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Improved allele-specific PCR assays for detection of clarithromycin and fluoroquinolone resistant of *Helicobacter pylori* in gastric biopsies: identification of N87I mutation in GyrA

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Abstract

Molecular testing can rapidly detect *Helicobacter pylori* susceptibility using gastric biopsies. Allele-specific polymerase chain reaction (ASP-PCR) was used to identify *H. pylori* 23S rRNA and *gyrA* mutation using gastric biopsies from Colombian patients and confirmed by PCR and sequencing of the 23S rRNA and *gyrA* genes. The sensitivity and specificity of ASP-PCR were compared with susceptibilities measured by agar dilution. Samples included gastric biopsies from 107 biopsies with *H. pylori* infections and 20 *H. pylori* negative. The sensitivity and specificity of ASP-PCR for the 23S rRNA gene were both 100%. The sensitivity and specificity of ASP-PCR for the *gyrA* gene, published in 2007 by Nishizawa et al., were 52% and 92.7%, respectively; the lower sensitivity was due to the presence of mutation N87I in our samples, which were not detected by the test. In this study, we designed new primers to detect the mutation N87I in GyrA. The ASP-PCR was performed with the original primers plus the new primers. The molecular test with the new primers improved the sensitivity to 100%. In conclusion, ASP-PCR provides a specific and rapid means of predicting resistance to clarithromycin and levofloxacin in gastric biopsies.

Keywords

SP-PCR; Mutations, *gyrA*; 23S rRNA; Sequencing; Agar dilution

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1. Introduction

Helicobacter pylori is an important human pathogen that causes gastroduodenal inflammation and is etiologically associated with duodenal ulcer disease, gastric ulcer disease, gastric adenocarcinoma, and primary B-cell gastric lymphoma (Chisholm et al., 2001; Graham, 2009; Heo and Jeon, 2014). The National Institutes of Health in the United States, the Maastricht Consensus in Europe, and the Canadian Consensus all recommend *H. pylori* eradication for the treatment or prevention of these disorders and reduction of the occurrence of new gastric cancers after endoscopic resection (Fontana et al., 2002; Malfertheiner et al., 2012). The most common method for the eradication of *H. pylori* infections consists of the administration of a proton pump inhibitor and several antimicrobial agents such as amoxicillin, clarithromycin, metronidazole, fluoroquinolone, or tetracycline (Furuta and Graham, 2010). Antimicrobial resistance is now the most important factor determining the outcome of *H. pylori* eradication therapy (Fontana et al., 2002). Resistances to clarithromycin and fluoroquinolones are particularly important, as they cannot be overcome by increasing the dose or duration of therapy (Agudo et al., 2010; Furuta and Graham, 2010; Sugimoto et al., 2014). In both, phenotypic resistance is correlated with clinical and microbiological failure. Mutations leading to resistance have been described for macrolides and fluoroquinolones (Cambau et al., 2009; Papastergiou et al., 2014; Wang et al., 2001). Resistance to clarithromycin results from structural changes in the 23S rRNA molecule caused by mutation of the 23S rRNA gene (Papastergiou et al., 2014; Wang et al., 2001). Most common mutations are A-G transitions at position 2143 (A2143G) and 2142 (A2142G) (Ahmad et al., 2009; Cambau et al., 2009; Garrido and Toledo, 2007; Ho et al., 2010; Kim et al., 2002); these mutations have been confirmed to confer resistance by mutagenesis studies (Taylor et al., 1997; Marais et al., 1999). Other mutations observed in low-level clarithromycin-resistant *H. pylori* isolates include A2142C, A2144G (De Francesco et al., 2014; Xiong et al., 2013), and C2694A (Rimbara et al., 2008).

Fluoroquinolone resistance is caused by point mutations in the quinolone resistance-determining region (QRDR) of the *gyrA* gene, which encodes subunit A of DNA gyrase (Miyachi et al., 2006). The amino acid substitutions observed in clinical strains have been primarily reported at position 87 (Asn to Lys) or 91 (Asp to Gly, Asp to Asn, or Asp to Tyr) (Alfizah et al., 2014; Cattoir et al., 2007; Hung et al., 2009; Nishizawa et al., 2007), although resistant strains lacking these mutations have also been described (Wang et al., 1999, 2010).

In routine clinical practice, the detection of clarithromycin and levofloxacin resistance is based on phenotypic methods such as E-test or agar dilution; however, these methods are time consuming, as they require up to 2 weeks for completion. Molecular methods to detect the point mutations conferring resistance have the potential advantage of providing rapid results. A simple method for detection of antibiotic susceptibility using polymerase chain reaction (PCR) would promote the use of “tailored treatment” in the era of increasing prevalence of antimicrobial resistance (Heo and Jeon (2014). Allele-specific PCR (ASP-PCR) is especially useful to determine single nucleotide polymorphism in DNA samples, and this technique allows the identification of mutations without direct sequencing or digestion with restriction enzymes (Furuta et al., 2007; Nakamura et al., 2007; Nishizawa et

al., 2007). This study used ASP-PCR to identify mutations predictive of clarithromycin and fluoroquinolone resistance in DNA from gastric mucosal biopsy samples from Colombian patients infected with *H. pylori*. The concordance, sensitivity, and specificity for the ASP-PCR assay were determined by comparing the results of agar dilution (phenotypic test) for each clinical isolate of *H. pylori* and the molecular detection of the mutant in respective biopsy.

2. Materials and methods

2.1. Clinical samples

Gastric biopsies from 127 patients referred for gastroscopy at the Gastroenterology Unit of Clínica Fundadores, Bogotá, Colombia, were entered, including 107 patients with active *H. pylori* infections and 20 *H. pylori*-negative patients. Written informed consent for participation was obtained from each of the patients before entry into the study. The protocol was approved by ethical committee of Javeriana University and Clínica Fundadores. These patients were part of a clinical trial to assess the efficacy of a triple therapy containing levofloxacin. The status of the infection was confirmed by rapid urease test and histopathology (Giemsa stain). Two antral biopsies specimens were obtained of each patient, and 1 of the tissue specimens was cultured for *H. pylori* and the other was used for DNA extraction.

2.2. Bacterial strains, culture conditions, and determination of susceptibility to clarithromycin and levofloxacin

Biopsies samples were crushed in 0.5 mL of Phosphate buffered saline (PBS) and cultured in Wilkins Chalgren Agar (Becton Dickinson, Heidelberg, Germany) containing 7% horse blood, vancomycin (10 mg/L), and trimethoprim (5 mg/L). The plates were incubated at 37 °C under microaerophilic conditions for up to 14 days, and isolates were identified as *H. pylori* by Gram stain, urease, catalase, and oxidase reactions (Kist, 1991).

Susceptibility to clarithromycin was assessed using dilution method according to the CLSI breakpoints: S 0.25 µg/mL; I 0.5 µg/mL; R 1.0 µg/mL (CLSI, 2010). Breakpoint for levofloxacin was determined as R 1.0 µg/mL agrees with EUCAST clinical breakpoints for *H. pylori* (EUCAST, 2011; Hung et al., 2009).

2.3. DNA sequencing of 23S rRNA gene and gyrA gene of *H. pylori* isolates

Total genome DNA was extracted from *H. pylori* isolates using DNAzol® kits (Invitrogen, Carlsbad, CA, USA). The DNA samples were stored at -20 °C until use. Primers 23S rRNA F (5'-CCACAGCGATGTGGTCTCAG-3') corresponding to position 2191 to 2210 and 23S rRNA R (5'-CTCCATAAGAGCCAAAGCCC-3') corresponding to position 2596 to 2615 were used to amplify a fragment of 425 bp of the peptidyltransferase region of the 23S rRNA in *H. pylori* (GenBank accession number [U27270](#)) and described by Kim et al. (2002). PCR amplification of DNA was performed using a MyCycler thermal cycler (Biorad, Foster City, California, USA) in a final volume of 50 µL containing 1 µg of *H. pylori* genomic DNA, 1 pmol/L concentration of primers, and 42 µL of Super Mix

(Invitrogen). The cycling program was 1 cycle at 95 °C for 5 min; 35 cycles of 95 °C for 30 s, 54 °C for 30 s, 72 °C for 30 s, and a final extension step at 72 °C for 10 min.

Primers corresponding to regions flanking the 428-bp coding sequence of the QRDR of *gyrA* (codons 38–154) were used for the amplification of the *gyrA* gene. The *gyrA* primer sequences were *gyrA* F (5'-TTTRGCTTATTCMATGAGCGT-3') and *gyrA* R (5'-GCAGACGGCTTGGTARAATA-3'). The PCR mixture (50-μL final volume) contained 1 μg of *H. pylori* genomic DNA, Super Mix (Invitrogen), and 0.5 μmol/L (each) primer. PCR was performed under the following conditions: initial denaturation at 94 °C for 4 min, followed by 26 cycles of denaturation at 94 °C for 60 s, annealing at 56 °C for 60 s, and extension at 72 °C for 30 s, with a final extension at 72 °C for 5 min.

PCR products (23S rRNA and *gyrA*) were purified by Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, Wisconsin, USA) and sequenced by MacroGen (Korea, Seoul, Republic of Korea).

2.4. DNA preparation and PCR analysis for gastric biopsy specimens

DNA was isolated from the gastric tissue specimens using QIAmp DNA mini kits (Qiagen, Hilden, Germany). DNA preparations were subjected to PCR for *ureA* and *vacA* genes to confirm the presence of *H. pylori* DNA. PCR for the *ureA* gene was performed using primers: *ureA*-F (5'-AACCGGATGATGTGATGGAT-3') and *ureA*-R (5'-GGTCTGTCGCCAACATTTT-3') reported by Kim et al. (2002). The amplification was conducted using GoTag polymerase (WI, USA) under the following conditions: initial denaturation at 94 °C for 2 min, followed by 40 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 20 s, and extension at 72 °C for 30 s, with final extension 72 °C for 5 min. PCR for the *vacA* gene was performed using GoTag polymerase (WI, USA) and the primers *vacA*-F (5'-TACAACAAACACACCGCAAAA-3') and *vacA*-R (5'-TGTAGCGATACCCCAACAA-3') reported in 2004 by Ayala et al. The PCR condition for the *vacA* gene amplifications was initial denaturation at 94 °C for 2 min, followed by 40 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 20 s, and extension at 72 °C for 30 s, with final extension 72 °C for 5 min.

2.5. ASP-PCR to determine 23S rRNA mutation of *H. pylori* from DNA extracted from gastric biopsies

ASP-PCR for 23S rRNA gene was performed with 4 primers described previously by Furuta et al. (2007). The primer sequences were FP-1 (5'-TCGAAGGTTAAGAGGATGCGTCAGTC-3'), FP2143G (5'-CCGCGGCAAGACAGAGA-3'), RP-1 (5'-GACTCCATAAGAGCCAAAGCCCTTAC-3'), and RP2142G (5'-AGTAAAGGTCCACGGGGTATTCC-3'). ASP-PCR was performed with KOD Xtreme™ Hot Start DNA Polymerase (Toyobo, Osaka, Japan) and obtained a band of 320 bp for both wild type (wt) and mutant, 238 bp for A2142G mutation, and 118 bp for A2143G mutation.

The mix for KOD Xtreme™ Hot Start DNA Polymerase was performed using 2.4 μL of distilled water, 10 μL of 2X reaction Xtreme buffer, 4 μL of dNTPs (200 μmol/L each), 0.3 μmol/L each primer, 0.4 μL KOD Xtreme Hot Start DNA polymerase, and 2 μL of DNA

template. The amplification was conducted under the following conditions: 1 cycle at 94 °C for 2 min; 40 cycles of 98 °C for 10 s, 65 °C for 30 s, 68 °C for 20 s, with a final extension at 72 °C for 2 min.

2.6. ASP-PCR for to determine *gyrA* mutation of *H. pylori* from DNA extracted from gastric biopsies

ASP-PCR for the *gyrA* gene was performed with the primers described previously by Nishizawa et al. (2007) (Table 1). Three new primers were designed to determine mutations at positions A260T, T261C, related with N87I mutation in the GyrA protein. The design of the primers was based on the *gyrA* sequence of *H. pylori* 26695 (Table 1).

ASP-PCR amplification of the *gyrA* gene using the new primers set was performed in a final volume of 20 µL containing 0.8 µ of distilled water, 10 µL of 2X reaction Xtreme buffer, 2 µL of dNTPs (200 µmol/L each), 5.8 µL of primer mixture (8 pmol of *gyrA* primer R, 2 pmol of primer F261A1, 2 pmol of primer F261G1, 8 pmol of primer F271A5, 10 pmol of primer F271A9, 6 pmol of primer F271T9, 6 pmol of primer F272G1, 6 pmol of primer F272G9, 6 pmol of primer F87ATC, 2 pmol of primer F87ATT, and 2 pmol of primer F87ATA), 0.4 µL KOD Xtreme Hot Start DNA polymerase (Toyobo), and 1 µL of DNA template. The amplification was conducted under the following conditions: 1 cycle at 94 °C for 2 min; 40 cycles of 98 °C for 10 s, 65 °C for 30 s, 68 °C for 20 s, with a final extension at 68 °C for 2 min. ASP-PCR amplification with original primers was performed by the method described by Nishizawa et al. (2007). Samples were defined as positive for mutation of *gyrA* gene when 254-bp band was detected.

2.7. Evaluation of sensitivity and specificity

The proportion of positives by ASP-PCR among the true positives by agar dilution defined the sensitivity, and the proportion of negatives by ASP-PCR among the true negatives by agar dilution defined the specificity. Agar dilution was the reference method. The proportion of samples with positive results by ASP-PCR who were correctly diagnosed defined the positive predictive value (PPV). Negative predictive value (NPV) indicates the proportion of samples with negative results by ASP-PCR who are correctly diagnosed. 95% confidence intervals (CIs) that were calculated for sensitivity, specificity, PPV, and NPV agree with the publication of Riddle and Stratford (1999).

3. Results

3.1. Susceptibilities to clarithromycin and levofloxacin

The MICs for 107 strains were determined by the agar dilution method ; 42 isolates were resistant to clarithromycin. The range of susceptible strain MICs was 0.016–0.25 µg/mL, and the clarithromycin-resistant isolate MICs ranged between 1 and 16 µg/mL. The isolates with the mutation A2142G showed high resistance to clarithromycin (MIC 16 µg/mL), while the mutant A2143G MIC ranged from 1 to 8 µg/mL.

MICs for levofloxacin-resistant strains had MICs from 1 to 32 µg/mL. The MICs of the resistant strains with N87I mutation ranged from 1 to 32 µg/mL; MICs among N87K

resistance strains ranged from 2 to 16 µg/mL; for D91G mutations, the MICs ranged between 2 and 32 µg/mL; the strain with double mutation (N87Y and D91G) had an MIC of 32 µg/mL; and for D91N mutation, the MICs ranged between 1 and 2 µg/mL.

The MIC distributions of susceptible strains to levofloxacin were 0.032–0.5 µg/mL. Four susceptible strains with MICs of 0.5 µg/mL to levofloxacin included 2 strains with D91G mutations and 2 with N87I mutations. Fifty-five isolates were levofloxacin susceptible, and 52 were resistant to levofloxacin by the agar dilution method.

3.2. DNA sequencing analysis of DNA from *H. pylori* strains

The sequences for the peptidyltransferase region of the 23S rRNA and QRDRs of *gyrA* were analyzed for susceptible and resistant *H. pylori* strains. Analysis of the 23S rRNA sequence detected 38 strains with A2143G mutation and 4 strains with the A2142G mutation; the sequences of the remaining 65 strains were wild type. Analysis in the QRDR of the *gyrA* gene showed 51 strains with wild-type sequence and 56 strains with mutations, N87I (25/56), N87K (7/56), D91G (20/56), D91N (3/56), and a double mutation N87Y, D91G (1/56).

3.3. PCR for the *ureA* gene and *vacA* gene of *H. pylori* in biopsies samples

Both the *ureA* gene and *vacA* gene were detected in 107 biopsies in which culture was positive; the remaining 20 biopsies were negative by both PCRs.

3.4. ASP-PCR for mutations in 23S rRNA gene of *H. pylori* in biopsies samples

In the 107 *H. pylori*-positive biopsies, ASP-PCR classified 65 biopsies as wild type and 42 as resistant, which included 4 with the A2142G mutation and 38 with the A2143G mutation. ASP-PCR for mutations in 23S rRNA was performed using mixtures in various proportions of DNA from the wild-type and A2143G or A2142G mutant samples. ASP-PCR was able to detect the mutant or wild-type DNA at concentrations as low as 2 ng/µL (data not shown).

3.5. ASP-PCR for mutations in *gyrA* gene of *H. pylori* in biopsies samples

Two different sets of primers were used to identify mutations in the *gyrA* gene. The original set of primers described by Nishizawa et al. (2007) classified 76 of DNA biopsy samples as wild-type and 31 as resistant genotypes. The distribution of genotypes detected by ASP-PCR with a new primer plus the original primers was 51 wild type and 56 resistant. The ASP-PCR with the new primer detected 25 samples with *gyrA* mutations at codon 87 (N87I) (Table 2). No amplification was observed in 20 DNA biopsy samples of patients whose status was negative for *H. pylori*. Mixtures in various proportions of DNA strain samples from the wild-type and N87I, D91G, and D91N mutant samples were performed, and the ASP-PCR was able to detect the mutant DNA concentration as low as 5 ng/µL (data not shown).

3.6. Sensitivity and specificity of ASP-PCR for mutations in 23S rRNA gene of *H. pylori*

There was complete agreement between the different techniques; additionally, the molecular test correctly classified all those negative for *H. pylori* infection (i.e., they showed no amplification in any sample). Using agar dilution as the “gold standard”, both of the

sensitivity and specificity of ASP-PCR of 23S rRNA gene were 100% (95% CI 89–100 and 95% CI 93–100, respectively). The same result was obtained when ASP-PCR was compared with the DNA sequencing analysis.

3.7. Sensitivity and specificity of ASP-PCR for mutations in *gyrA* gene of *H. pylori*

ASP-PCR *gyrA* using the original primers and the new primer showed no amplification in samples negative for *H. pylori*. ASP-PCR of *gyrA* gene with original primers had sensitivity of 52% (95% CI 38–66) and specificity of 92.7% (95% CI 81–97). The ASP-PCR with new primer set improved the sensitivity to 100% (95% CI 91–100%), while specificity remained 92.7% (95% CI 82–97) (Table 3).

4. Discussion

Accurate and rapid detection of antibiotic resistance has an important role in the selection of optimal antibacterial agents for the treatment of patients infected by *H. pylori* (Furuta et al., 2007; Lehours et al., 2011; van Doorn et al., 2001). However, such information is rarely available at time of starting therapy in part because conventional antimicrobial susceptibility testing requires culture of the bacterium, which is time consuming and often unavailable. In addition, approximately 10% of culture attempts fail. Overall, phenotyping methods are both expensive and cumbersome (Heo and Jeon (2014); Lehours et al., 2011). In Colombia, as in most of the world, culture and susceptible test are not available in routine *H. pylori* clinical practice. Genetic tests are simple and straightforward and, theoretically, are good tools to rapidly detect resistance to macrolides and fluoroquinolones using direct detection with DNA from gastric biopsy samples.

In the present study, we used ASP-PCR methods to detect mutations in the 23S rRNA and *gyrA* genes of *H. pylori*. The methods were useful for easily identifying susceptible organisms from those resistant to clarithromycin and/or levofloxacin in gastric mucosal biopsies. Our results showed a good correlation between ASP-PCR and both agar dilution and direct sequencing (i.e., 100% concordance, sensitivity, and specificity for ASP-PCR of 23S rRNA gene were obtained). However, this results could be different if mutations in addition to A2142G or A2143G are present in the samples, as the methods used in this study were designed to detect only the 2 most common described mutations. Colombian samples in this study had only A2142G and A2143G mutations, and thus, the molecular test was able to discriminate mutations from wild types perfectly. Additionally, the molecular test correctly classified all negative patients as no sample showed any amplification.

The analysis of sequences for GyrA of Colombian *H. pylori*-resistant strains showed N87I, D91G, N87K, and D91N mutations, and the susceptible strains showed that 18 strains had N87(AAT), 8 N87(AAC), and 25 N87T(ACC) sequence. We used ASP-PCR with the original primers described by Nishizawa et al. (2007) to detect mutations C261A, C261G, G271A, G271T, and A272G in the *gyrA* gene that correspond to the following GyrA amino acid changes, N87K(AAA), N87K(AAG), D91N(AAT), D91Y(TAT), D91G(GGT), respectively. No amplification was observed in negative samples for *H. pylori*. The sensitivity and specificity of this molecular test were 52% and 92.7%, respectively. The reason of lower sensitivity was due to the presence of mutation N87I in our samples, which

were not detected by the original test as 44.6% of resistant strains carried this mutation. Importantly, false-negative results would have a major clinical impact in that patients infected with resistant strains would not be detected and might be given fluoroquinolones for treatment of their *H. pylori* infection. When other similar techniques for the detection of levofloxacin resistance were reviewed, including Genotype Helicobacter DR (a commercial test), we found that none of them are designed to detect the N87I mutation. As such, because the N87I mutation was common in Colombian strains resistant to levofloxacin, the failure to detect these resistance strains would likely result in failure of the treatment. We, therefore, designed new primers to detect the mutation N87I in GyrA. The ASP-PCR was performed with the original primers plus the new primers. The new primers improved the sensitivity of the molecular test to 100%, although the specificity remained 92.7% when used to evaluate resistance to fluoroquinolones among Colombian *H. pylori* strains using gastric biopsy specimens. With respect to specificity, the discordant results between the genotyping and phenotyping methods for levofloxacin were most likely related to the presence of mixed infections with both wild-type and mutant genotypes in 4 of the gastric biopsy specimens. Because it is often difficult to culture *H. pylori* in all gastric biopsies and cultures do not always recover all the strains present in the sample, molecular test is a good alternative to detect resistant genotypes in gastric biopsy specimens. However, molecular methods have some difficulties including requiring use of many primers, which requires longer standardization, and the test detects only mutations currently known to confer resistance. For this reason, the details of testing may need to be adapted in accordance with the mutations found in each area. Once implemented, molecular testing is both time saving and useful for routine clinical practice as rapid DNA from biopsy samples theoretically would allow patients to receive appropriate treatment resulting in few treatment failures due to unrecognized resistance.

In conclusion, we developed a new primer for detection of N87I mutation in GyrA in *H. pylori* related to levofloxacin resistance and evaluated 2 ASP-PCR previously described for detection of mutations that confer clarithromycin and levofloxacin resistance. These assays provide a specific and rapid means of predicting resistance to clarithromycin and levofloxacin, and their application could be of enhanced value in the management of *H. pylori* infections. The optimization in gastric biopsy tissue without the need for culture allows the implementation of this test in Colombia, a country where culture and susceptibility tests are not routinely performed.

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Table 1

Oligonucleotide sequences of primers used in ASP-PCR method to determine *gyrA* mutation of *H. pylori* at positions 260, 261, 271, and 272.

Primer name	Primer direction	Mutation	Sequence ^a
F261A1 ^a	Forward	C261A	CCCCCATGGCGAGAAaG
F261G1 ^a	Forward	C261G	CCC CCA TGG CGAGAAgG
F271A5 ^a	Forward	G271A	GCGATAACGCGGTTTAGaA
F271A9 ^a	Forward	G271A	GCGATAATGCGGTTTAGaA
F271T9 ^a	Forward	G271T	GGCGATAATGCGGTTAAtA
F272G1 ^a	Forward	A272G	GCGATAACGCGGTTTAGgT
F272G9 ^a	Forward	A272G	GCGATAATGCGGTTTAGgT
F87ATC ^b	Forward	A260T, T261C	CCCCCATGGCGAGAtcG
<i>gyrA</i> R	Reverse		GTTAGGCAGACGGCTTGGTARAATA

^aPrimer sequence described by Nishizawa et al. (2007). In the primer sequence, the penultimate nucleotide to distinguish between wild-type and mutant sequences is shown in lowercase. The 1-bp mismatch at another nucleotide is underlined.

^bNew primer used in ASP-PCR method to determine *gyrA* mutations A260T and T261C.

Table 2

Results of fluoroquinolone susceptibility tests by agar dilution method, DNA sequencing analysis, and ASP-PCR for the *gyrA* gene.

		<u>Agar dilution method</u>		<u>DNA sequencing analysis</u>	
		Resistant	Susceptible	Resistant	Susceptible
ASP-PCR with original primers ^a	Resistant	27	4	31	0
	Susceptible	25	51	25	51
ASP-PCR with new primers set ^b	Resistant	52	4	56	0
	Susceptible	0	51	0	51

^aOriginal primers set was reported by Nishizawa et al., 2007.

^bNew primer set includes original primers and new primers to determine *gyrA* mutations A260T and T261C.

Sensitivity, specificity, PPV, and NPV of ASP-PCR for the detection of fluoroquinolone resistance compared to the agar dilution method.

Table 3

	Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)
ASP-PCR with original primers ^a	52(38–66)	92.7 (81–97)	87(69–96)	67(55–77)
ASP-PCR with new primers set ^b	100.0 (91–100)	92.7 (82–97)	92.8 (82–97)	100.0 (91–100)

^aOriginal primer set was reported by Nishizawa et al. (2007).

^bNew primer set includes original primers and new primers to determine *gyrA* mutations A260T and T261C.