enterococci* ATCC 29212, and *K. pneumoniae* ATCC 27853 were used as quality control strains for antimicrobial susceptibility testing.

### Phenotypic identification of $\beta$ -lactamase strains

$\beta$ -Lactamase-producing strains were identified by resistance to third generation cephalosporins by using a VITEX-60 system (bioMerieux) and by production of  $\beta$ -lactamase utilizing the confirmatory double-disk combination test (17). *K. pneumoniae* ATCC 700603 was used as the  $\beta$ -lactamase positive control.  $\beta$ -lactamase producers were further characterized for the presence of different  $\beta$ -lactamase genes.

### Extraction of total DNA from $\beta$ -lactamase-producing strains

Whole genomic DNA was extracted from colonies grown overnight (14–16 h) at 37°C on blood agar (Remel, Lenexa, KS) using the QIAamp DNA Mini Kit and the QIAcube instrument (Qiagen, Valencia, CA) according to the manufacturers' instructions. Total DNA was extracted and stored at –20°C for further assays.

### Genotypic characterization of $\beta$ -lactamase and 16S rRNA methylase genes

*bla* genes were detected in  $\beta$ -lactamase-producing isolates by PCR using the previously reported oligonucleotide primers for *bla*<sub>TEM-1</sub>, *bla*<sub>SHV-12</sub>, *bla*<sub>CTX-M-14</sub> (18), and the *armA*, *rmtA*, *rmtB*, *rmtC*, *rmtD*, and *npmA* genes by using the following previously described primers (9, 13, 19–21):

Primer	Sequence
<i>armA</i> -f	5' TATGGGGGTCTTACTATTCTGCCTAT
<i>armA</i> -r	5' TCTTCCATTCCTTCTCCTTT
<i>rmtA</i> -f	5' CTAGCGTCCATCCTTTCCTC
<i>rmtA</i> -r	5' TTTGCTTCCATGCCCTTGCC
<i>rmtB</i> -f	5' TCAACGATGCCCTCACCTC
<i>rmtB</i> -r	5' GCAGGGCAAAGGTAAATCC
<i>rmtC</i> -f	5' GCCAAAGTACTACAAGTGG
<i>rmtC</i> -r	5' CTCAGATCTGACCCAACAAG
<i>rmtD</i> -f	5' CTGTTTGAAGCCAGCGGAACGC
<i>rmtD</i> -r	5' GCGCCTCCATCCATTGGAATAG
<i>npmA</i> -f	5' CTCAAAGGAACAAGACGG
<i>npmA</i> -r	5' GAAACATGGCCAGAAACTC

### Sequencing analysis of multiplex PCR products

Isolates positive for *bla* or 16S rRNA methylase genes were identified by PCR. The identities of the genes were confirmed by DNA sequencing on an ABI PRISM 3730 automated sequencer (Applied Biosystems, Foster City, CA) following the standard protocol, and the sequences were compared with those in the GenBank nucleotide database at <http://www.ncbi.nlm.nih.gov/blast/>. Thereafter, positive isolates were used as positive controls for the rest of the respective PCR reactions.

### Results

Of the 330 isolated strains of Enterobacteriaceae, 218 isolates were identified as  $\beta$ -lactamase producers by the double-disk combination test: 50 (22.9%) *E. coli*, 92 (42.2%) *K. pneumoniae*, 44 (20.2%) *C. freundii*, and 32 (14.7%) *Enterobacter* spp. All 330 isolates showed the highest resistance to ciprofloxacin (70%) followed by tobramycin (68%), gentamicin (58%), aztreonam (57%), amikacin (54%), and co-trimoxazole (54%). A significantly higher resistance to nitrofurantoin was seen in *K. pneumoniae* strains (82%) than in *E. coli* (24%). Strong resistance was noted for carbapenems: meropenem (49%) and imipenem (38%). Most of the isolates were susceptible to colistin (95%) and tigecycline (93%). MIC values of all 330 isolates were  $\geq 16$   $\mu\text{g/ml}$  for gentamicin and tobramycin, and more than 32  $\mu\text{g/ml}$  for amikacin.

### $\beta$ -Lactamase characterization

The major  $\beta$ -lactamase-producing isolates were of the *bla*<sub>TEM-1</sub> type: at least one *bla*<sub>TEM-1</sub> gene was present in 83.5% (182/218) of the isolates. All 218 isolates of *E. coli* and *K. pneumoniae* possessed one or more  $\beta$ -lactamase gene, whereas 14 of the *C. freundii* and 16 *Enterobacter* spp were negative for all tested  $\beta$ -lactamase genes.

In total 70 isolates (32%) harbored only *bla*<sub>TEM-1</sub>: 26 *E. coli* and 44 *K. pneumoniae*. We detected the presence *bla*<sub>TEM-1</sub> in combination with *bla*<sub>CTX-M-14</sub> in 51% of the isolates (111/218) and in combination with *bla*<sub>SHV-12</sub> in 39.4% (86/218). Other important combinations were *bla*<sub>CTX-M-14</sub>+*bla*<sub>TEM-1</sub>+*bla*<sub>SHV-12</sub> (39%; *n* = 85) and *bla*<sub>CTX-M-14</sub>+*bla*<sub>TEM-1</sub> (12%; *n* = 26) (Table 1).

**Table 1.** Coexistence of different  $\beta$ -lactamase genes in isolates phenotypically positive for  $\beta$ -lactamase

	<i>bla</i> <sub>TEM-1</sub>	<i>bla</i> <sub>TEM-1</sub> +CTX-M 14	<i>bla</i> TEM-1+SHV-12 like	<i>bla</i> <sub>TEM-1</sub> +SHV 12 like+CTX-M 14	<i>bla</i> negative*	Total
<i>E. coli</i>	26	3	1	20	00	50
<i>K. pneumoniae</i>	44	9	0	39	00	92
<i>C. freundii</i>	0	10	0	16	14	44
<i>Enterbacter</i>	0	4	0	10	18	32
Total	70	26	1	85	36**	218

\**bla*-negative isolates are attributed to presence of *bla* genes other than those analyzed.

\*\*Six isolates were positive for 16S rRNA methylase genes (*armA* and *rmtB*).

### 16S rRNA methylase characterization of $\beta$ -lactamase

Among the 218 isolates, 122 isolates (56%) were found to produce 16S rRNA methylase genes, including 24 *E. coli*, 52 *K. pneumoniae*, 30 *Citrobacter*, and 16 *Enterobacter* spp. *armA*, *rmtB*, *rmtC*, and *npmA* were found exclusively in 72, 10, 6, and 4 isolates, respectively. *armA* coexisted with *rmtB* in 26 isolates and with *rmtC* in 4 isolates. *rmtA* and *rmtD* were not detected in any of the isolates (Table 2).

### Coexistence of $\beta$ -lactamase and 16S rRNA methylase genes

A total of 116 isolates harbored both  $\beta$ -lactamase and 16S rRNA methylase genes, including 24 *E. coli*, 52 *K. pneumoniae*, 26 *C. freundii*, and 14 *Enterobacter* spp. Six isolates were positive for 16S rRNA methylase genes alone. Sixty-six  $\beta$ -lactamase-producing isolates did not harbor any methylase genes (Tables 3 and 4).

### Discussion

We noted that almost all (83.5%) Enterobacteriaceae isolates were  $\beta$ -lactamase positive. This rate is even higher than reported in some other developing countries (60–70%) (2, 5, 13, 22). By contrast, the prevalence of ESBL-producing isolates among Enterobacteriaceae in developed nations is much lower: <1% in Sweden (23) and 1.7% in France (24). One review reported that the prevalence of ESBLs in Europe was higher than in the US but much lower than in Asia and South America (25). This issue is evidently a challenge in developing nations, and our study clearly shows that it is a matter of urgency in our region.

The isolates were tested only for the *bla* genes reported to be prevalent in this region (26) (*bla*<sub>CTX-M-14</sub>+*bla*<sub>TEM-1</sub> and *bla*<sub>SHV</sub>), and 83.5% of them were positive for *bla*<sub>TEM-1</sub>. An Indian study (2) reported that 61.1% of MBL producers carried *bla*(*VIM*) and 2 (3%) carried *bla*(*IMP*). However, in our study we did not examine these two genes but only the major ESBL genes. This is probably the reason why some phenotypically  $\beta$ -lactamase positive isolates were genotypically negative for *bla* genes.

**Table 2.** Coexistence of different 16sRNA methylase genes in isolates phenotypically positive for  $\beta$ -lactamase

	<i>armA</i>	<i>rmt B</i>	<i>rmt C</i>	<i>npmA</i>	<i>armA+rmtB</i>	<i>armA+rmtC</i>	<i>arm</i> negative*	Total
<i>E. coli</i>	16	2	2	0	2	2	26	50
<i>K. pneumoniae</i>	34	6	0	2	8	2	40	92
<i>C. freundii</i>	16	2	0	0	12	0	14	44
<i>Enterobacter</i>	6	0	4	2	4	0	16	32
Total	72	10	6	4	26	4	96	218

\*Isolates negative for 16S rRNA methylase genes may be attributed to absence of methylase genes or to presence of methylase genes other than those that were analyzed in this study, such as *rmtE* and *rmtF*.

Previous study showed that the most prevalent micro-organism was *E. coli*, followed by *K. pneumoniae*, and other *Enterobacteriaceae* (5, 6, 21, 27). A study from France in 2008 reported that the most prevalent species were *E. coli* (48.5%), *Enterobacter aerogenes* (23.7%), and *K. pneumoniae* (14.8%) (24). In our study, the contribution of *K. pneumoniae* to the  $\beta$ -lactamase strains was higher (42.2%, 92/218), whereas that of *E. coli* was lower (22.9%, (50/218). Noteworthy, most of the isolates (72%) were obtained from urinary tract specimens, and they showed strong resistance to aminoglycosides (amikacin, tobramycin, and gentamicin), thus limiting the treatment options.

$\beta$ -Lactamase-producing *Enterobacteriaceae* often harbor other genes (*ampC*, *MBL*, and 16S rRNA methylase genes) that confer resistance to other antibiotics, including carbapenams, cepheims, and aminoglycosides (3, 4, 9). 16S rRNA methylase genes have been reported worldwide in *Enterobacteriaceae*, *P. aeruginosa* and *Acinetobacter*

*baumannii* (4, 14, 16, 28). A previously published report suggested the spread of *armA* and *rmtB* in *Enterobacteriaceae* isolates globally (29), and these genes were often reported to coexist with other resistance determinants, such as *bla* and *MBL* genes (12, 13, 30, 31). Newer 16S ribosomal RNA methylases have been identified recently and added to the database of aminoglycoside resistance mechanisms (4, 14, 16).

Only a few studies are available on the prevalence of 16S rRNA methylase genes in clinical *Enterobacteriaceae* isolates in our region (4). Therefore, we investigated and characterized the association of 16S rRNA methylase genes with  $\beta$ -lactamase genes among clinical *Enterobacteriaceae* isolates in a Saudi hospital. We observed that the coexistence of the 16S rRNA methylase genes and  $\beta$ -lactamase was very common: 56% of isolates that were genotypically positive for  $\beta$ -lactamase possessed one of the 16S rRNA methylase genes, predominantly *armA*. This rate is higher than those reported in a Taiwanese

**Table 3.** Coexistence of *bla* genes with different 16S rRNA methylase genes in isolates phenotypically positive for  $\beta$ -lactamase

Positive for genes	<i>n</i>
$\beta$ -Lactamase + <i>armA</i>	64
$\beta$ -Lactamase + <i>armA+rmt B</i>	23
$\beta$ -Lactamase + <i>rmt B</i>	10
$\beta$ -Lactamase + <i>rmt C</i>	6
$\beta$ -Lactamase + <i>armA+rmt C</i>	3
$\beta$ -Lactamase + <i>npmA</i>	4
$\beta$ -Lactamase + <i>armA</i>	4
$\beta$ -Lactamase + <i>armA+rmt C</i>	1
$\beta$ -Lactamase + <i>armA+rmt B</i>	1
<i>armA</i> only	4
<i>armA+rmt B</i> only	2
Positive for <i>bla</i> gene but no 16S rRNA detected but	66
No <i>bla</i> gene or 16S rRNA methylase gene detected*	30
Total	218

\*Negative isolates may attributed to presence of *bla* or methylase genes that were not analyzed in this study.

**Table 4.** Coexistence of 16S rRNA genes with different ESBL genes in isolates phenotypically positive for  $\beta$ -lactamase

Positive for gene	<i>n</i>
<i>TEM-1+SHV 12 like+CTX-M 14+16S rRNA methylases</i>	64
<i>TEM-1+CTX-M 14+16S rRNA methylases</i>	23
<i>TEM-1+SHV 12 like+CTX-M 14+16S rRNA methylases</i>	10
<i>TEM-1+SHV 12 like+CTX-M 14+16S rRNA methylases</i>	6
<i>TEM-1+16S rRNA methylases</i>	4
<i>TEM-1+SHV 12 like+CTX-M 14+16S rRNA methylases</i>	4
<i>TEM-1+CTX-M 14+16S rRNA methylases</i>	3
<i>TEM-1+SHV 12 like</i>	1
<i>TEM-1+16S rRNA methylases</i>	1
<i>TEM-1+SHV 12 like+CTX-M 14+16S rRNA methylases</i>	1
<i>TEM-1</i> only	65
Positive for 16S rRNA but no <i>bla</i> gene detected	6
No <i>bla</i> or 16S rRNA methylase gene detected*	30
Total	218

\*Negative isolates may be attributed to presence of *bla* or methylase genes that were not analyzed in this study.

study (21) and a Belgian study (28). But like previous reports (4, 16, 28), we also observed greater prevalence of *armA* than *rmtB*. We also report the presence of *rmtC* in four *E. coli* and four *Enterobacter* isolates. It is noteworthy that we identified *npmA* in two *K. pneumoniae* and two *Enterobacter* isolates. This is a novel finding in this region, because *rmtC* and *npmA* have been detected in *Proteus mirabilis* and *E. coli*, respectively, in Japan (13, 20). We did not detect any *rmtA* or *rmtD* gene in our isolates.

In agreement with previous reports (26, 32),  $\beta$ -lactamase positive isolates were more susceptible to colistin and tigecycline, which were the only available therapeutic options for them.

In our study, 35.2% (64/182), 5.5% (10/182), 3.3% (6/182), and 2.2% (4/182) of the isolates harboring *bla*<sub>TEM-1</sub> also contained *armA*, *rmtB*, *rmtC*, and *npmA*, respectively, in association with *bla*<sub>TEM-1</sub> + SHV 12 like + CTX-M 14. One isolate produced *armA* and *rmtB* along with *bla*<sub>TEM-1</sub> + SHV 12 like + CTX-M 14. This points to horizontal as well as clonal dissemination of these genes in our hospital. We also found strains harboring two different 16S rRNA methylase genes in combinations, including two strains carrying *armA* + *rmtB* and one strain carrying *armA* + *rmtC*.

In conclusion,  $\beta$ -lactamase-producing isolates of Enterobacteriaceae were common in our hospital and they usually harbored 16S rRNA methylase genes, predominantly *armA* and *rmtB*. The major co-producers of  $\beta$ -lactamase and 16S rRNA methylases were *K. pneumoniae*, followed by *E. coli*. Our findings underline the emerging threat of pan-drug resistant pathogens that produce both  $\beta$ -lactamase and 16S rRNA methylase disseminating in this region. Further work to evaluate other  $\beta$ -lactamase types and novel antibiotic resistance mechanisms among Enterobacteriaceae and other nosocomial pathogens is needed.

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## Conflict of interest and funding

The authors certify that there is no financial or other conflict of interest regarding the material discussed in the manuscript.

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