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Detection of A2143G mutation in 23S rRNA gene associated with clarithromycin resistant *Helicobacter pylori* by Loop mediated isothermal amplification

Thanaporn Champathai[1], Sutep Gonlachanvit[2] and Nuntaree Chaichanawongsaroj[1]*

[1]*Department of Transfusion Medicine and Clinical Microbiology, Faculty of Allied Health Sciences, Chulalongkorn University, Bangkok, Thailand*
[2]*Department of Gastroenterology, Faculty of Medicine, Chulalongkorn University, Thialand*

ABSTRACT

The clarithromycin resistance is the major problem of treatment failure of Helicobacter pylori infection in many countries. The point mutation in 23S rRNA is the main reason and the most common is A-G transitions at position 2143 (A2143G). In the present study, the loop mediated isothermal amplification-restriction fragment length polymorphism (LAMP-RFLP) was developed to detect A2143G mutation in 23S rRNA of H. pylori. The A2143G mutation was examined from 449 *positive urease test samples collected between March 2012 and January 2014 using PCR-RFLP method. The LAMP primers were designed to specifically target 23S rRNA of H. pylori and subsequently digested with BsaI. Mutation at A2143G was detected from 10 of 82 (12.2%) glmM gene PCR positive samples. Ladder pattern of DNA bands could be amplified at 65°C within 60 min in the presence of 0.2 μM each of F3 and B3, 1.6 μM each of FIP and BIP, 0.8 μM each of LF and LB, 1.4 mM of deoxynucleoside triphosphates, 0.8 M betaine and 6 mM $MgSO_4$. The ladder DNA bands of wild type and A2143G mutant strains could be distinguished from different sizes of DNA fragments after restriction enzyme analysis. Thus, our LAMP-RFLP assay is a rapid and simple method for the detection of A2143G mutation in 23S rRNA gene in H. pylori which will be useful for primary antimicrobial resistance screening in countries with a high prevalence of clarithromycin resistance.*

Keywords: *Helicobacter pylori*, clarithromycin, 23S rRNA, Loop mediated isothermal amplification

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INTRODUCTION

The high incidence rate of *Helicobacter pylori* infection, the main etiologic agent of peptic ulcer diseases, gastric cancer and mucosa-associated lymphoid tissue lymphoma (MALT) remains the serious healthcare problem. According to *H. pylori* antibiotic resistance is dramatically increasing worldwide, this contributes to the eradication failure. The overall resistance rates were 17.2% for clarithromycin, 26.7% for metronidazole, 11.2% for amoxicillin, 16.2% for levofloxacin, 5.9% for tetracycline, 1.4% for rifabutin, 9.6% for two or more antibiotics [1]. Several treatment regimens have been used to overcome the antibiotic resistance problem including standard triple therapy, quadruple, and sequential therapy [2]. The triple regimen according to the Maastricht IV consensus report is consisting of a proton pump inhibitor (PPI) or ranitidine bismuth citrate plus clarithromycin and amoxicillin or metronidazole [3]. Although, the cure rate of standard triple therapy is dramatically reduced in recent years in many geographical areas, it is still recommended for first line treatment of *H. pylori* [4]. One major cause of eradication failure resulted from clarithromycin resistance, a key drug in this regimen. The clarithromycin resistance of *H.*

pylori was significantly different between three geographical areas (17.5% in Europe, 18.9% in Asia and 29.3% in America). Among Asian countries, the high resistance rate of 84.9% and 40.7% was detected in China and Japan, respectively [1, 5]. The mechanism of resistance is mainly related to the point mutations in the peptidyltransferase region of domain V of the 23S rRNA which inhibit the binding between clarithromycin and the ribosomal subunit [6]. The mutation sites of 23S rRNA associated with clarithromycin resistance in *H. pylori* were determined at A2142G (5.8%), A2143G (76.5%), T2182C (5.8%), A2143G plus T2182C (8.8%) and A2142G plus T2182C (2.9%) in Spanish patients [7]. The predominant mutation in many countries was A-G transitions at position 2143 in 23S rRNA of *H. pylori* [7-9]. The presence of the A2143G mutation, rather than A2142G or A2142C, significantly reduced the *H. pylori* eradication rate [10]. The routine antimicrobial testing either agar dilution or E-test is depended on bacterial culture, which is laborious and time consuming. Several molecular methods have been developed mostly based on polymerase chain reaction principle such as PCR-restriction fragment length polymorphism (PCR-RFLP) [7], real-time PCR [11] and dual-priming oligonucleotide (DPO)-based multiplex PCR [12]. The disadvantage of PCR-based technology is the requirement of thermal cycler, which normally does not use in small clinics, urban hospitals, resource-limited laboratories and rapid point of care testing. The development of loop mediated isothermal amplification (LAMP) has been widely applied for infectious diseases detection in the past 10 years because of its rapidity, sensitivity, specificity, and simplicity. Moreover, the amplification could be conducted in general equipment such as heat block or water bath. The LAMP reaction employs a set of 4-6 primers specifically binding to 8 distinct regions of target genes and amplifies under isothermal condition within 1 hour. In addition, the initial denaturation step can be omitted and no loss of time for temperature shifts as in conventional PCR. The LAMP products could easily be visualized by turbidity, SYBR Green I colour, or investigated by agarose gel electrophoresis and real-time turbidimeter [13, 14]. Although, LAMP method for *H. pylori* detection has already been established [15], determination of genotypic drug resistances is still not developed. In this study, LAMP based assay with restriction enzyme analysis was developed for determination of A2143G mutations in 23S rRNA associated with clarithromycin resistance of *H. pylori* which will be useful for monitoring the treatment regimens and increasing the eradication rate.

EXPERIMENTAL SECTION

H. pylori strain and Clinical samples

In this study, 449 samples of positive urease test samples (267 *Campylobacter*-like organism tests (CLO tests) and 182 in house urease tests) were collected from the Department of Gastroenterology Chulalongkorn Hospital during March 2012 and lasted till January 2014. *H. pylori* ATCC 43504 was grown on brain heart infusion agar containing 7% (v/v) sheep blood and were incubated at 37°C for 3-5 days under microaerophilic conditions using gas generating kit.

DNA Extraction

The agar from each positive urease test samples was picked up in sterile 1.5 ml tube and was homogenized with sterile pestle. Three hundred microliters of sterile distilled water was added and boiled for 10 min. Then, the sample was centrifuged at 4,000 rpm for 1 min and the supernatant was collected in a new tube. The genomic DNA was precipitated by adding double volume of absolute ethanol and kept at -80°C for 10 min. The sample was centrifuged at 12,000 rpm for 10 min, washed with 70% ethanol and air-dried. The extracted DNA was suspended in 50 µl of TE buffer. For *H. pylori* ATCC 43504, the genomic DNA was extracted from a fresh culture using Wizard genomic DNA purification kit according to manufacturer's instructions. All DNA samples were quantified using a NanoDrop spectrophotometer and stored at -20°C for further analysis.

PCR assay for *glm*M gene amplification

The extracted DNA from positive urease test samples was subjected to PCR amplification of *glm*M gene for confirmation of *H. pylori* identification. PCR reactions were carried out in a total volume of 50 µl containing 5 µl of 10X buffer, 0.2 mM dNTP, 1.25 unit Taq DNA polymerase, 10 ng of template DNA and 0.5 µM of each primer (forward primer 5'AAGCTTTTAGGGGTGTTAGGGGTTT-3', backward primer: 5'-AAGCTTACTTTCTAACACTAACGC-3'). PCR was performed using a thermal cycler under the following conditions: an initial denaturation for 5 min at 93°C; and 35 cycles for 1 min at 93°C, 1 min at 55°C, 1 min at 72°C, and a final extension at 72°C for 10 min. The PCR products were electrophoresed through a 1.5% agarose gel using TBE buffer (45 mM Tris-borate, 1 mM EDTA pH 8.0) at 100 V. The gel was stained with 0.01% ethidium bromide (10 mg/ml). A 100 bp DNA ladder was used to detect the molecular weight of observed bands under a UV lamp and

photo was taken using G: BOX Chemi SD. The expected size of PCR product was 294 bp. The genomic DNA of *H. pylori* ATCC 43504 was used as positive DNA template and distilled water was used as negative control.

Determination of point mutations in 23S rRNA

Presence of an A2143G point mutation in 23S rRNA was analyzed by PCR-RFLP method. The sequences of specific oligonucleotide primers derived from the 23S rRNA gene of reference strain of *H. pylori* ATCC 26695 were used including 5'CCACAGCGATGTGGTCTCAG3' (sense, positions 1891 to 1911) and 5'CTCCATAAGAGCCAAAGCCC3' (antisense, positions 2200 to 2220) [16]. PCR reactions were performed in a total volume of 50 µl containing 10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$ pH 8.3, 0.2 mM dNTP, 1.25 unit of Taq DNA polymerase, 10 ng of template DNA and 0.5 µM of each primer. Amplification was carried out in a thermal cycler with the following conditions: pre-denature at 95°C, 5 min followed by 35 cycles of 95°C for 1 min, 54°C for 1 min, 72°C for 1 min and a final extension at 72°C for 5 min. A PCR product of 425 bp was digested with *Bsa*I which allow discrimination between the wild type and the A2143G mutant. Five microliters of the PCR products was digested in a total volume of 20 µl containing 1XCutSmart™ buffer and 0.5 µl of *Bsa*I, incubated at 37°C for 24 h. The restriction products were analyzed by electrophoresis on 1.5% agarose gel. For A2143G mutant, two fragments of 304 bp and 101 bp were visualized while one fragment of 425 bp was seen for wild type.

LAMP primers design

Six LAMP primers, two outer primers (F3 and B3) four inner primers (FIP and BIP, LF and LB) were designed from the published sequence of 23S rRNA of *H. pylori* (GenBank accession number U27270) at position 2281 to 2640 by using Primer Explore V4 software program (http://Eiken Chemical Co. Ltd., Tokyo, Japan). The specificity of each designed primer was initially confirmed by BLAST analysis on the National Center for Biotechnology Information server (http://www.ncbi.nlm.nih.gov/). All primers were synthesized from Biodesign Company, Thailand.

LAMP reaction

The LAMP reaction was carried out in a 25 µl reaction mixture containing 0.2 µM each of F3 and B3, 1.6 µM each of FIP and BIP, 0.8 µM each of LF and LB, 1.4 mM of each deoxynucleoside triphosphates, 0.8 M betaine, 20 mM Tris-HCl, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 0.1% triton X-100, 6 mM $MgSO_4$, 8 units of *Bst* DNA polymerase large fragment and 10-100 ng of genomic DNA. The reaction was carried out at 65°C for 1 h and heated at 80°C for 2 min to terminate. The LAMP products were analyzed on 1.5% agarose gel. The expected ladder like pattern from 195 bp was observed.

Restriction enzyme analysis of LAMP products

A LAMP product was digested with *Bsa*I which allow discrimination between the wild type and the A2143G mutant. Five microliters of the LAMP products was digested in a total volume of 20 µl containing 1XCutSmart™ buffer and 0.5 µl of *Bsa*I followed by incubated at 37°C for 2 h. The restriction products were analyzed by electrophoresis on 1.5% agarose gel. LAMP products of wild type and A2143G mutant were confirmed for 23S rRNA sequence by subjecting to *Ava*II digestion and combination of *Bsa*I + *Ava*II. The restriction reactions of *Ava*II or *Bsa*I +*Ava*II were set as same as *Bsa*I.

RESULTS AND DISCUSSION

Prevalence of A2143G point mutation

Due to the standard testing of clarithromycin resistance of *H. pylori* is based on phenotypic detection by agar dilution or E-test. The disadvantages of the conventional testing are need of gastric biopsy for culture of *H. pylori*, which is invasive for patients and the bacterium is too fastidious to grow. Although, the prevalence of clarithromycin resistance in *H. pylori* trended to increase worldwide, standard triple therapy is recommended for countries with less than 20% resistance [17]. In this study, we developed a direct detection of clarithromycin resistance from samples of urease testing, which is rapid routine screening of *H. pylori* infection. Our detection of clarithromycin resistance is mainly focused on the A2143G point mutation in the peptidyl transferase of the 23S rRNA gene, which has been found to be significantly linked to eradication rate rather than other mutations [10]. All positive urease test samples were confirmed for *H. pylori* by PCR of *glm*M gene. Of all 449 positive urease test samples, 82 could be amplified for *H. pylori glm*M gene and 23S rRNA. The failure of the rest might result from gastric biopsies in urease test samples containing a small amount of bacterial DNA. Moreover, the samples also had

interference of human DNA and inhibitors from urease testing. The sensitivity and specificity of *H. pylori* detection depended on the methods as the following: culture (55.9%, 100%), Gram stain (89.3%, 93.5%), histology (93.5%, 90.4%), serology (96.8%, 96.8%), CLO test (99.0%, 91.9%), in-house urease test (100%, 88.9%) [18]. The sensitivity and specificity for *H. pylori* PCR detection also varied among target genes, *glm*M gene is the most superior with 100% sensitivity and 96% specificity [19].

PCR-RFLP analysis of 23S rRNA as shown in Fig 1 found 12.2% (10 strains) of A2143G point mutation. The report of prevalence of clarithromycin resistance in Thailand in 2012 was 13.8% with 36.4% of A2142G mutation, 18.2% of multiple mutations at G2111A, A2115G and A2144G [20]. In Jeju Island, Korea, A2142G and A2143G mutations were 7.9% and 18.1%, respectively, and higher failure of eradication rate (87.5%) of A2143G mutation in *H. pylori* was observed [21]. The sensitivity and specificity for detection of clarithromycin resistance in *H. pylori* was 89% and 100% by PCR-RFLP, and 90.62% and 95.83% by real-time PCR assay, respectively [22, 23]. High-level of clarithromycin resistance in *H. pylori* is correlated to the presence of A2143G mutation with different MIC values widely ranging from 0.016 to 256 mg/L [24]. Recent study found that the rate of clarithromycin phenotypic resistance was significantly lower than that of genotypic resistance (18.4% *vs* 37.6%, $P < 0.001$) and the lowest eradication rate was genetically linked to the A2143G point mutation [25].

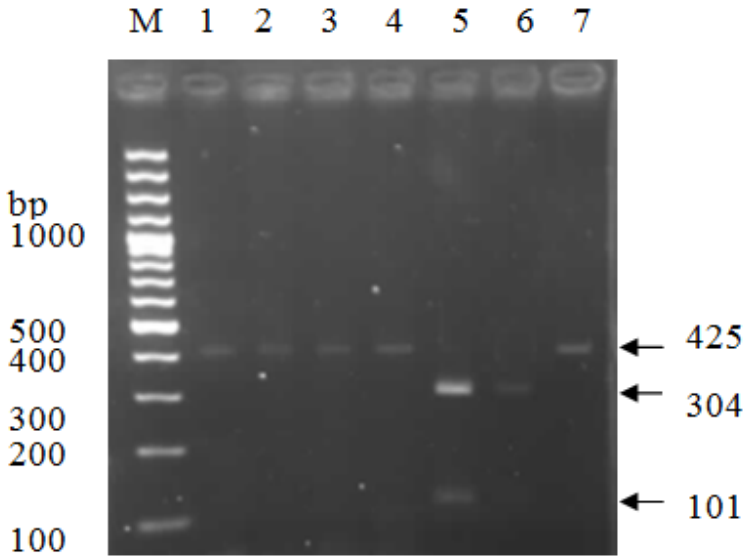

Fig 1. PCR-RFLP analysis of 23S rRNA gene of *H. pylori*. M: 50 bp DNA ladder; 1: sample No 24; 2: sample no.23B; 3: sample no.16B; 4: sample no.15B; 5: sample no.12; 6: sample no.9; 7: sample no.14

Table1. Sequence of LAMP primers used for specific amplification of 23S rRNA in this study

primer	Sequence	Length
F3	5'-ACCGACCTG CATGAATGG-3'	18-mer
B3	5'-AGCCAAAGCCCTTACTTCAA-3'	20-mer
LF	5'-CCTCCACTACAATTTCACTGAATCT-3'	25-mer
LB	5'-ACTACAACTTAGCACTGCTAATGGG-3'	25-mer
FIP	5'-GCCGCGGGTAGGAGGAATTTTC-GTAACGAGATGGGAGCTGTC-3'	42-mer (F1C: 22-mer, F2: 20-mer)
BIP_WT	5'-CGGAAAGACCCCGTGGACCT-AGCCTCCCACCTATCCTG-3'	38-mer (B1C: 20-mer, B2: 18-mer)
BIP_MT	5'-GAGACCCCGTGGACCTTT-AGCCTCCCACCTATCCTG-3'	36-mer (B1C: 18-mer, B2: 18-mer)
BIP_MT-2	5'-ACCCCGTGGACCTTTAC-AGCCTCCCACCTATCCTG-3'	35-mer (B1C: 17-mer, B2: 18-mer)
LB+2	5'-ACAACTTAGCACTGCTAATGGGAAT-3'	25-mer

Design LAMP primers of 23S rRNA and condition optimization

The LAMP primers were designed based on the criteria of standard primer design in PrimerExplorer V4 program to amplify 23S rRNA in *H. pylori*. The common primer set of F3, B3, LF, LB, FIP and BIP_WT (Table 1) were generated and tested for LAMP reaction as shown in Table 1 and Fig 2. The optimum condition for the LAMP reaction was as following: 0.2 μM each of F3 and B3, 1.6□ μM each of FIP and BIP and 0.8□ μM each of LF and

LB. The reaction was carried out at 65°C for 1 h and heated at 80°C for 2 min to terminate. The specific amplification products were observed from both wild type and A2143G mutant DNA with the ladder-like pattern of DNA bands, sizes ranging from 195 bp up to the loading wells. No amplification was observed in distilled water.

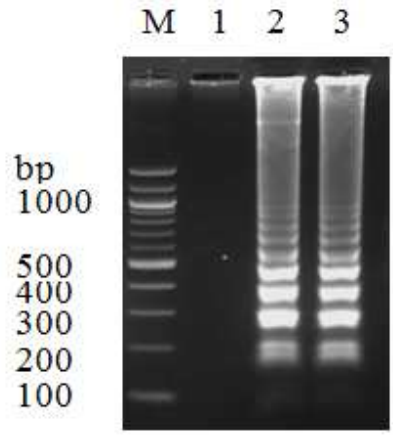

Fig 2. LAMP products of common primers with BIP_WT. M: 100 bp DNA ladder; 1: distilled water; 2: *H. pylori* ATCC 43504; 3: A2143G mutant

Due to, the first set of common primers could not exclude mutation site from the primer region, BIP primer was redesigned with G mutation at the 5' end of B1C (BIP_MT) using advance primer design in PrimerExplorer V4 program. If the primers include a mutation in this location, the mutant type is generally amplified while the wild type amplification is hindered. Various LAMP conditions were tested to determine optimal incubation time and concentration of dNTPs which could differentiate A2143G 23S rRNA mutant and wild type. Various dNTP concentrations ranging from 0.2, 0.6, 1, 1.4 mM and incubation times of 35, 45 and 55 min failed to distinguish wild type and mutant as shown in Fig 3A and 3B, respectively. The optimum primers and betaine concentrations were also tested and similar results were observed (data not shown). In order to exclude mutation from the primer region, the new BIP primer (BIP_MT-2) was designed by deletion of 3 nucleotide at the 5' end of BIP_MT and new LB primer (LB+2) was replaced. The successful LAMP amplification was achieved from both wild type and A2143G mutant as shown in Fig 4.

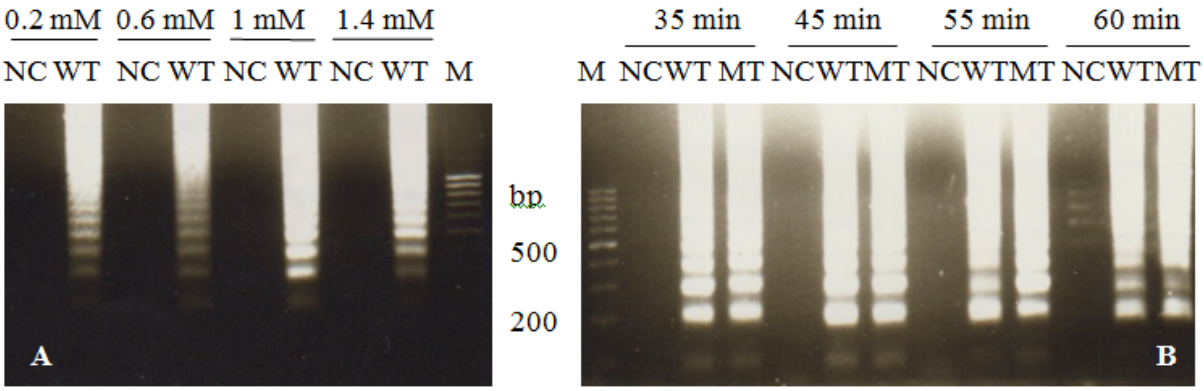

Fig 3. LAMP products of common primers with BIP_MT at various dNTP concentrations (A) and times (B). M: 100 bp DNA ladder; NC: distilled water; WT: *H. pylori* ATCC 43504; MT: A2143G mutant

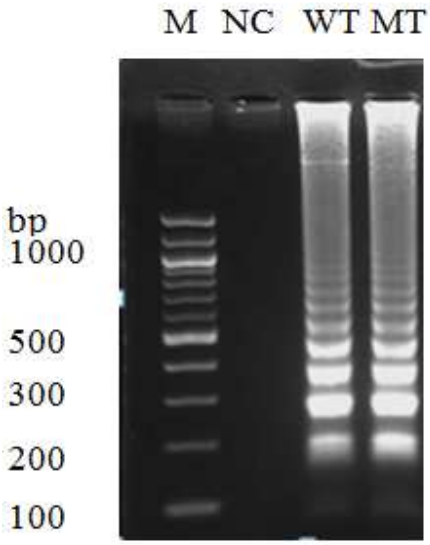

Fig 4. LAMP products of common primers with BIP_MT-2. M: 100 bp DNA ladder; NC: distilled water; WT: *H. pylori* ATCC 43504; MT: A2143G mutant

LAMP-RFLP of wild type *H. pylori* and A2143G mutant strains

LAMP-RFLP, similar to PCR-RFLP, is based on the presence or absence of the restriction enzyme recognition sequences within the amplified DNA fragment. Thus, the *Bsa*I enzyme was used to digest the LAMP products amplified from BIP_MT-2 primer set in order to detect the presence of A2143G point mutation in 23S rRNA associated with clarithromycin resistance of *H. pylori* strains. The *Bsa*I enzyme was able to digest only LAMP product of A2143G mutant, while the ladder-like pattern still be seen in wild type (Fig 5). The *Ava*II enzyme locating in the B1 region was chosen to confirm the 23S rRNA of *H. pylori* and similar sizes of DNA fragments could be seen from both wild type and A2143G mutant as shown in Fig. 6 (lane 2 and 5). While combination of *Ava*II and *Bsa*I resulted in different DNA patterns between wild type and A2143G mutant as shown in Fig 6 (lane 3 and 6).

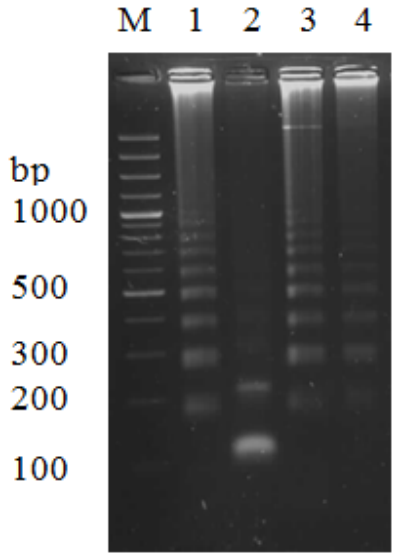

Fig 5. LAMP-RFLP analysis using *Bsa*I restriction enzyme. M: 100 bp DNA ladder; 1: A2143G mutant; 2: A2143G mutant digested with *Bsa*I; 3: *H. pylori* ATCC 43504; 4: *H. pylori* ATCC 43504 digested with *Bsa*I

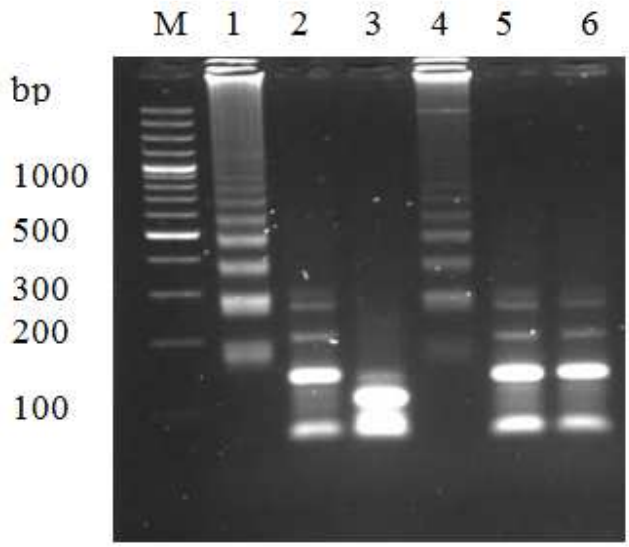

Fig 6. LAMP-RFLP analysis using *Ava*II and *Bsa*I+*Ava*II. M: 100 bp DNA ladder; 1: A2143G mutant; 2: A2143G mutant digested with *Ava*II; 3: A2143G mutant digested with *Bsa*I+*Ava*II; 4: *H. pylori* ATCC 43504; 5: *H. pylori* ATCC 43504 digested with *Ava*II; 6: *H. pylori* ATCC 43504 digested with *Bsa*I+*Ava*II

The lowest detection limit of *H. pylori* was 10^2 CFU by LAMP assay in combination with endoscopic brushing technique [15]. The multiplex LAMP-RFLP was developed for simultaneous detection of *Salmonella* strains and *Shigella* strains in foods [26]. A rapid genotyping of human herpesvirus 6 (HHV-6) was distinguished between HHV-6 A and B using LAMP method and *Acc*I digestion [27]. For our LAMP-RFLP can be used for rapidly screening of A2143G mutation in 23S rRNA gene associated with clarithromycin resistant *H. pylori* which would be helpful for effective treatment. Moreover, the assay could be tested directly from positive urease test samples, which are routinely used for screening of *H. pylori* infection. Thus, culture of *H. pylori* could be omitted, which save times and budgets for the conventional assay.

CONCLUSION

In this study, A2143G point mutation in 23S rRNA of *H. pylori* could be rapidly detected from positive urease test samples by LAMP-RFLP. Screening of 23S rRNA mutations associated with clarithromycin resistance before starting standard triple regimen would be helpful for appropriate antibiotics treatment of *H. pylori*.

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