Detection of mixed clarithromycin-resistant and -susceptible *Helicobacter pylori* using nested PCR and direct sequencing of DNA extracted from faeces

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The major cause of chemotherapy failure in patients with chronic gastritis and peptic ulcers caused by *Helicobacter pylori* is clarithromycin (CAM) resistance due to a mutation in the 23S rRNA gene. This study describes a non-invasive and accurate method for the detection of mixed CAM-resistant and -susceptible *H. pylori* by sequencing of the *H. pylori* 23S rRNA gene. Faeces were crushed with beads and the 23S rRNA gene was amplified using a nested PCR on the extracted DNA. Mutation analysis of this gene using this method showed that 20.4 % of patients carried mixed CAM-susceptible (wild type) and -resistant (A2142G or A2143G mutant) *H. pylori*. Furthermore, it was found that 66.6 % of patients who had been treated unsuccessfully carried one of these mutations in the 23S rRNA gene (including the mixed type), whilst standard culture detected CAM-resistant isolates in only 22.2 % of patients with unsuccessful treatment. These data suggest that, for successful therapy, the diagnosis method described here would more accurately detect CAM-resistant *H. pylori*, including mixed infections.

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INTRODUCTION

Helicobacter pylori is a Gram-negative, spiral bacterium found in the human stomach. This micro-organism causes chronic gastritis and peptic ulcers (Kuipers, 1997) and is linked to gastric cancer and other non-gastrointestinal diseases (Kusters *et al.*, 2006; Leong & Sung, 2002). To treat *H. pylori* infections, a triple therapy is used comprising a combination of a proton pump inhibitor and two antimicrobial agents. This is reported to be the most successful method for eradication of infection (Malfertheiner *et al.*, 2002). In Japan, a combination of lansoprazole, amoxicillin and clarithromycin (CAM) (the LAC regimen) is commonly used to eliminate *H. pylori* (Asaka *et al.*, 2001). Although most patients are treated successfully with this triple therapy, some failure has necessitated the use of additional drugs such as levofloxacin (Asaka *et al.*, 2001; Kato *et al.*, 2000). The major cause of therapy failure in patients is resistance to CAM (Kato *et al.*, 2000; Rimbara *et al.*, 2005a). Therefore, a reliable

diagnostic test to detect macrolide resistance would be useful in planning a therapeutic regimen. Standard culture is generally used to determine the susceptibility of *H. pylori* to antimicrobial agents; however, acquiring a test sample is invasive for the patient, as endoscopy is required to obtain gastric specimens. Faecal culture is not possible, as *H. pylori* coccoid forms are unculturable; however, there are some reliable non-invasive tests for the diagnosis of *H. pylori* infections (Graham *et al.*, 1987; Leodolter *et al.*, 2003; Shuber *et al.*, 2002; Vaira *et al.*, 1999).

Resistance to CAM in *H. pylori* is due to a mutation in the 23S rRNA subunit in the 50S ribosome. The two most common mutations are an adenine-to-guanine transition at position 2142 or 2143, the latter leading to an adenine-to-cytosine transversion at position 2142 (Alarcon *et al.*, 2003; van Doorn *et al.*, 2001). Resistance due to mutations at other positions has also been reported (Fontana *et al.*, 2002; Khan *et al.*, 2004; Toracchio *et al.*, 2004). Several methods to detect CAM-resistant *H. pylori* from faecal samples have used RFLP (Alarcon *et al.*, 2003), real-time PCR (Schabereiter-Gurtner *et al.*, 2004) or other methods

Abbreviations: CAM, clarithromycin; HpSA, *H. pylori* stool antigen.

(Morris *et al.*, 2005; Prokhorenko *et al.*, 2006; van Doorn *et al.*, 2001).

In the above methods, it is important that the DNA of all of the micro-organisms in the faeces is isolated efficiently. *H. pylori* converts from a spiral shape in the stomach to a coccoid form in the faeces, which is unculturable *in vitro* (Kusters *et al.*, 1997; Thomas *et al.*, 1992). Therefore, isolation of CAM-resistant *H. pylori* using coccoid-form DNA from faeces is required for mutation analysis of the 23S rRNA gene of *H. pylori*.

Here, we developed an improved method to extract DNA from faeces together with an efficient and inexpensive PCR procedure. Previously, we reported a method for extracting bacterial DNA, including that of *H. pylori*, from faeces using a combination of enzymic lysis and crushing with beads. To diagnose CAM-resistant *H. pylori* infection, we detected mutations in the *H. pylori* 23S rRNA gene with DNA specifically amplified using a nested PCR (Rimbara *et al.*, 2005b). Chemical cell lysis including enzyme treatment and physical crushing are generally used as methods for extracting DNA from cells (Akiyama *et al.*, 2005). Chemical lysis is a simple method and is used in commercial kits (McOrist *et al.*, 2002; Monteiro *et al.*, 2001). Compared with chemical lysis, physical crushing is able to isolate DNA uniformly from various species and different cell types (seeds, Gram-positive bacteria and spores), although a special homogenizer or grinder is necessary (Akiyama *et al.*, 2005; Matsuki *et al.*, 2004). Previously used methods needed improvement as the procedures were complicated and time-consuming, such as the long incubation times required for enzymic reactions. Furthermore, the nested PCR in our previous study required about 4 h for amplification using DNA polymerase. Therefore, in this study, we improved our previous method for detection of mutations in the *H. pylori* 23S rRNA gene from faeces and compared this improved method with the *H. pylori* stool antigen (HpSA) ELISA and culture methods. We also correlated our results with data from patients using the triple therapy to help design a more successful treatment for patients using the LAC regimen.

METHODS

Patients and materials. A total of 146 stool specimens was obtained from 18 healthy subjects (12 males and 6 females; mean age 22.9 ± 1.4 years, range 22–26 years) and 128 patients (101 males and 27 females; mean age 51.5 ± 14.8 years, range 20–81 years) who visited Tokyo Medical University Hospital, Tokyo, Japan, or Kyorin University Hospital, Tokyo, Japan, between 1998 and 2004. Diagnoses were made in patients having gastric cancer ($n=25$), peptic ulcers ($n=82$), chronic gastritis ($n=19$), gastric polyps ($n=1$) and oesophageal cancer ($n=1$). Faeces taken from all patients and healthy subjects were stored at $-80\text{ }^{\circ}\text{C}$ until used. Upper endoscopy procedures were performed on 114 patients and gastric specimens from the antrum or body were used for *H. pylori* culture. Informed consent was obtained from all patients and healthy subjects.

HpSA ELISA. An enzyme immunoassay to detect *H. pylori* antigen in stool specimens was performed using a Premier Platinum HpSA kit (Meridian Diagnostics) as reported previously (Vaira *et al.*, 1999). Measuring the absorbance at 450/630 nm, the cut-off levels for the HpSA test were <0.100 for a negative result, $0.100 < A_{450/630} < 0.120$ as equivocal and >0.120 for a positive result, as recommended by the manufacturer.

DNA extraction from faeces. DNA was extracted from faeces using a bead crushing method developed in this study. The tube contained 250 mg silica powder (63–210 μm), 32.5 mg ceramic beads (1–2 mm diameter) and 75 mg glass beads. Approximately 50 mg faeces was added to the tube with 980 μl sodium phosphate buffer and 180 μl 7.5 M guanidine solution containing 5% sarcosine and homogenized for 20 s at level 6 using a FastPrep FP120 instrument (Qbiogene). The homogenized solution was centrifuged at 14 000 g for 30 s. The supernatant was transferred into a new tube and 250 μl of 3.5 M sodium acetate (pH 5.2) was added. After mixing, the solution was centrifuged at 14 000 g for 5 min and 350 μl of the supernatant was purified using Wizard SV Gel and PCR Clean-up systems (Promega). The final volume for each DNA preparation was 50 μl .

Nested PCR of the *H. pylori* 23S rRNA gene. Nested PCR for detection of mutations in the *H. pylori* 23S rRNA gene was performed using the primers shown in Table 1. PCR primers were designed using regions with little sequence similarity to other bacterial species. Nested PCR was performed in a thermal cycler using Ex Taq (Takara Biomedicals) and PCR master mix (Promega) for the first and second PCRs, respectively. The conditions for the first PCR were as follows: initial denaturation at $95\text{ }^{\circ}\text{C}$ for 2 min; five cycles of $94\text{ }^{\circ}\text{C}$ for 30 s, $57\text{ }^{\circ}\text{C}$ for 30 s and $72\text{ }^{\circ}\text{C}$ for 30 s; and 30 cycles of $94\text{ }^{\circ}\text{C}$ for 15 s, $57\text{ }^{\circ}\text{C}$ for 15 s, $72\text{ }^{\circ}\text{C}$ for 20 s. A second PCR was performed using

Table 1. Primers used for nested PCR of the *H. pylori* 23S rRNA gene

Primer positions are shown relative to the 23S rRNA gene sequence of *H. pylori* strain 26695.

Primer	Sequence (5'→3')	T_m ($^{\circ}\text{C}$)	Position (nt)	Amplicon (bp)
First-round				
Hp23S 1835F	GGTCTCAGCAAAGAGTCCCT	62.4	1835–1854	493
Hp23S 2327R	CCCACCAAGCATTGTCCT	63.6	2327–2310	
Second-round				
Hp23S 1942F	AGGATGCGTCAGTCGCAAGAT	68.2	1942–1962	367
Hp23S 2308R	CCTGTGGATAACACAGGCCAGT	67.1	2308–2287	

2 µl of the first PCR product with initial denaturation at 95 °C for 2 min, followed by 25 cycles of 94 °C for 10 s and 63 °C for 20 s. The size of final PCR products was confirmed by electrophoresis in 2.5 % agarose gels. DNA sequencing was performed using an ABI PRISM 3100 DNA sequencer (Applied Biosystems) with a BigDye Terminator version 3.1 cycle sequencing kit (Applied Biosystems). The primers Hp23S 1942F and Hp23S 2308R were used for DNA sequencing (Table 1).

***H. pylori* culture and antibiotic susceptibility testing.** Gastric biopsy specimens obtained from 114 patients were cultured on modified Skirrow agar (Nissui Pharmaceutical) under microaerophilic conditions (5 % O₂, 10 % CO₂ and 85 % N₂) for approximately 7 days at 37 °C (Rimbara *et al.*, 2005a). *H. pylori* was identified using oxidase production and the API Campy test (bioMérieux). The isolates were stored in 20 % glycerol/brain heart infusion broth at –80 °C until used for the antibiotic susceptibility test. For *H. pylori* isolated prior to 2001, susceptibility to CAM was tested using the Dry Plate test (Eiken Chemical), as reported previously (Hoshiya *et al.*, 2000), and an agar dilution method was used thereafter according to the Clinical and Laboratory Standards Institute, as reported previously (CLSI, 2002; Rimbara *et al.*, 2005a). *H. pylori* isolates were considered resistant when the MIC for CAM was $\geq 1 \mu\text{g ml}^{-1}$ (CLSI, 2002).

RESULTS

Detection of *H. pylori* infection from faeces using nested PCR and HpSA ELISA and from gastric specimens by culture

Nested PCR and HpSA ELISA tests for the detection of *H. pylori* from faeces were performed on 146 samples taken from 128 patients and 18 healthy subjects. *H. pylori* was detected in 118/146 samples (80.8 %) from 116 patients and two healthy subjects using nested PCR, and in 122/124 samples (84.9 %) from 122 patients and two healthy subjects using HpSA ELISA. Of the 124 samples with positive results using HpSA ELISA, eight were found to be negative using nested PCR, and two samples with negative results using HpSA ELISA were found to be positive using nested PCR. Thus, the detection sensitivity and specificity of the nested PCR method compared with the HpSA ELISA were 93.5 and 90.9 %, respectively.

Detection of *H. pylori* by culture was performed for 114 patients and compared with the results from the nested PCR and HpSA ELISA (Table 2). Using nested PCR, *H. pylori* was detected in 92.5 % (99/107) of the patients with positive results by both HpSA ELISA and culture. All patients with positive results using either HpSA or culture were found to be positive using nested PCR. Of the eight patients with false-negative faecal samples using nested PCR, seven of the samples were collected before 2000 and more than 5 years before the DNA extraction. Furthermore, the amount of faeces was insufficient for the extraction of DNA in three of the eight patients. *H. pylori* was detected in one of the patients with negative results by both HpSA ELISA and culture, and was positive using the ¹³C-urea breath test.

Table 2. Detection of *H. pylori* from faeces by nested PCR and HpSA ELISA and from gastric biopsies by culture

Values are numbers of patients.

HpSA ELISA	Culture	Nested PCR result		
		Positive	Negative	Total
Positive	Positive	99	8	107
Negative	Positive	1	0	1
Positive	Negative	3	0	3
Negative	Negative	1*	2	3
Total		104	10	114

**H. pylori* infection was confirmed by ¹³C-urea breath test.

Comparison of 23S rRNA gene mutants from faeces with susceptibility to CAM

Of 100 patients with positive results by both culture and nested PCR, *H. pylori* culture isolates from two patients could not be tested for their susceptibilities to CAM due to poor growth. Therefore, the comparison of mutations of the *H. pylori* 23S rRNA gene detected by nested PCR from faeces with the susceptibility of *H. pylori* culture isolates to CAM was performed in 98 patients. The results of DNA sequencing of the *H. pylori* 23S rRNA gene amplified by nested PCR using DNA from faeces are shown in Table 3. The 23S rRNA gene without mutation (wild type) was detected in 58/98 patients (59.2 %). The 23S rRNA gene with an A2142G or A2143G mutation was detected in nine (9.2 %) and 31 (31.6 %) patients, respectively. Of the nine patients infected with *H. pylori* with the A2142G mutation, seven had a mixed-type infection, being simultaneously infected with the wild type (A2142G/wild type). Similarly, a mixed-type infection comprising both the A2143G mutation and wild type (A2143G/wild type) was detected in 13 of the 31 patients with the 23S rRNA gene mutation A2143G.

A comparison of the presence of a mutation in the *H. pylori* 23S rRNA gene from faeces with the susceptibility of *H. pylori* isolates to CAM is shown in Fig. 1. In 81 patients with CAM-susceptible *H. pylori* isolates, the mutation

Table 3. Mutations in the 23S rRNA gene of *H. pylori* in faeces from 98 *H. pylori*-infected patients

Values in parentheses are numbers of patients in which both wild type and mutant (A2142G or A2143G) 23S RNA genes were detected simultaneously.

Gene sequence	Number of patients
Wild type	58
A2142G	9 (7)
A2143G	31 (13)

Fig. 1. Comparison of mutations in the *H. pylori* 23S rRNA gene isolated from faeces with CAM susceptibility of *H. pylori* isolates. Mixed type indicates that both wild type and mutant (A2142G or A2143G) 23S RNA genes were detected simultaneously in these patients. The total number of patients is shown in parentheses.

(A2142G or A2143G) and mixed-type infection were detected in five (6.2%) and 18 (22.2%) patients, respectively; thus, overall the mutation was detected in 23 of the patients (28.4%) with CAM-susceptible *H. pylori* isolates. Conversely, the wild type alone was not detected in 17 patients with CAM-resistant *H. pylori* isolates, but mixed-type infection was detected in two of these patients (11.8%).

Correlation between successful therapy and detection of the resistant mutant *H. pylori* 23S rRNA gene from faeces

In a total of 128 patients who had had no previous therapy, 49 patients were treated with an LAC regimen consisting of 30 mg lansoprazole, 750 mg amoxicillin and 400 mg CAM, each given twice daily for 7 days. This regimen is generally used for *H. pylori* eradication chemotherapy in Japan. Of the 49 patients, 40 (81.6%) were treated successfully, whilst in nine (18.4%) the treatment was unsuccessful. Fig. 2 shows the susceptibility to CAM and the mutations in the 23S rRNA gene from faecal samples among the patients treated with the LAC regimen. Of the 40 patients treated successfully, CAM-resistant *H. pylori* culture isolates were detected in two patients (5.0%), and a mutation (A2142G, A2143G or mixed type) in the *H. pylori* 23S rRNA gene was detected in faecal samples from ten patients (25.0%). Similarly, CAM-resistant *H. pylori* culture isolates were detected in two of the nine patients (22.2%) in whom treatment was unsuccessful, whereas the mutated *H. pylori* 23S rRNA gene was detected in faecal samples from six of the nine patients (66.6%).

DISCUSSION

In this study, we developed an improved method to detect CAM-resistant *H. pylori* from faeces and compared our method with HpSA ELISA and culture methods using 146 faecal samples.

We previously reported a method for the extraction of DNA from faeces using a combination of enzymic lysis and physical crushing of the faeces (Rimbara *et al.*, 2005b). In

Fig. 2. Analysis of mutations in the 23S rRNA gene isolated from faeces and susceptibility to CAM among patients treated using the LAC regimen. Values are percentages. See Fig. 1 legend for explanation of mixed-type infection.

this study, we improved the composition of the beads and lysis buffer and omitted the enzymic incubation for cell lysis used in our previous method. This resulted in a more efficient and simpler method for isolation of DNA from the coccoid *H. pylori* cells in faeces. Furthermore, by reducing the amplification time for the first PCR in the nested PCR, we improved the efficiency of the nested PCR and were able to use a less expensive DNA polymerase. Generally, *H. pylori* DNA is extracted from faeces by chemical cell lysis using a commercial kit (Booka *et al.*, 2005; Fontana *et al.*, 2003). Compared with the commercial chemical cell lysis kit method, our improved method was able to extract DNA efficiently from a small amount of faeces and to amplify the 23S rRNA gene of *H. pylori* with greater sensitivity than the commercial kit (Fig. 3). Therefore, this method, which efficiently crushed the *H. pylori* coccoid form to yield detectable DNA, may be useful for the isolation of bacterial DNA, including that of *H. pylori*, from faeces.

Although many methods for the diagnosis of *H. pylori* infections have been reported, including culture (Goodwin *et al.*, 1985), the ^{13}C -urea breath test (Graham *et al.*, 1987) and HpSA ELISA (Vaira *et al.*, 1999), a combination of two methods is recommended for a more reliable diagnosis (Ito

et al., 2005; Nurgalieva *et al.*, 2006). To reduce the inconvenience for patients, we recommend non-invasive methods for the diagnosis of *H. pylori* infection. We found that the method developed in this study was able to detect CAM-resistant *H. pylori* in a non-invasive way with a sensitivity and specificity equal to HpSA ELISA and the invasive culture method.

It was also shown that false-negative results using the nested PCR were the result of the faecal samples containing a significant amount of moisture or fibre, or of an insufficient amount of faeces. We found that the false negatives could be resolved by replacement with another sample from the same patient (data not shown). Therefore, it is possible to select a successful therapy for the eradication of *H. pylori* using the method developed here.

For rapid detection of the mutation in the 23S rRNA gene, we performed a PCR-RFLP assay using *Mbo*II and *Bsa*II (data not shown). Whilst a simple mutation of either A2142G or A2143G could be detected, it was difficult to demonstrate clear restriction patterns in the case of mixed infections. In the diagnosis of CAM resistance in *H. pylori*, accuracy is more important than rapidity, as the progress of *H. pylori* infection is gradual. Therefore, we detected the mutation in the 23S rRNA gene by DNA sequencing.

It was shown that mixed infections with both CAM-susceptible and -resistant *H. pylori* occurred in individual patients. We reported previously using culture that 39 % of patients with CAM-resistant *H. pylori* were infected with both CAM-resistant and -susceptible *H. pylori* (Rimbara *et al.*, 2005a). Wong *et al.* (2001) also report a high prevalence of mixed infections with metronidazole-resistant and -susceptible strains using culture. This indicates the need to test for the susceptibility of *H. pylori* isolates using more than two gastric specimens obtained from different sites in the stomach of a single patient to diagnose the presence of CAM-resistant *H. pylori* correctly. We showed that mixed infections could be detected non-invasively using the method developed here. Schabereiter-Gurtner *et al.* (2004) also reported mixed infections with CAM-susceptible and -resistant *H. pylori* in 4.4 % of patients (2/45) using a real-time PCR assay from faeces. In this study, a mixed-type infection was detected in 20/98 patients (20.4 %). These data strongly suggest that our method may detect mixed infections with high sensitivity, as the *H. pylori* DNA was extracted from the coccoid cells of *H. pylori* in faeces containing both sensitive and resistant cells. Furthermore, our nested PCR specifically amplified the 23S rRNA gene from a small amount of *H. pylori* DNA compared with the number of other bacteria in faeces. The data shown here may explain the highly sensitive detection rate in mixed infections.

In the case of mixed infections, CAM-resistant *H. pylori* may be selected using therapy including CAM, which consequently results in failure of the therapy. Although there have been several reports of mixed infections, the influence of mixed infections on the results of eradication

Fig. 3. Comparison of methods for the isolation of DNA from faeces in *H. pylori*-positive (1 and 2) and -negative (3) samples. (a) Electrophoresis of DNA isolated from faeces (1 % agarose gel). Lane M1, *Hind*III-digested λ DNA; lanes a and b, DNA isolated from 200 and 50 mg faeces, respectively, using a QIAamp DNA Stool Mini kit; lanes c, DNA isolated from 50 mg faeces using the method developed in this study. (b) Agarose gel (2.5 %) electrophoresis of the nested PCR *H. pylori* 23S rRNA gene amplification product (arrow) from DNA isolated as described in (a). Lane M2, 100 bp DNA ladder.

therapy has not been well studied. In this study, we evaluated the influence of mixed infections in patients treated using an LAC regimen, which is the regimen used most frequently in Japan (Asaka *et al.*, 2001). We found that the proportion of patients with a mutation in the 23S rRNA gene (including mixed type) was 66.6 % of patients who had been treated unsuccessfully. Recently, it has been reported that 16.4 % of treatment failures showed mixed infections with antibiotic-susceptible and -resistant *H. pylori* (Kim *et al.*, 2006). The data suggest that the existence of both CAM-resistant and -susceptible *H. pylori* may be the reason for therapy failure.

Moreover, CAM-susceptible *H. pylori* was detected in 33.3 % of patients treated unsuccessfully using the LAC regimen. It has been reported that single nucleotide polymorphisms of cytochrome P450 2C19 are significantly related to eradication rates of *H. pylori* by triple therapy as well as susceptibility to CAM (Furuta *et al.*, 2001). Although single nucleotide polymorphism analysis was not performed in this study, such polymorphisms may be the reason for the failure of therapy in these patients.

In conclusion, we have developed a method for the detection of CAM-resistant *H. pylori* from faeces, including mixed infection with CAM-susceptible and -resistant *H. pylori*. Use of this method showed that mixed infections of resistant and susceptible *H. pylori* were prevalent. Therefore, as mixed infections are common and may be the reason for unsuccessful therapy, an accurate test for the presence of CAM-resistant *H. pylori* is necessary for successful eradication therapy.

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