

PAPERS

Helicobacter pylori specific nested PCR assay for the detection of 23S rRNA mutation associated with clarithromycin resistance

S Maeda, H Yoshida, K Ogura, F Kanai, Y Shiratori, M Omata

Abstract

Background—Clarithromycin is one of the most important antibiotics for *Helicobacter pylori* eradication. However, 5–10% of strains are reported to be resistant. It has been shown that one point mutation in the 23S rRNA gene is associated with resistance to clarithromycin.

Aims—To establish a polymerase chain reaction (PCR) system which amplifies a segment of the 23S rRNA gene containing the mutation points with primers specific for *H pylori*, so that *H pylori* infection and the mutation associated with clarithromycin resistance can be examined simultaneously.

Methods—To detect *H pylori* infection and the mutation simultaneously, primers specific for the *H pylori* 23S rRNA gene were designed based on sequence conservation among *H pylori* strains and sequence specificity as compared with other bacteria. DNA from 57 cultured strains and from 39 gastric juice samples was amplified in the seminested 23S rRNA PCR. Clinical applicability was evaluated in 85 patients.

Results—DNA samples from 57 cultured strains were all amplified. The novel assay and the urease A PCR agreed in 37/39 gastric juice samples with no false positives. The assay did not amplify the DNA of bacteria other than *H pylori*. Eight of 85 samples had the mutation before treatment. In clarithromycin based treatment, eradication was achieved in 2/5 (40%) with the mutation and 29/34 (85%) without the mutation.

Conclusion—The assay using gastric juice is quick (within 12 hours) and non-invasive (endoscopy not required), enabling rapid initiation of appropriate antibiotic treatment.

(Gut 1998;43:317–321)

Keywords: *Helicobacter pylori*; eradication; clarithromycin; resistance; point mutation

Helicobacter pylori is a Gram negative bacterium which resides in the human stomach.¹ The organism plays an important role in the pathogenesis of not only persistent gastritis but also peptic ulcers and gastric cancer.^{2–5} Eradication of *H pylori* may reduce the risk of peptic

ulcer and gastric carcinoma.⁶ The National Institutes of Health Consensus Development Conference has recommended the addition of antimicrobial agents to antisecretory drugs (proton pump inhibitors or H₂ blockers) to treat patients with *H pylori* infection associated peptic ulcer disease. These antibiotics include amoxicillin, clarithromycin, and metronidazole and the eradication rates achieved by one week of triple therapy were above 90%.^{7–10} All *H pylori* strains were shown to be susceptible to amoxicillin, but 5–10% of strains are resistant to clarithromycin and 10–50% to metronidazole.^{11–14} In the analysis of clarithromycin based treatment failure, Cayla *et al* reported that eradication was achieved in 3/10 (30%) clarithromycin resistant strains compared with 77/93 (83%) clarithromycin susceptible strains.¹⁵ Debets-Ossenkopp *et al* reported that 11/15 cases of unsuccessful eradication were due to clarithromycin resistance.¹⁶ These observations suggest the importance of evaluating drug resistance before and after treatment.

Recently, Versalovic *et al* showed that an adenine (A) to guanine (G) mutation at 2143 or 2144 in domain V of the 23S rRNA gene of *H pylori* is associated with resistance to clarithromycin. They also established a polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assay using primers conserved among most bacteria.¹⁷ However, the assessment of clarithromycin resistance with this assay requires culture of the bacterium beforehand, and takes about five to six days. The 23S rRNA gene is well conserved among various bacterial species and contamination will interfere with the assay.

The purpose of the current study was to establish a PCR system which amplifies a segment of the 23S rRNA gene containing the mutation points with primers specific for *H pylori*, so that *H pylori* infection and the mutation associated with clarithromycin resistance can be examined simultaneously.

Materials and methods

BACTERIAL STRAINS AND GROWTH MEDIA

Fifty seven *H pylori* strains were isolated from gastric biopsy specimens from pretreatment Japanese patients and the biopsy specimens were cultured on Columbia agar with 5% (vol/vol) horse blood and Dent antibiotic supplement (Oxoid, Basingstoke, UK) at 37°C for five days under microaerobic conditions

Second Department of Internal Medicine, University of Tokyo, Japan
S Maeda
H Yoshida
K Ogura
F Kanai
Y Shiratori
M Omata

Correspondence to:
Dr S Maeda, Second Department of Internal Medicine, Faculty of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113, Japan.

Accepted for publication
4 March 1998

Table 1 Treatment regimens

Regimen	No of patients	Week	Lansoprazole (mg)	Amoxicillin (mg)	Clarithromycin (mg)
LA	39	1	60	2000	—
LAC	39	1	60	2000	400

LA, lansoprazole + amoxicillin; LAC, lansoprazole + amoxicillin + clarithromycin.

(Campy-Pak Systems; BBL, Cockeysville, Maryland). The organisms were identified as *H pylori* by Gram stain morphology, colony morphology, and positive urease, catalase, and oxidase activities. The isolates were kept at -80°C in Brucella broth with 5% (vol/vol) fetal bovine serum (FBS) containing 16% (vol/vol) glycerol. In addition, 31 clarithromycin resistant and 27 sensitive strains which had been previously determined by minimum inhibitory concentration (MIC), were recovered from frozen stocks and used.

GASTRIC JUICE SAMPLES

Gastric juice samples were aspirated with disposable nasogastric tubes from 39 patients to compare the results of urease A (URA) PCR^{18,19} and 23S rRNA PCR. More samples from 28 pretreatment and 85 post-treatment patients were used for clinical application.

PREPARATION OF DNA

The culture medium was centrifuged for five minutes at 10 000 *g*, and genomic DNA from *H pylori* isolates was extracted from the pellets using SepaGene (Sanko Junyaku, Japan). From

the gastric juice samples, to avoid possible interference from substances in the gastric juice, *H pylori* was first captured on rabbit anti-*H pylori* antibody (Dako A/S, Denmark) immobilised on polystyrene beads. After elution from the beads, DNA was extracted using the SepaGene. DNA was stored at -20°C until use as PCR templates.

SEQUENCE ANALYSIS OF 23S rRNA GENE

To examine the nucleotide conservation in the region flanking 23S rRNA position 2143 and 2144, we amplified a segment of approximately 330 bp in seven *H pylori* strains by using PCR primers 23SF2 (5'-GTGCGAAATTCCTTGTCGG-3', corresponding to *H pylori* 23S rRNA position 2015–2034) and 23SR (5'-GACCGCCCCAGTCAAAC-3', position 2343–2326). The PCR products were cloned into pCRII using the original TA cloning kit (Invitrogen, San Diego, California). The nucleotide sequence of the insert was determined by the dideoxy chain termination procedure.²⁰

SPECIFIC PRIMERS FOR *H PYLORI* AND AMPLIFICATION

After analysing the sequence conservation among the seven clinically isolated *H pylori* strains and the sequence specificity for *H pylori* compared with other bacteria reported in GenBank, we chose one sense primer, CRF-4 (5'-AGTGGAGGTGAAAATTCC-3', corresponding to *H pylori* 23S rRNA position 2105–2123), and two antisense primers, CRR-1 (5'-TAAGAGCCAAAGCCCTTAC-3', position 2239–2221), and CRR-3 (5'-ATTCCCATTAGCAGTGCT-3', position 2189–2172). A novel seminested PCR assay was thus designed with CRF-4/CRR-1 as the first and CRF-4/CRR-3 as internal PCR primers (23S rRNA PCR assay). A PCR cycle consisted of denaturation at 94°C for 30 seconds, annealing at 50°C for 30 seconds, and extension at 72°C for one minute: 30 cycles were performed for the first and 20 cycles for the second PCR amplification.

DETECTION OF URE A GENE FROM GASTRIC JUICE SAMPLES

DNA from gastric juice samples was also analysed using primers set in *ureA* gene described previously (URA PCR)^{18,19} and the results were compared with those of the 23S rRNA PCR assay.

SPECIFICITY OF PRIMERS

Specificity for *H pylori* of the novel PCR primers, CRF-4/CRR-1 and CRF-4/CRR-3, was evaluated by testing cross reaction with DNA samples extracted from 12 bacterial species other than *H pylori*. These bacterial species were *Staphylococcus aureus*, *Staph epidermidis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Streptococcus pyogenes*, *Strept mitis*, *Haemophilus parainfluenzae*, *Campylobacter jejuni*, *Aspergillus flavus*, *Proteus mirabilis*, *Klebsiella pneumoniae*, and *Branhamella catarrhalis*.

23S rRNA gene sequence homology (2042–2281bp)

```

23S reported 1:CCGACCTGCATGAAATGGCGTAACGAGATGGGAGCTGTCTCAACCAGAGATTCACTGAAAT 60
TO-1 1:..... 60
TO-64 1:..... 60
TO-66 1:..... 60
TO-76 1:..... 60
TN-1 1:..... 60
TN-5 1:..... 60
TN-7 1:..... 60
*****
CRF-4 →
23S reported 61:TGTAGTGGAGGTGAAAATTCCCTCTACCCGCGCAAGAGCGAAAGACCCCGTGGACCTTT 120
TO-1 61:..... 120
TO-64 61:..... 120
TO-66 61:..... 120
TO-76 61:..... 120
TN-1 61:..... 120
TN-5 61:.....G..... 120
TN-7 61:..... 120
*****
←CRR-3
23S reported 121:ACTAGCAACTTAGCACTGCTAATGGGAATATCATGCCGAGGATAGTGGGAGGCTTTGAAAG 180
TO-1 121:.....C..... 180
TO-64 121:.....C..... 180
TO-66 121:.....C..... 180
TO-76 121:.....C..... 180
TN-1 121:.....C..... 180
TN-5 121:.....C..... 180
TN-7 121:.....C.....C..... 180
*****
←CRR-1
23S reported 181:TAGGGCTTTGGCTCTTATGGAGICATCCTTGAGATAACCAACCTTGATGTTCTGTGTAC 240
TO-1 181:.....C..... 240
TO-64 181:.....C..... 240
TO-66 181:.....C..... 240
TO-76 181:.....C..... 240
TN-1 181:.....C..... 240
TN-5 181:.....C..... 240
TN-7 181:.....C..... 240
*****

```

Figure 1 23S rRNA gene sequence in seven *H pylori* strains. 23S reported was the sequence reported in GenBank (accession number U27270). Colons denote identity.

Table 2 Comparison between the 23S rRNA PCR and URA PCR assays using gastric juice samples

URA ^{rs}	23S rRNA		Total
	Positive	Negative	
Positive	29	2	31
Negative	0	8	8
Total	29	10	39

DETECTION OF 23S rRNA MUTATION ASSOCIATED WITH CLARITHROMYCIN RESISTANCE

To detect the A to G mutations at 23S rRNA gene positions 2143 and 2144, PCR products were digested with 5 U of MboII and BsaI at 37°C and 55°C respectively for two hours. PCR products containing the 2143 A to G mutation showed two smaller products after digestion with MboII, those containing the 2144 A to G mutation showed two smaller products after digestion with BsaI, and those containing no mutation revealed undigested amplicon.

DETERMINATION OF MIC TO CLARITHROMYCIN

The susceptibility of *H pylori* isolates to clarithromycin was evaluated by the agar dilution method. Twenty seven strains without mutation and six strains with mutation were tested and the MIC was determined. The MIC was also determined in 31 strains from frozen stocks.

CLINICAL APPLICATION

Clinical applicability of the 23S rRNA PCR was evaluated in a total of 85 patients who received anti-*H pylori* treatment. *H pylori* infection was confirmed before treatment by culture, microscopy, rapid urease test, and URA PCR assay. The 23S rRNA-PCR detected the gene, with or without mutations, in all subjects before treatment. Seventy eight patients were treated; table 1 summarises the regimens. The outcome of antimicrobial therapy was assessed more than eight weeks after the completion of treatment. Gastric juice samples were aspirated with endoscopes or nasogastric strings, and the mutations at positions 2143 and 2144 of the *H pylori* 23S rRNA gene were evaluated as described earlier.

STATISTICAL ANALYSIS

Fisher's exact probability test was used. A value of $p < 0.05$ was considered statistically significant.

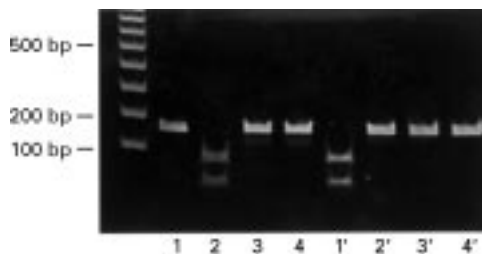


Figure 2 Detection of A to G mutation. PCR products from four strains were digested with MboII (lanes 1–4) and with BsaI (lanes 1'–4'). Strain 2 had the A to G mutation at 2143 (lane 2), strain 1 had the A to G mutation at 2144 (lane 1'), and strains 3 and 4 had no mutations at either 2143 or 2144.

Results

SEQUENCE ANALYSIS OF 23S rRNA GENE

Sequence analysis between 2042 to 2281 of the *H pylori* 23S rRNA gene in seven clinical isolates revealed that a few positions had frequent nucleotide mutations while others were well conserved. Nucleotides were different from the standard at two positions in all clinical isolates. One strain (TN-5) had the resistance associated 2144 A to G mutation (fig 1).

PCR AMPLIFICATION

The primers used for the 23S rRNA PCR assay, CRF-4, CRR-1, and CRR-3, were designed to target the regions conserved in *H pylori* strains but not shared by other bacteria. Using 10 ng of DNA extracted from culture, all DNA samples from 57 *H pylori* isolates revealed positive amplification by the first step PCR. The results of the 23S rRNA PCR on gastric juice samples agreed with those of the URA PCR assay in 37/39 samples. Importantly, eight samples negative by the URA PCR assay were negative also by the 23S rRNA PCR assay. Thus, there were no apparent false positives (table 2). The specificity of the 23S rRNA PCR assay was also confirmed by the absence of cross reaction with DNA samples obtained from 12 bacterial species other than *H pylori*.

DETECTION OF MUTATIONS

The 23S rRNA PCR products were incubated with two restriction enzymes, MboII and BsaI. Eighty five pretreatment patients were investigated, and overall, 8/85 (9%) had a point mutation associated with clarithromycin resistance. Only one sample was digested with MboII, indicating the 2143 A to G mutation. Seven samples were digested with BsaI, indicating the 2144 A to G mutation. Figure 2 shows typical examples. Six of the eight strains were shown to be clarithromycin resistant by the agar dilution method as expected. The remaining two were not tested because culture was not available. Twenty nine of 31 strains which were recovered from frozen stocks and previously determined as clarithromycin resistant by MIC had the 2144 mutation and only one strain had the 2143 mutation. The remaining strain had no mutation at either 2143 or 2144. Twenty seven strains susceptible to clarithromycin by the agar dilution method did not have a mutation at either 2143 or 2144 (fig 3).

CLINICAL APPLICATION

The mutations in the *H pylori* 23S rRNA gene were revealed in 8/85 (9%) samples obtained before treatment. Thirty nine patients, including three infected with mutant strains, received regimens without clarithromycin. Eradication was achieved in 1/3 (33%) patients with mutant infection and 12/36 (33%) patients with wild type infection (NS). After treatment, sustained mutant infection was confirmed in two patients who had mutant strains before treatment, but all 24 patients with wild type infection before treatment still harboured wild types. Thirty nine other patients were treated

Table 3 Result of clinical application

Regimen	No of patients	Mutation present		Acquired mutation	Treatment success
		Before	After		
LA	39	3/39	2/26	0	12 (33%)
LAC	39	5/39	5/8	2	31 (79%)

LA, lansoprazole + amoxicillin; LAC, lansoprazole + amoxicillin + clarithromycin.

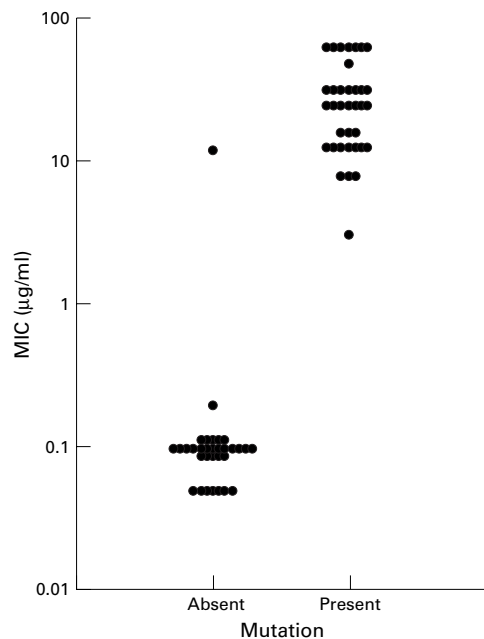


Figure 3 Relation between the A to G mutation at 2143/2144 and MIC. All strains with the mutation at 2143/2144 were resistant to clarithromycin by MIC. One strain was resistant by MIC but had no mutation. Those without the mutations were susceptible to clarithromycin by MIC.

with regimens containing clarithromycin; five had mutant strains before treatment. Eradication was achieved in 2/5 (40%) patients with mutant infection and in 29/34 (85%) patients with wild type infection ($p < 0.05$). Post-treatment 23S rRNA PCR assay revealed the clarithromycin resistance mutations in 3/3 (100%) patients with mutant infection before treatment and 2/5 (40%) patients with wild type infection before treatment. Thus, the proportion of mutant infection increased from 5/39 (13%) to 5/8 (63%) among this group (table 3).

Discussion

We have developed an assay that can detect *H pylori* infection with high sensitivity and simultaneously evaluate clarithromycin resistance. As 23S rRNA genes are fairly conserved among various bacterial species, PCR amplification of DNA directly extracted from gastric biopsy or gastric juice samples may be affected by the 23S rRNA gene of other bacteria if non-specific primers are used. Thus, the detection of the mutations in the 23S rRNA gene associated with clarithromycin resistance with consensus primers requires culture before extraction of DNA and PCR-RFLP; this takes about 10 days. In contrast, our novel assay does not require culture and can be completed in only

12 hours, assessing *H pylori* infection and clarithromycin resistance simultaneously. The high sensitivity of the assay was due to the good conservation of the sequences targeted by its primers among *H pylori* strains. The seminesting of PCR amplification was also essential for its high sensitivity. The novel 23S rRNA PCR and the URA PCR agreed in 37/39 gastric juice samples. Importantly, the 23S rRNA PCR resulted in no false positive results.

Clarithromycin resistance of *H pylori* was reported to be due to the point mutation of 23S rRNA by Versalovic *et al.*¹⁹ We confirmed that this finding also applies to *H pylori* isolates in Japan. We tested 37 clarithromycin resistant strains and 27 susceptible strains as determined by MIC; 36/37 resistant strains and 0/27 susceptible strains had the mutation. These results show that most strains resistant to clarithromycin have the mutations in the 23S rRNA gene. However, one clarithromycin resistant strain has no mutation at positions 2143 or 2144 by both our assay and sequence analysis (data not shown) and the mechanism of resistance remains to be investigated.

In the present study, 36/38 (95%) strains with the mutation associated with clarithromycin resistance revealed the A to G mutation at position 2144, and only 2/38 (5%) had that at 2143. Recently, Stone *et al* showed that a higher level of resistance was associated with the A to G mutation at 2143.²¹ Seven strains examined in this study had MIC values greater than 50 µg/ml, and the MIC of other resistant strains ranged from 3 to 32 µg/ml. The relatively low level of MIC in the isolates may be related to the high prevalence of the A to G mutation at position 2144 in Japan. Although the overall prevalence of clarithromycin resistant strains is similar in Japan and Western countries, the difference in the mutation position may have affected the eradication rates of clarithromycin based treatments. To confirm these observations, further clinical trials using this assay will be necessary.

Recently it was reported that the A to C mutation at position 2143 was also associated with clarithromycin resistance.²¹ The authors also reported that 7% of clarithromycin resistant strains had this mutation. Our assay is not able to detect the A to C mutation. However, in 37 clarithromycin resistant isolates determined by MIC, we found no strain with the A to C mutation, and the sensitivity of the assay seems to be practically sufficient, at least in Japan. The clinical applicability of this assay in other countries needs to be investigated. To establish a more sensitive detection system, we are developing an assay to detect all mutations associated with clarithromycin resistance.

Anti-*H pylori* treatment with clarithromycin, together with amoxicillin and a proton pump inhibitor, is one of the current standard protocols. However, several studies have indicated that *H pylori* may become clarithromycin resistant after an unsuccessful clarithromycin based treatment.^{15, 16} Indeed, the present study confirmed genotypically the appearance of clarithromycin resistance. It has also been shown that the eradication rates achieved by

clarithromycin based regimens significantly differed between mutant and wild type infections. Thus, regimens not containing clarithromycin may be preferable in cases with mutant *H pylori* infections. The prevalence of mutant infection is likely to be high among cases where the initial clarithromycin based treatment failed to achieve eradication, and the assessment of mutation will be more important in those cases. The 23S rRNA PCR, which does not require culture and can use gastric juice samples, may be useful under these circumstances.

In conclusion, we have established a highly sensitive seminested PCR assay for both the detection of *H pylori* infection and the evaluation of the 23S rRNA mutation associated with clarithromycin resistance. By using gastric juice samples, the assay is rapid (overnight) and less invasive (no need to perform biopsy), and patients can be treated with adequate antibiotics, based on knowledge of the resistance of the infecting organism.

Presented in part at the American Gastroenterological Association meeting in Washington, DC, 10–16 May 1997.

- 1 Warren JR, Marshall BJ. Unidentified curved bacilli on gastric epithelium in active chronic gastritis. *Lancet* 1983;i:1273–5.
- 2 NIH consensus conference. Helicobacter pylori in peptic ulcer disease. NIH Consensus Development Panel on Helicobacter pylori in Peptic Ulcer Disease. *JAMA* 1994;272:65–9.
- 3 Graham DY, Lew GM, Klein PD, et al. Effect of treatment of Helicobacter pylori infection on the long-term recurrence of gastric or duodenal ulcer: a randomized, controlled study. *Ann Intern Med* 1992;116:705–8.
- 4 Parsonnet J, Friedman GD, Vandersteen DP, et al. Helicobacter pylori infection and the risk of gastric carcinoma. *N Engl J Med* 1991;325:1127–31.
- 5 Nomura A, Stemmermann GN, Chyou PH, et al. Helicobacter pylori infection and gastric carcinoma among Japanese Americans in Hawaii. *N Engl J Med* 1991;325:1132–6.
- 6 Walsh JH, Peterson WL. The treatment of Helicobacter pylori infection in the management of peptic ulcer disease. *N Engl J Med* 1995;333:984–91.
- 7 Lind T, Veldhuyzen S, Unge P, et al. Eradication of Helicobacter pylori using one-week triple therapies combining omeprazole with two antimicrobials: the MACH I study. *Helicobacter* 1996;1:138–44.
- 8 Chiba N. Omeprazole and clarithromycin with and without metronidazole for the eradication of Helicobacter pylori. *Am J Gastroenterol* 1996;91:2139–43.
- 9 Labenz J, Stolte M, Ruhl GH, et al. One-week low-dose triple therapy for the eradication of Helicobacter pylori infection. *Eur J Gastroenterol Hepatol* 1995;7:9–11.
- 10 Penston JG, McColl KE. Eradication of Helicobacter pylori: an objective assessment of current therapies. *Br J Clin Pharmacol* 1997;43:223–43.
- 11 Gotoh A, Kawakami Y, Akahane T, et al. Susceptibility of Helicobacter pylori isolates against agents commonly administered for eradication therapy and the efficacy of chemotherapy. *Micobiol Immun* 1997;41:7–12.
- 12 Van Zwet AA, Deboer WA, Schneeberger PM, et al. Prevalence of primary Helicobacter pylori resistance to metronidazole and clarithromycin in The Netherlands. *Eur J Clin Microbiol Infect Dis* 1996;15:861–4.
- 13 Midolo PD, Lambert JR, Turmidge J. Metronidazole resistance: a predictor failure of Helicobacter pylori eradication by triple therapy. *J Gastroenterol Hepatol* 1996;11:290–2.
- 14 Weldon MJ, Broadbent A, Chambers S, et al. A seven-day Helicobacter pylori treatment regimen using clarithromycin, omeprazole and tripotassium dicitrato bismuthate. *Aliment Pharmacol Ther* 1996;10:279–83.
- 15 Cayla R, Zerbib F, Talbi P, et al. Pre and post-treatment clarithromycin resistance of Helicobacter pylori strains: key factor of treatment failure [abstract]. *Gut* 1996;37(suppl 1):A55.
- 16 Debets-Ossenkopp YJ, Sparrius M, Kusters JG, et al. Mechanism of clarithromycin resistance in clinical isolates of Helicobacter pylori. *FEMS Microbiol Lett* 1996;142:37–42.
- 17 Versalovic J, Shortridge D, Kibler K, et al. Mutations in 23S rRNA are associated with clarithromycin resistance in Helicobacter pylori. *Antimicrob Agents Chemother* 1996;40:477–80.
- 18 Kawamata O, Yoshida H, Hirota K, et al. Nested-polymerase chain reaction for the detection of Helicobacter pylori infection with novel primers designed by sequence analysis of urease A gene in clinically isolated bacterial strains. *Biochem Biophys Res Commun* 1996;219:266–72.
- 19 Yoshida H, Hirota K, Shiratori Y, et al. Use of a gastric juice-based PCR assay to detect Helicobacter pylori infection in culture-negative patients. *J Clin Microbiol* 1998;36:317–20.
- 20 Sanger F, Nicklen S, Coulson AR. DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci USA* 1977;74:5463–7.
- 21 Stone GG, Shortridge D, Versalovic J, et al. PCR oligonucleotide ligation assay to determine the prevalence of 23S rRNA gene mutations in clarithromycin resistant Helicobacter pylori. *Antimicrob Agents Chemother* 1997;41:712–14.