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Full Length Research Paper

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

Fangyou Yu[1], Jingye Pan[2], Baixing Ding[2], Lehe Yang[3], Xueqing Zhang[1] and Liangxing Wang[3]*

[1]Department of Laboratory Medicine, the First Affiliated Hospital of Wenzhou Medical College, Wenzhou.325000, China.
[2]Department of Intensive Care Unit, the First Affiliated Hospital of Wenzhou Medical College, Wenzhou.325000, China.
[3]Department of Respiratory Medicine, the First Affiliated Hospital of Wenzhou Medical College, Wenzhou.325000, China.

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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 μg/ml). One of five isolates harboring 16S rRNA methyalse genes were extended-spectrum β-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 acetyl-transferases, 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 β-lactamases (ESBLs) have spread in many countries (Endimiani et al., 2005). In addition to resistance to β-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: wzyxywlx@163.com.

specimens in hospitalized patients in this hospital. Bacterial identification was based on the results of the Vitek GNI card (bioMe´rieux 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 (bioMerieux, Marcy l'E´ toile, 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 µl of sterile distilled water and boiled at 100℃ for 10 min. After centrifugation at 15000 rpm for 15 min at 4℃, the supernatant was removed and stored at -20℃ 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/).

β-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 β-lactamase genes in ESBL-producing isolates with the previously reported oligonucleotide primers, including blaTEM, blaSHV, blaCTX-M, blaVEB, and blaPER (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* DH5α 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℃ 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 (bioMerieux, Marcy l'E´ toile, France). Aminoglysides-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).

β-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 blaCTX-M-14 determined by PCR and DNA sequencing. All 16S rRNA methylase gene-positive isolates were positive for β-lactamase gene, blaTEM-1.

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

Strain	MIC (µg/ml)			*rmtB*	*armA*	ESBL	*bla*TEM	*bla*CTX-M
	GEN	AMK	TOB					
P4	>1024	1024	512	+[b]	-[a]	+	TEM-1	CTX-M-14
E. coli P4	256	256	256	+	-	+	TEM-1	CTX-M-14
P7	>1024	>1024	1024	-	+	-	TEM-1	-
E. coli P7	256	512	256	-	+	-	TEM-1	-
P14	>1024	>1024	512	+	-	-	TEM-1	-
P16	>1024	>1024	512	+	-	-	TEM-1	-
E. coli P16	512	512	256	+	-	-	TEM-1	-
P17	>1024	>1024	1024	+	-	-	TEM-1	-
E. coli 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.

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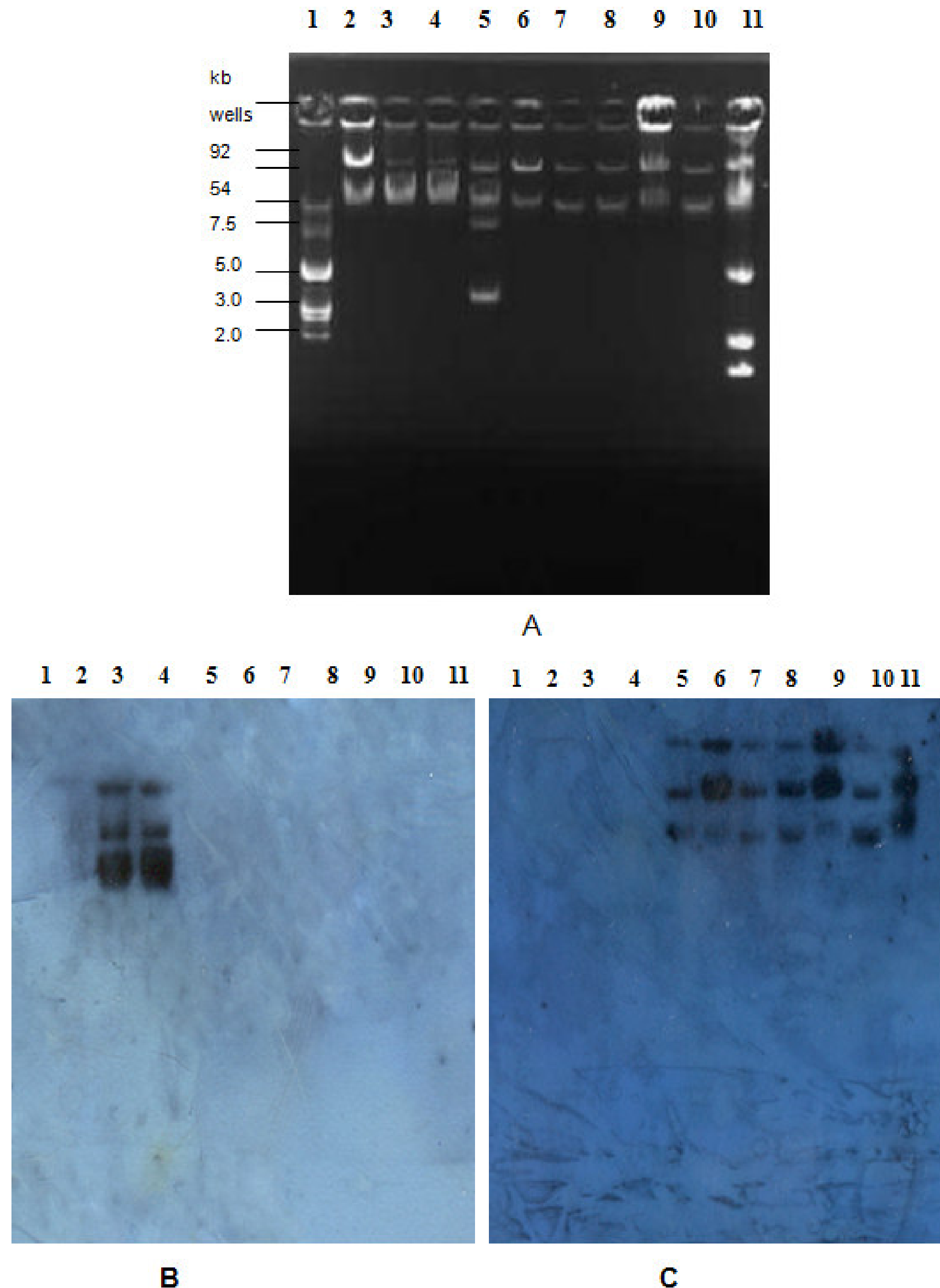

Figure 1. (A) Plasmid extractions from reference strains of *E. coli*,5 *P. mirabilis* isolates carrying armA and rmtB and their transconjugants, (B) Southern hybridization with armA-specific probe and (C) Southern hybridization with rmtB-specific probe.Lanes: 1, *E. coli* V517; 2, *E. coli* J53/R1;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

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