

Full Length Research Paper

Prevalence of plasmid-mediated 16S rRNA methylase genes among *Proteus mirabilis* isolates from a Chinese hospital

Fangyou Yu¹, Jingye Pan², Baixing Ding², Lehe Yang³, Xueqing Zhang¹ and Liangxing Wang^{3*}

¹Department of Laboratory Medicine, the First Affiliated Hospital of Wenzhou Medical College, Wenzhou.325000, China.

²Department of Intensive Care Unit, the First Affiliated Hospital of Wenzhou Medical College, Wenzhou.325000, China.

³Department of Respiratory Medicine, the First Affiliated Hospital of Wenzhou Medical College, Wenzhou.325000, China.

Accepted 11 November, 2018

16S rRNA methylase-mediated high-level resistance to aminoglycosides has been reported recently in clinical isolates of Gram -negative bacilli from several countries. Five (2.5%, 5/198) of 198 isolates of *Proteus mirabilis* from a teaching hospital in Wenzhou, China, were positive for 16S rRNA methylase genes (one for *armA*, four for *rmtB*) and highly resistant to gentamicin, amikacin and tobramycin (MICs, 256 g/ml). One of five isolates harboring 16S rRNA methylase genes were extended-spectrum - lactamases (ESBL) producer. The plasmids harboring 16S rRNA methylase genes from four out of five donors were transferred into the recipients, *Escherichia coli* J53. Among five isolates harboring *armA* and *rmtB*, the *armA* gene and the *rmtB* genes were located on the plasmids, as determined by Southern hybridization. The present study investigated the prevalence of 16S rRNA methylase genes in clinical isolates of *P. mirabilis* in China for the first time.

Key words: *Proteus mirabilis*, 16S rRNA methylase, plasmid.

INTRODUCTION

Increasing resistance to aminoglycosides is becoming a clinical problem (Kotra et al., 2000; Yamane et al., 2005). The antimicrobial activity of aminoglycosides depends on its binding affinity to a highly conserved motif of 16S rRNA. The mechanism of resistance to aminoglycosides is frequently due to the acquisition of modifying enzymes that vary in their substrate ranges, such as acetyltransferases, phosphorylases, and adenylyltransferases. Recently, 16S rRNA methylase-mediated high-level resistance to aminoglycosides has been reported in clinical isolates of Gram-negative bacilli from several countries (Yokoyama et al., 2003). Six plasmid-mediated 16S rRNA methylases (*ArmA*, *RmtA*, *RmtB*, *RmtC*, *RmtD* and *NpmA*) have been identified in clinical isolates of Gram-negative bacilli (Doi et al., 2004, 2007; Galimand et al., 2003; Wachino et al., 2006; Yamane et al., 2007).

Proteus mirabilis is one of the most common Gram-negative pathogens causing urinary tract, wound, and

bloodstream infections. This organism is usually susceptible to antimicrobial agents except nitrofurantoin and tetracycline. But the isolates producing extended-spectrum -lactamases (ESBLs) have spread in many countries (Endimiani et al., 2005). In addition to resistance to -lactams, the clinical isolates of *P. mirabilis* are resistant to aminoglycosides, fluoroquinolones, and trimethoprim-sulfamethoxazole. In our previous study, 16S rRNA methylase-producing isolates of *Klebsiella pneumoniae* were commonly identified in our hospital (Yu et al., 2009) . However, the prevalence of these 16S rRNA methylases in clinical isolates of *P. mirabilis* is not known. The aim of the present study was to investigate the prevalence of 16S rRNA methylases in this organism from a Chinese teaching hospital in Wenzhou.

MATERIALS AND METHODS

Clinical isolates

Between January, 2004 and December, 2007, a total of 198 non-duplicate isolates of *P. mirabilis* were isolated from clinical

*Corresponding author. E-mail: wzyxyw@163.com.

specimens in hospitalized patients in this hospital. Bacterial identification was based on the results of the Vitek GNI card (bioMérieux Vitek Inc.,) as well as conventional biochemical tests.

Antimicrobial susceptibility testing

Initial antimicrobial susceptibilities were determined by using GNS cards on the VITEK-60 system (bioMérieux, Marcy l'Etoile, France). Amikacin-, tobramycin-, or gentamicin-resistant isolates were screened for further antimicrobial susceptibilities. The antimicrobial susceptibility was determined by the disk diffusion test according to the criteria recommended by the Clinical and Laboratory Standards Institute (CLSI, 2007).

Escherichia coli (ATCC 25922) was used as the control strain for antimicrobial susceptibility testing. Minimum Inhibitory Concentrations (MICs) of amikacin, tobramycin, and gentamicin were determined by the agar dilution method in accordance with the CLSI guidelines (CLSI, 2007).

Extraction of total DNA and plasmid DNA

Total DNA was extracted by boiling. Briefly, a fresh bacterial colony was suspended in 150 l of sterile distilled water and boiled at 100°C for 10 min. After centrifugation at 15000 rpm for 15 min at 4°C, the supernatant was removed and stored at -20°C for further assays. Plasmid DNA of the donors, transconjugants, and transformants was extracted with the QIAGEN (Hilden, Germany). Plasmid Midi kit according to the manufacturer's instructions. Plasmid size was estimated as previously described (Wang et al., 2004).

Screening for 16S rRNA methylase genes

The *armA*, *rmtA*, *rmtB*, *rmtC*, *rmtD* and *npmA* genes were detected by Polymerase Chain Reaction (PCR) with total DNA from potential producers and a series of primers as previously reported (Doi et al., 2004, 2007; Galimand et al., 2003; Yan et al., 2004; Yokoyama et al., 2003). *P. mirabilis* isolates with 16S rRNA methylase genes identified in our previous study were used as positive control in every test for detecting 16S rRNA methylase genes (Yu et al., 2009). All PCR products were sequenced on an ABI PRISM 3730 automated sequencer (Applied Biosystems, Foster City, Calif). The nucleotide sequences were analyzed with software available over the Internet (www.ncbi.nlm.nih.gov/).

-lactamase characterization

All 16S rRNA methylase gene-positive isolates were tested for ESBL production by the CLSI-recommended confirmatory double disk combination test (CLSI, 2007). *K. pneumoniae* (ATCC 700603) was used for the ESBL positive control. PCR was performed for the detection of -lactamase genes in ESBL-producing isolates with the previously reported oligonucleotide primers, including *bla*TEM, *bla*SHV, *bla*CTX-M, *bla*VEB, and *bla*PER (Jiang et al., 2005; Pagani et al., 2003). All PCR products were directly sequenced and the sequences were compared with those in the GenBank nucleotide database at www.ncbi.nlm.nih.gov/blast/.

Transfer of aminoglycoside resistance genes

In order to determine if aminoglycoside resistance was transferable in *K. pneumoniae* isolates with 16S rRNA methylase-bearing plasmids, a conjugation experiment was carried out in Luria-Bertani

broth with *E. coli* J53 as the recipient as previously described (Wang et al., 2004). Transconjugants were selected on tryptic soy agar plates containing sodium azide (100 µg/ml) for counter selection and amikacin (30 µg/ml) for plasmid-mediated resistance selection. The plasmid extracts were electroporated into *E. coli* DH5 by using a Gene Pulser II apparatus (Bio-Rad), and transformants were selected on Luria-Bertani agar plates containing amikacin (30 µg/ml).

Southern hybridization for determining localization of 16S rRNA methylase genes, Southern hybridization was performed by standard methods with *armA*-specific and *rmtB*-specific digoxigenin (DIG)-labeled probes formed by the PCR DIG detection system (Roche Diagnostics GmbH). Briefly, plasmid DNA was subjected to electrophoresis in 0.7% agarose gels. After depurination, denaturation, and neutralization of the agarose gels, DNA was transferred to a positive-charged nylon membrane (Millipore) by capillary action. Plasmid DNA was hybridized successively with *armA*-specific and *rmtB*-specific DIG-labeled probes at 42°C under highly stringent conditions.

RESULTS

Prevalence of 16S rRNA methylase genes and antimicrobial susceptibility

Twenty-one (10.6%) of 198 isolates were resistant to gentamicin, tobramycin or amikacin determined by using GNS cards on the VITEK-60 system (bioMérieux, Marcy l'Etoile, France). Aminoglycosides-resistant isolates were further determined by agar dilution test and selected for screening 16S rRNA methylase genes. Four (2.0%, 4/198) and one (0.5%, 1/198) isolate were confirmed to harbor *rmtB* and *armA* by PCR and DNA sequencing. The prevalence of 16S rRNA methylase genes in clinical isolates of *P. mirabilis* was 2.5% (5/198) in the present study. The *armA* amplicon showed 100% identity with the *armA* gene in *K. pneumoniae* BM4536 (GenBank AY220558), and the *rmtB* amplicons showed 100% identity with the *rmtB* in *Serratia marcescens* S-95 (GenBank AB103506), *rmtA*, *rmtD*, and *npmA* genes were not detected in any of tested isolates. The five 16S rRNA methylase gene-positive isolates (three from wound exudate, one each from sputum and urine) were isolated from inpatients of four different wards. The first isolate was isolated from wound exudate in a 71-year inpatient in September, 2005. All five 16S rRNA methylase gene-positive isolates were highly resistant to gentamicin, amikacin, tobramycin, and kanamycin (MICs, 256 mg/l).

-Lactamase characterization

Only one *rmtB*-positive *P. mirabilis* was found to be ESBL producer determined by the CLSI-recommended confirmatory tests. The genotype of the ESBL gene was *bla*CTX-M-14 determined by PCR and DNA sequencing. All 16S rRNA methylase gene-positive isolates were positive for -lactamase gene, *bla*TEM-1.

Table 1. Characteristics of the 5 *P. mirabilis* isolates harboring 16S rRNA methylase genes.

Strain	MIC ($\mu\text{g/ml}$)			<i>rmtB</i>	<i>armA</i>	ESBL	<i>bla</i> _{TEM}	<i>bla</i> _{CTX-M}
	GEN	AMK	TOB					
P4	>1024	1024	512	+ ^b	- ^a	+	TEM-1	CTX-M-14
<i>E. coli</i> P4	256	256	256	+	-	+	TEM-1	CTX-M-14
P7	>1024	>1024	1024	-	+	-	TEM-1	-
<i>E. coli</i> P7	256	512	256	-	+	-	TEM-1	-
P14	>1024	>1024	512	+	-	-	TEM-1	-
P16	>1024	>1024	512	+	-	-	TEM-1	-
<i>E. coli</i> P16	512	512	256	+	-	-	TEM-1	-
P17	>1024	>1024	1024	+	-	-	TEM-1	-
<i>E. coli</i> P17	512	512	512	+	-	-	TEM-1	-

a+, positive; -, negative.

Transfer of aminoglycoside resistance genes and southern hybridization

The plasmids of 4 out of the 5 isolates harboring 16S rRNA methylase genes were transferred to the recipients, *E. coli* J53. The *armA* and *rmtB* genes were transferable by conjugation with frequencies between 10^{-5} and 10^{-7} . All transconjugants were highly resistant to amikacin, gentamicin, and tobramycin (MICs, 256 mg/l). Characteristics of 4 transconjugants were listed in Table 1. Plasmids of one *armA*-positive isolate, four *rmtB*-positive isolates, and their transconjugants were extracted and hybridized with digoxigenin-labeled *armA*- and *rmtB*-specific probes. The hybridization signals were obtained in approximately 80 Kb plasmids of all 5 isolates and their transconjugants, which indicated that the *armA* and *rmtB* genes located on the plasmids of *P. mirabilis* (Figure 1).

DISCUSSION

16S rRNA methylases can confer high-level resistance to aminoglycosides. Since *armA* was first identified in *K. pneumoniae* BM4536 isolated from a French patient in 2000 and *rmtB* was first found in *S. marcescens* S-95 isolated from a Japanese patient in 2002 (Doi et al., 2004; Galimand et al., 2003), these genes have been found in *Enterobacteriaceae*, *Pseudomonas aeruginosa*, and *Acinetobacter baumannii* in many areas (Doi et al., 2004; Galimand et al., 2003; Yan et al., 2004; Yokoyama et al., 2003; Yu et al., 2009). In Asia, *armA* and *rmtB* have been found in many species of Gram-negative bacilli in Japan, Korea, Taiwan, and China (Wachino et al., 2006; Yan et al., 2004; Yu et al., 2007, 2009). In the present study, the *rmtB* gene was more prevalent than the *armA* gene in *P. mirabilis* isolates, which was in accordance with those in *K. pneumoniae* isolates reported by our previous study (Yu et al., 2009) but was contrast to previous reports in *E. coli* isolates and *K. pneumoniae*

isolates with more *armA* than *rmtB* (Bogaerts et al., 2007; Wu et al., 2008; Yan et al., 2004). The present study and our previous study indicated that the *armA* and *rmtB* genes were spreading among *Enterobacteriaceae* and the *rmtB* gene was more prevalent than the *armA* in our hospital. Although, *rmtC* was first found in *P. mirabilis* strain ARS68 from Japan in 2003 (Wachino et al., 2006), *rmtC* was not detected in this species in the present study. Other than in Japan, only *armA* and *rmtB* were found in the Gram-negative bacilli isolates in Asia.

The dissemination mechanisms of 16S rRNA methylase genes are of clinical significance. 16S rRNA methylase-bearing strains usually co-produced ESBLs. A recent study reported the prevalence of the *rmtB* gene and the *armA* gene in ESBL-producing *P. mirabilis* isolates (Wu et al., 2008). The prevalence of 16S rRNA methylase genes in non-ESBL-producing *P. mirabilis* isolates is not reported before. However, four non-ESBL-producing *P. mirabilis* isolates were found to harbor 16S rRNA methylase genes in the present study. To best of our knowledge, 16S rRNA methylase genes and CTX-M-14 type ESBLs among *P. mirabilis* were first reported in China. As reported previously, the *armA* gene and the *rmtB* gene in *P. mirabilis* located self-transmissible conjugative plasmids.

In conclusion, the present study investigated the prevalence of 16S rRNA methylase genes in clinical isolates of *P. mirabilis* in China for the first time. 16S rRNA methylase genes usually do not coexist with ESBL genes in *P. mirabilis* isolates.

ACKNOWLEDGEMENTS

The author are grateful to George A. Jacoby for providing reference strains *E. coli* J53AzR and *E. coli* V517. This work was supported by Medical Science Fund of Zhejiang Province, China (2008A116) and Wenzhou Municipal Science and Technology Bureau, China (H20080064).

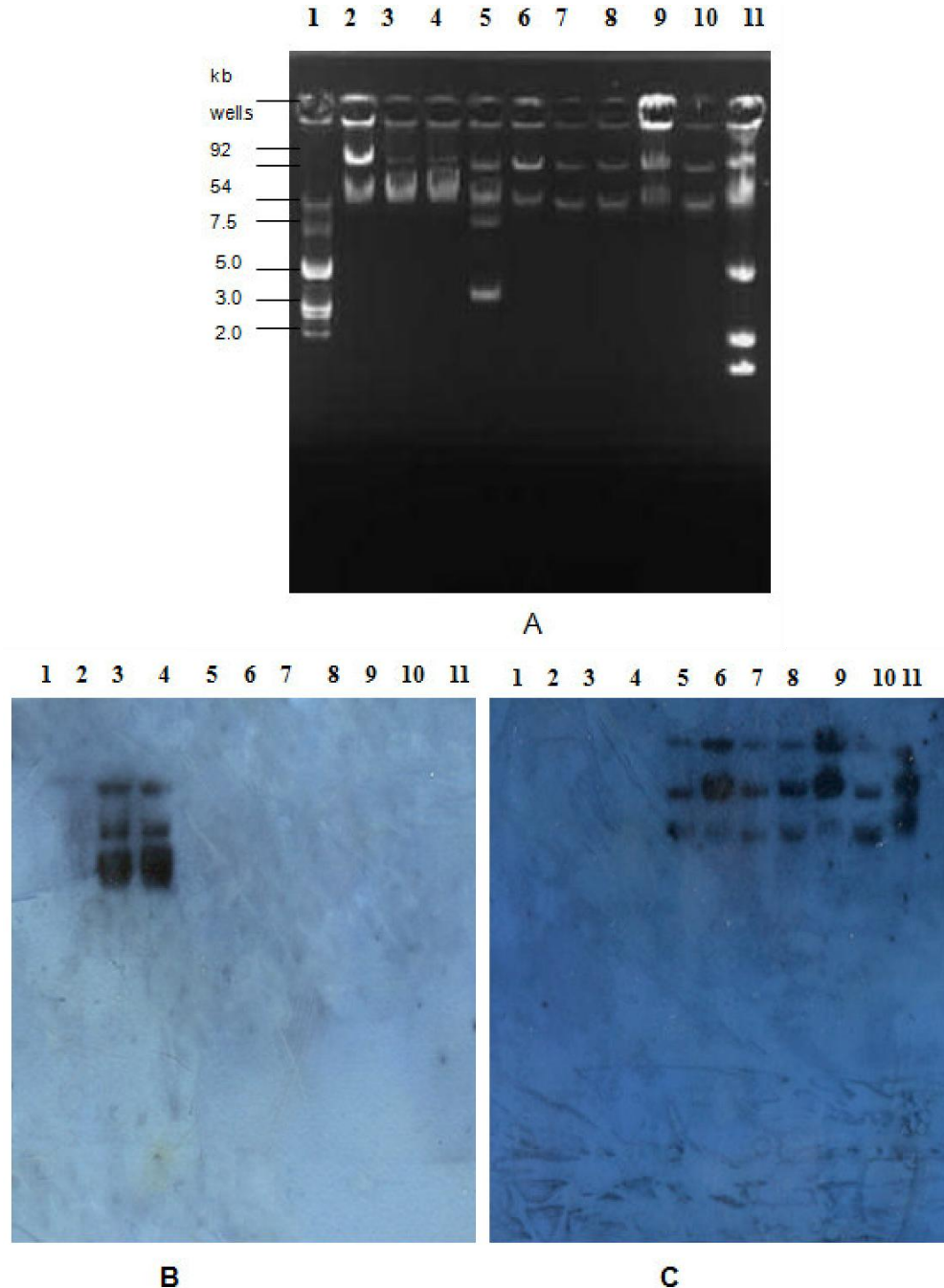


Figure 1. (A) Plasmid extractions from reference strains of *E. coli*, *P. mirabilis* isolates carrying *armA* and *rmtB* and their transconjugants, (B) Southern hybridization with *armA*-specific probe and (C) Southern hybridization with *rmtB*-specific probe. Lanes: 1, *E. coli* V517; 2, *E. coli* J53/R1; 3, P7; 4, P7 transconjugant; 5, P4; 6, P4 transconjugant; 7, P16; 8, P16 transconjugant; 9, P17; 10, P17 transconjugant; 11, P14.

REFERENCES

Bogaerts P, Galimand M, Bauraing C, Deplano A, Vanhoof R, De Mendonca R, Rodriguez-Villalobos H, Struelens M, Glupczynski Y (2007). Emergence of *ArmA* and *RmtB* aminoglycoside resistance 16S rRNA methylases in Belgium. *J. Antimicrob. Chemother.*, 59: 459-464.

Doi Y, de Oliveira Garcia D, Adams J, Paterson DL (2007).

Coproduction of novel 16S rRNA methylase *RmtD* and metallo-beta-lactamase *SPM-1* in a panresistant *Pseudomonas aeruginosa* isolate from Brazil. *Antimicrob. Agents Chemother.*, 51: 852-856.

Doi Y, Yokoyama K, Yamane K, Wachino J, Shibata N, Yagi T, Shibayama K, Kato H, Arakawa Y (2004). Plasmid-mediated 16S rRNA methylase in *Serratia marcescens* conferring high-level resistance to aminoglycosides. *Antimicrob. Agents Chemother.*, 48: 491-496.

- Endimiani A, Luzzaro F, Brigante G, Perilli M, Lombardi G, Amicosante G, Rossolini GM, Toniolo A (2005). *Proteus mirabilis* bloodstream infections: risk factors and treatment outcome related to the expression of extended-spectrum beta-lactamases. *Antimicrob. Agents Chemother.*, 49: 2598-2605.
- Galimand M, Courvalin P, Lambert T (2003). Plasmid-mediated high-level resistance to aminoglycosides in Enterobacteriaceae due to 16S rRNA methylation. *Antimicrob. Agents Chemother.*, 47: 2565-2571.
- CLSI (2007). Performance standards for antimicrobial susceptibility testing, 17th informational supplement (M100-S17). Clinical and Laboratory Standards Institute, Wayne, PA, USA.
- Jiang X, Ni Y, Jiang Y, Yuan F, Han L, Li M, Liu H, Yang L, Lu Y (2005). Outbreak of infection caused by *Enterobacter cloacae* producing the novel VEB-3 beta-lactamase in China. *J. Clin. Microbiol.*, 43: 826-831.
- Kotra LP, Haddad J, Mobashery S (2000). Aminoglycosides: perspectives on mechanisms of action and resistance and strategies to counter resistance. *Antimicrob. Agents Chemother.*, 44: 3249-3256.
- Pagani L, Dell'Amico E, Migliavacca R, D'Andrea MM, Giacobone E, Amicosante G, Romero E, Rossolini GM (2003). Multiple CTX-M-type extended-spectrum beta-lactamases in nosocomial isolates of Enterobacteriaceae from a hospital in northern Italy. *J. Clin. Microbiol.*, 41: 4264-4269.
- Wachino J, Yamane K, Shibayama K, Kurokawa H, Shibata N, Suzuki S, Doi Y, Kimura K, Ike Y, Arakawa Y (2006). Novel plasmid-mediated 16S rRNA methylase, RmtC, found in a *proteus mirabilis* isolate demonstrating extraordinary high-level resistance against various aminoglycosides. *Antimicrob. Agents Chemother.*, 50: 178-184.
- Wang M, Sahn DF, Jacoby GA, Hooper DC (2004). Emerging plasmid-mediated quinolone resistance associated with the qnr gene in *Klebsiella pneumoniae* clinical isolates in the United States. *Antimicrob. Agents Chemother.*, 48: 1295-1299.
- Wu JJ, Chen HM, Ko WC, Wu HM, Tsai SH, Yan JJ (2008). Prevalence of extended-spectrum beta-lactamases in *Proteus mirabilis* in a Taiwanese university hospital, 1999 to 2005: identification of a novel CTX-M enzyme (CTX-M-66). *Diagn Microbiol. Infect. Dis.*, 60: 169-175.
- Yamane K, Wachino J, Doi Y, Kurokawa H, Arakawa Y (2005). Global spread of multiple aminoglycoside resistance genes. *Emerg. Infect. Dis.*, 11: 951-953.
- Yamane K, Wachino J, Suzuki S, Kimura K, Shibata N, Kato H, Shibayama K, Konda T, Arakawa Y (2007). New plasmid-mediated fluoroquinolone efflux pump, QepA, found in an *Escherichia coli* clinical isolate. *Antimicrob. Agents Chemother.*, 51: 3354-3360.
- Yan JJ, Wu JJ, Ko WC, Tsai SH, Chuang CL, Wu HM, Lu YJ, Li JD (2004). Plasmid-mediated 16S rRNA methylases conferring high-level aminoglycoside resistance in *Escherichia coli* and *Klebsiella pneumoniae* isolates from two Taiwanese hospitals. *J. Antimicrob. Chemother.*, 54: 1007-1012.
- Yokoyama K, Doi Y, Yamane K, Kurokawa H, Shibata N, Shibayama K, Yagi T, Kato H, Arakawa Y (2003). Acquisition of 16S rRNA methylase gene in *Pseudomonas aeruginosa*. *Lancet* 362: 1888-1893.
- Yu F, Wang L, Pan J, Yao D, Chen C, Zhu T, Lou Q, Hu J, Wu Y, Zhang X, Chen Z, Qu D (2009). Prevalence of 16S rRNA methylase genes in *Klebsiella pneumoniae* isolates from a Chinese teaching hospital: coexistence of rmtB and armA genes in the same isolate. *Diagn. Microbiol. Infect. Dis.*, 64: 57-63.
- Yu YS, Zhou H, Yang Q, Chen YG, Li LJ (2007). Widespread occurrence of aminoglycoside resistance due to ArmA methylase in imipenem-resistant *Acinetobacter baumannii* isolates in China. *J. Antimicrob. Chemother.*, 60: 454-455.