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Original article

Loop-mediated isothermal amplification assay for 16S rRNA methylase genes in Gram-negative bacteria

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ABSTRACT

Using the loop-mediated isothermal amplification (LAMP) method, we developed a rapid assay for detection of 16S rRNA methylase genes (*rmtA*, *rmtB*, and *armA*), and investigated 16S rRNA methylase-producing strains among clinical isolates. Primer Explorer V3 software was used to design the LAMP primers. LAMP primers were prepared for each gene, including two outer primers (F3 and B3), two inner primers (FIP and BIP), and two loop primers (LF and LB). Detection was performed with the Loopamp DNA amplification kit. For all three genes (*rmtA*, *rmtB*, and *armA*), 10^2 copies/tube could be detected with a reaction time of 60 min. When nine bacterial species (65 strains saved in National Institute of Infectious Diseases) were tested, which had been confirmed to possess *rmtA*, *rmtB*, or *armA* by PCR and DNA sequencing, the genes were detected correctly in these bacteria with no false negative or false positive results. Among 8447 clinical isolates isolated at 36 medical institutions, the LAMP method was conducted for 191 strains that were resistant to aminoglycosides based on the results of antimicrobial susceptibility tests. Eight strains were found to produce 16S rRNA methylase (0.09%), with *rmtB* being identified in three strains (0.06%) of 4929 isolates of *Enterobacteriaceae*, *rmtA* in three strains (0.10%) of 3284 isolates of *Pseudomonas aeruginosa*, and *armA* in two strains (0.85%) of 234 isolates of *Acinetobacter* spp. At present, the incidence of strains possessing 16S rRNA methylase genes is very low in Japan. However, when Gram-negative bacteria showing high resistance to aminoglycosides are isolated by clinical laboratories, it seems very important to investigate the status of 16S rRNA methylase gene-harboring bacilli and monitor their trends among Japanese clinical settings.

1. Introduction

Some Gram-negative bacilli are highly resistant to aminoglycosides (AGs), including strains of *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and *Serratia marcescens*. In Japan, such resistant strains were first reported in 2003 [1]. These AG-resistant bacteria acquire genes similar to the 16S rRNA methylase genes of actinomyces, which confer resistance to clinically used AGs antibiotics such as gentamicin and amikacin.

So far, at least eleven 16S rRNA methylase genes (*rmtA*, *rmtB*, *rmtC*, *rmtD*, *rmtD2*, *rmtE*, *rmtF*, *rmtG*, *rmtH*, *armA*, and *npmA*) have been identified [1–8], and since these genes are usually present on plasmids, they can easily transfer to other bacteria. When multiple drug-resistant *P. aeruginosa* acquires a 16S rRNA methylase gene, antibiotic therapy would become ineffective. Accordingly, the emergence and spread of such bacteria should be carefully monitored, and investigating the acquisition of 16S rRNA methylase genes by clinical isolates is important for both prevention and treatment of their infections, so development of a rapid and convenient detection method would be desirable.

Detection of AGs-resistant strains can be done by antimicrobial susceptibility testing with methods such as broth microdilution method in accordance with the CLSI's protocol, but such procedures

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Table 1
LAMP primers (F3, B3, FIP, BIP, LF, and LB) for the target 16S rRNA methylase genes (*rmtA*, *rmtB*, and *armA*) were designed by Primer Explorer Ver.3 software (Eiken Chemical Co., Ltd. Tokyo, Japan).

16S rRNA methylase	Amplified region (GenBank accession number)	Primer	Type	Sequence (5′–3′)
rmtA	7118–7328 (AB120321)	rmtA-F3	F3	GGATTGGACTTCACGTTCGC
		rmtA-B3	B3c	TTGCTTCCATGCCCTTGC
		rmtA-FIP	F2–F1c	CTCGCTCCAGCAAAGGCAGTAATGCAGGATGTGATGTGTACG
		rmtA-BIP	B1–B2c	GGCGCTACTGCAGGCACTAGTAAACTGCGGGTGGGGAA
		rmtA-LB	LB	TACCCCTCGGATTGCCGT
rmtB	1776–2001 (AB103506)	rmtB-F3	F3	GGCATTGCATCCGTGTGG
		rmtB-B3	B3c	GCGGGGTATTGAGGGATTG
		rmt-FIP	F2–F1c	CACATCCTGCAGGGCAAAGGTAAGGGATTGGGGGATGTCAT
		rmt-BIP	B1–B2c	GGCGACCTGGCGCTGATTTTAAGTGCCATGGCAGAACC
		rmtB-LF	LFc	CCAATCTTTTTCCCTAGCAAAGGG
		rmtB-LB	LB	TTTTGCCCCTGCTGGAGCG
armA	2281–2539 (AB116388)	armA-F3	F3	GGAAATATCAAACATGTCTCATCT
		armA-B3	B3c	GCTGTTTTAGCACAGGAAG
		armA-FIP	F2–F1c	CTCAGCTCTATCAATATCGTATGCATGGCTTCAATCCATTAGCTTTA
		armA-BIP	B1–B2c	AGCATTATTGGGAAGTTAAAGACGAACATCATAAGTACCTTTGTAGAC
		armA-LF	LFc	TTTTTCATTTTCATTCCATTGGT
		armA-LB	LB	AGGTTTTTGAATAAAGAGAGTGA

are time-consuming because bacterial culture in broth media containing some AGs is required after identification of bacterial species. In addition, the presence or absence of 16S rRNA methylase genes cannot be determined by the routine microbiology laboratory testing. Although tests based on the polymerase chain reaction (PCR) have been developed for detection of resistance genes, PCR requires special equipment and is unsuitable for an ordinary hospital laboratory. In contrast to PCR, loop-mediated isothermal amplification (LAMP) is an easy and rapid method for genetic testing [9,10].

Accordingly, we designed LAMP primers to detect three 16S rRNA methylase genes (*rmtA*, *rmtB,* and *armA*) that have been reported to be associated with AG resistance in Japan. Then we employed the LAMP method to investigate the frequency of 16S rRNA methylase gene-positive isolates among the clinical isolates identified in clinical microbiology laboratories around Japan.

2. Materials and methods

2.1. Design of primers and LAMP assay method

For specific detection of three 16S rRNA methylase genes (*rmtA*, *rmtB,* and *armA*), a set of LAMP primes (two outer primers; F3 and B3, two inner primers; FIP and BIP, and two loop primers; LF and LB) was designed for each gene using Primer Explorer Ver.3 software (Eiken Chemical Co., Ltd. Tokyo, Japan). The GenBank accession numbers of *rmtA*, *rmtB*, and *armA* which were used for the design are AB120321, AB103506, and AB116388, respectively.

The LAMP reaction was carried out with the Loopamp DNA amplification kit (Eiken Chemical Co., Ltd.). The reaction was performed in a volume of 25 μl containing 40 pmol each of the FIP and BIP primers, 5 pmol each of the F3 and B3 primers, 20 pmol each of the LF and LB primers, 2× reaction mixture (12.5 μl), *Bst*-DNA polymerase (1 μl), and template DNA (2 μl). For *rmtA*, an LF primer was not used because it could not be designed by the Primer Explorer Ver.3. Extraction of template DNA was conducted by incubating bacterial strains at 95 °C for 10 min and 2 μL of the supernatant was obtained by 10,000 rpm and 1 min of centrifugation. The reaction was performed at 65 °C for 60 min, and the amplified products in the reaction tube were detected by visual inspection using Loopamp fluorescent detection reagent (Eiken Chemical Co., Ltd.).

Primers were synthesized by a contract laboratory (Nihon Gene Research Laboratory Inc., Miyagi, Japan).

3. Evaluation of LAMP assay performance

The detection sensitivity and reaction time were investigated by using serial dilutions of PCR products for *rmtA, rmtB,* and *armA*. For real-time assay performance, a real-time turbidimeter (Loopamp LA-320C, Eiken Chemical Co., Ltd.) was used. The specificity of the LAMP assay was investigated by using nine bacterial species (65 strains) (Table 2) obtained from the National Institute of Infectious Diseases, which were verified to possess 16S rRNA methylase genes (*rmtA*, *rmtB*, and *armA*) by PCR and DNA sequencing.

4. Investigation of clinical isolates

At 33 medical institutions throughout Japan (3 in Hokkaido, 2 in Tohoku, 14 in Kanto, 4 in Chubu, 2 in Kinki, 3 in Chugoku, 4 in Shikoku, and 1 in Kyushu), agreement of the institutional head was obtained to investigate a total of 5998 strains isolated from January through December 2008, consisting of 3056 *Enterobacteriaceae* strains, 2885 *P. aeruginosa* strains, and 57 *Acinetobacter* spp. strains. From among these isolates, 132 strains with resistance to AGs (gentamicin and/or amikacin) were identified, including 52 *Enterobacteriaceae* strains, 77 *P. aeruginosa* strains, and 3 *Acinetobacter* spp. strains. 132 strains of AGs resistance were measured by the LAMP method.

Table 2
Specificity of the LAMP assay for bacterial strains confirmed to possess *rmtA*, *rmtB*, or *armA*.

16S rRNA methylase[a]	Strain	*n*	*rmtA*-LAMP	*rmtB*-LAMP	*armA*-LAMP
rmtA	*P. aeruginosa*	21	21	–	–
rmtB	*C. freundii*	1	–	1	–
	E. coli	7	–	7	–
	K. pneumoniae	5	–	5	–
	S. marcescens	1	–	1	–
armA	*Acinetobacter* sp.	3	–	–	3
	A. baumannii	4	–	–	4
	C. freundii	1	–	–	1
	E. aerogenes	1	–	–	1
	E. cloacae	3	–	–	3
	E. coli	3	–	–	3
	K. pneumoniae	10	–	–	10
	S. marcescens	5	–	–	5

[a] 16S rRNA methylase genes were verified by PCR and DNA sequencing.

In addition, another three institutions (Tohoku University Hospital, Yamagata University Hospital, and Miroku Medical Laboratory Co., Ltd. (Nagano, Japan)) provided a total of 2449 strains that were isolated from August 2013 through May 2014, consisting of 1873 *Enterobacteriaceae* strains, 399 *P. aeruginosa* strains, and 177 *Acinetobacter* spp. strains. Among these isolates, 59 strains with resistance to AGs (gentamicin and/or amikacin) were identified, consisting of 41 *Enterobacteriaceae* strains, 11 *P. aeruginosa* strains, and 7 *Acinetobacter* spp. strains. 59 strains of AGs resistance were measured by the LAMP method.

Evaluation of antimicrobial susceptibility was performed in accordance with CLSI M100 S-18, and resistance was defined as an MIC $\geq$16 μ/mL for gentamicin and an MIC $\geq$64 μ/mL for amikacin [11].

Information about the bacterial strains investigated in this study was limited to bacterial species name of each isolate and the results of antimicrobial susceptibility testing, with no clinical data being collected.

5. Results

5.1. LAMP primers

The sequences of the LAMP primers for the 16S rRNA methylase genes (*rmtA*, *rmtB*, and *armA*) are shown in Table 1. For *rmtA,* there was only a single loop primer (LB).

5.2. Assay performance

When the detection sensitivity of the LAMP assay was investigated, it was found that *rmtA*, *rmtB,* and *armA* could be detected at 10^2 copies/tube (Fig. 1).

The reaction time was 15 min for 1.0×10^7 copies/tube and 32 min for 1.0×10^2 copies/tube in the case of *rmtA*, while the respective times were 23 min and 52 min for *rmtB,* and 27 min and 41 min for *armA*.

The specificity of the assay was investigated using 9 species (65 strains) of bacteria known to possess 16S rRNA methylase genes *(rmtA*, *rmtB*, and *armA*) and the results are shown in Table 2. The LAMP assay for *rmtA* detected all of the strains possessing the *rmtA* gene (1 species, 21 strains), while strains with *rmtB* or *armA* were not detected. Similarly, the assays for *rmtB* and *armA* specifically detected strains possessing *rmtB* (4 species, 14 strains) and strains containing *armA* (8 species, 30 strains), respectively, with no false positive or false negative results.

5.3. Clinical isolates

The clinical isolates from 2008 and 2013–2014 included a total of 191 AG-resistant strains. Among the *Enterobacteriaceae* isolates, 3 strains were positive for *rmtB* (1 strain each of *Escherichia coli*, *Enterobacter cloacae,* and *Citrobacter freundii*). In addition, 3 strains of *P. aeruginosa* were positive for *rmtA* and 2 strains of *Acinetobacter* spp. were positive for *armA*. The 16S rRNA methylase gene detection rate was 0.06% for *Enterobacteriaceae*, 0.10% for *P. aeruginosa,* and 0.85% for *Acinetobacter* spp. (Table 3).

6. Discussion

Concerning 16S rRNA methylase-producing strains, *rmtA* was first discovered from *P. aeruginosa* in Japan as a gene for the enzyme that causes methylation of 16S rRNA at 1405G [1]. Thereafter, *armA*, *rmtB*, *rmtC*, *npmA*, *rmtD*, *rmtD2, rmtE, rmtF, rmtG, rmtH*, etc. have been identified [2–4,12–14]. Wachino and Arakawa has been reported that $bla_{\text{CTX-M-type}}$, $bla_{\text{OXA-type}}$, $bla_{\text{TEM-type}}$, $bla_{\text{SHV-type}}$, $bla_{\text{NDM-type}}$, or $bla_{\text{KPC-type}}$ positive isolate harbors 16S rRNA methylase encoding gene [15]. Such strains have been found among *K. pneumoniae*, *S. marcescens*, *P. aeruginosa*, *C. freundii,* and *Acinetobacter baumannii* [2–4,12–14]. Moreover, *Salmonella enterica* producing ArmA has been reported in Bulgaria, the USA, and the UK, while *Shigella flexneri* producing ArmA was reported in Bulgaria [15].

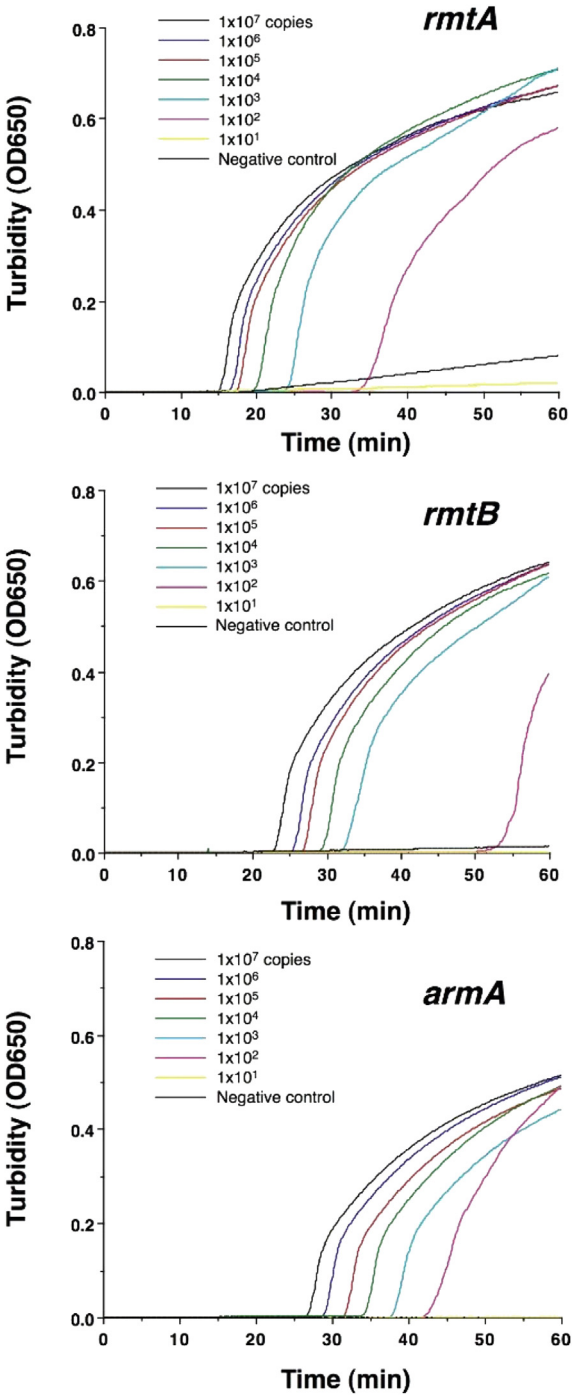

Fig. 1. Detection sensitivity and reaction time of the LAMP assays for *rmtA, rmtB,* and *armA*. Turbidity was monitored by a Loopamp real-time turbidimeter LA-320 at 650 nm.

In the present study, LAMP primers were designed to detect three 16S rRNA methylase genes (*rmtA, rmtB,* and *armA*) that have been reported to be associated with resistance in Japan. To reduce the reaction time, a loop primer (LF, LB) was also used [16].

Table 3
Detection of strains possessing *rmtA*, *rmtB*, or *armA* among 191 aminoglycoside-resistant strains identified from 8447 clinical isolates.

Bacterial species	Isolates	Resistance to AMK or GM		Strains with 16S rRNA methylase genes, *n*			% Of strains producing 16S rRNA methylase
		n	%	*rmtA*	*rmtB*	*armA*	
Enterobacteriaceae	4929	93[a]	1.89	0	3[b]	0	0.06
P. aeruginosa	3284	88	2.68	3	0	0	0.10
Acinetobacter sp.	234	10	4.27	0	0	2	0.85
Total	8447	191	2.26	3	3	2	0.09

AMK, amikacin; GM, gentamicin.
[a] *E. coli* (70 isoletes), *P. mirabilis* (6), *K. pneumonia* (6), *E. cloacae* (5), *P. stuartii* (2), *C. freundii* (1), *K. oxytoca* (1), *S. marsescens* (1), *S. plymuthica* (1).
[b] *E. coli* (1 isolete), *E. cloacae* (1), *P. stuartii* (1).

Assessment of the sensitivity of this assay showed that it could detect the target genes at 10^2 copies/tube in less than 60 min. In the future, detection of 16S rRNA methylase may be able to be directly carried out from clinical samples, such as a blood culture bottle. With regard to specificity, the LAMP assay for *rmtA*, *rmtB* and *armA* detected all strains, with no false positive or false negative results. Accordingly, this LAMP assay is easy to perform, can detect target genes for AG resistance within 60 min, and demonstrates high specificity, suggesting that it may be useful for clinical detection and surveillance of strains with high AG resistance. The LAMP method can be employed as an infection control test in medical institutions where PCR equipment is not available and it seems important to utilize this method as a rapid test for detecting 16S rRNA methylase producing strains.

The only previous surveillance of 16S rRNA methylase-producing strains was conducted by Yamane et al., in 2004 [17]. They investigated 87,626 Gram-negative clinical isolates for 16S rRNA methylase genes and identified *rmtB*, *armA* in 0.02% of *E. coli*, *rmtA* in 0.08% of *P. aeruginosa,* and *armA* in 0.13% of *Acinetobacter* spp. In the present investigation, 16S rRNA methylase genes were detected with *rmtB* being found in 0.06% of *Enterobacteriaceae*, *rmtA* in 0.10% of *P. aeruginosa,* and *armA* in 0.85% of *Acinetobacter* spp. These results were comparable to those reported by Yamane et al. in terms of the bacterial species, genes, and detection rates.

Currently, the prevalence of strains possessing 16S rRNA methylase genes is very low in Japan and there is no trend for a marked increase. However, it has been shown in other countries that the transmissible plasmid carrying the NDM-1 gene also carries the *armA*, *rmtB,* or *rmtC* genes. When Gram-negative bacteria showing high resistance to amikacin or gentamicin are isolated by clinical laboratories, it seems worth considering to perform the LAMP assay method to rapidly identify the 16S rRNA methylase gene-harboring Gram-negative bacilli and monitor their trends among Japanese clinical settings.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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