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Gastric Wash-Based Molecular Testing for Antibiotic Resistance in *Helicobacter pylori*

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Key Words

Helicobacter pylori · Gastric washes · Gastric cancer · Diagnosis

Abstract

Background: A number of noninvasive tests have been developed to establish the presence of *Helicobacter pylori* infection. However, thus far these tests have only been capable of detecting its presence. An increasing number of antibiotic-resistant *H. pylori* infections have been reported and they are known to be correlated with 23S rRNA single nucleotide polymorphisms (SNPs). We hypothesized that genomic analysis of *H. pylori* recovered from gastric washes could not only be less invasive, but also useful as a screening test and for assessing the outcome of eradication therapy. ***Methods:*** Biopsy specimens and gastric washes were collected from 100 patients during endoscopic examination. Then we analyzed 23S rRNA, *ureA*, and *cagA* genes using PCR and high-throughput pyrosequencing analysis. ***Results:*** Forty-five percent (44/97) of patients tested positive for *ureA* and 42.3% (41/97) tested positive by a rapid urease test. One hundred percent (35/35) of patients who tested positive by both methods were observed to have the *cagA* gene. Among these 35 patients, 23S rRNA SNPs were present in 34.3% (12/35). ***Conclusions:*** Gastric wash-based PCR and a pyrosequencing assay were used to rapidly detect and estimate the number of 23S rRNA SNPs in clinical isolates of *H. pylori*. Not only is this a less invasive technique, but it can also diagnose drug resistance.

Satoshi Baba and Yoshichika Oishi contributed equally to this work.

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Introduction

Helicobacter pylori is commonly known to be associated with gastritis and peptic ulcers. Patients can be tested for the presence of *H. pylori* via invasive or noninvasive methods, both of which have merits and demerits. Although serology for IgG often is chosen in the outpatient setting due to its convenience, it is less accurate (85% sensitivity, 79% specificity) than both the stool antigen (91–98% sensitivity, 94–99% specificity) and urea breath tests (95–100% sensitivity, 91–98% specificity) [1–7]. However, all of the above-mentioned methods are still insufficient because they only detect its presence and are unreliable in diagnosing 'sensitivity of *H. pylori* eradication'.

For '*H. pylori* eradication', all *H. pylori* strains were shown to be susceptible to amoxicillin, but 27.7–34.5% of the strains were resistant to clarithromycin [8, 9]. Nucleotide sequence comparisons revealed that clarithromycin resistance among isolates had a single base-pair mutation in 23S rRNA [10, 11]. These reports suggest the importance of evaluating drug resistance in patients before treatment. However, high-throughput and less invasive methods are still lacking [12].

In this study, we investigated pyrosequencing as a rapid and high-throughput method for genotyping single nucleotide polymorphisms (SNPs) associated with clarithromycin resistance in 23S rRNA using gastric washes. Pyrosequencing allows direct sequencing by the synthesis of short fragments of DNA by a novel enzymatic-cascade system [13]. Sequences generally do not require manual interpretation, which provides consistency in scoring and the interpretation of results [14, 15].

We hypothesized that the gastric wash-based assay by high-throughput pyrosequencing analysis (*ureA*, *cagA*, SNPs in 23S rRNA) can be useful not only for detection, but also in the assessment of the outcome of eradication therapy for *H. pylori*.

Materials and Methods

Patient Characteristics and Sample Collection

Gastric mucosa tissues and gastric wash samples were collected from Japanese patients who underwent endoscopic examination for screening purposes at St. Marianna University School of Medicine Hospital (Kanagawa, Japan) from March 2009 to July 2009. None of the patients had received antimicrobial therapy for eradication of *H. pylori* before, nor had any of them been subjected to long-term administration of any antibiotic in the previous year. Samples from 200 stomach biopsies [2 sites (body and antrum) per patient] in conjunction with 100 gastric washes were obtained from 100 patients who came in for endoscopic examination with informed consent.

To obtain gastric washes, patients were required to swallow a liquid solution [100 ml of water containing 80 mg of dimethylpolysiloxane (Gascon; Kissei Pharmaceutical Co. Ltd., Matsumoto, Japan), 1 g of sodium bicarbonate, and 20,000 units of pronase (Pronase MS; Kaken Pharmaceutical Co. Ltd., Tokyo, Japan)] approximately 10 min prior to endoscopic examination. Gastric washes were aspirated through the suction channel of the endoscope into specimen collection containers (No. 16200BZZ00045; Nippon Sherwood, Tokyo, Japan). The containers were directly connected to the endoscope modulator, and the washes were vacuumed manually. The samples were immediately centrifuged, and the pellets were frozen at –80°C. DNA was extracted using the standard phenol-chloroform method. The concentration and quantity of all DNA extracted from gastric washes were measured using the NanoDrop spectrophotometer (ND-1000 Spectrophotometer; Nano Drop Technologies, Wilmington, Del., USA).

After collection of gastric washes, biopsies were carried out using biopsy forceps (Radial Jaw; Boston Scientific Corp., Natick, Mass., USA) under endoscopic guidance with a GIF-Q260 endoscope using the EVIS LUCERA system (Olympus Inc., Tokyo, Japan). Mucosal samples (each approx. 5 mm in diameter) of the gastric body and antrum were collected by biopsy. The rapid urease test (RUT) was performed on 2 biopsy specimens using the PyloriTek test kit (Serim Research Corp., Elkhart, Ind., USA). We used disposable sample collection tubes, connector tubes, and endoscopic devices. The endoscope was washed with an automatic washing machine and disinfectant (DISOPA Solution 0.55%; Johnson and Johnson, Langhorne, Pa., USA) after each patient according to the guidelines.

This study protocol was approved by the institutional review board of St. Marianna University School of Medicine, and informed consent was obtained from each of the patients.

Detection of H. pylori *Using RUT and Serum Anti-*H. pylori *IgG Test*

We confirmed *H. pylori* infection using an approved RUT kit (PyloriTek test kit) as a reference analysis. Additionally, we confirmed *H. pylori* infection using a serum anti-*H. pylori* IgG test (SBS, Kanazawa, Japan) as an alternative method when there appeared to be a discrepancy between the results of RUT and gastric washes.

Detection of H. pylori*-Related Genes Using DNA from Gastric Washes*

PCR was performed using HPU1 and HPU2 primers to evaluate the presence of the *ureA* gene and to demonstrate *H. pylori* infection [16]. PCR for *cagA* was performed to type the *H. pylori* strains using cagA1 and cagA2 primers [17]. *ureA* and *cagA* PCR assays included a denaturation step at 95°C for 1 min, followed by an annealing step at various temperatures for 1 min, and an extension step at 72°C for 2 min (online suppl. table 1, see www.karger.com/doi/10.1159/000332570). To confirm positive results, we verified the appropriate band by using agarose gel electrophoresis.

Pyrosequencing Analysis of SNPs

One microliter of DNA was used as a template in subsequent PCR. Primers and PCR conditions used for amplifying appropriate sequences (targeted at A2143G, A2143C, and A2144G) of 23S rRNA are listed in online supplementary table 1. For this assay, we used biotinylated PCR. PCR assays included a denaturation step at 95°C for 30 s, followed by an annealing step at 60°C for 30 s, and an extension step at 72°C for 30 s. After PCR, the biotinylated strand was captured on streptavidin-coated beads (Amersham Bioscience, Uppsala, Sweden) and incubated with sequencing primers (online suppl. table 1).

Pyrosequencing was performed using PSQ HS 96 Gold SNP reagents on a PSQ HS 96 pyrosequencing machine (Biotage, Uppsala, Sweden). Pyrosequencing reactions were performed according to the manufacturer's instructions using the PSQ 96 SNP reagent kit, which contained enzymes, substrates, and nucleotides. The sample genotype was determined using SNP Software (Biotage, Uppsala, Sweden).

Eradication Therapy for Patients Infected with H. pylori

Fifteen patients who tested positive for *H. pylori* by both the biopsy-based RUT and gastric wash-based PCR assay *(ureA)* were treated with the standard *H. pylori* eradication regimen (rabeprazole 20 mg/day, amoxicillin 1,500 mg/day, and clarithromycin 400 mg/day for eradication of 7 days). A urea breath test was performed on each patient 4 weeks after treatment.

Statistical Analysis

All statistical analyses were performed using PRISM software for Windows, version 4 (GraphPad Prism Inc., San Diego, Calif., USA). To evaluate significant differences between the data of two independent groups of samples, the Mann-Whitney U test was employed. All reported p values were two-sided, and $p < 0.05$ was considered statistically significant.

Results

Clinical Features of Patients Investigated in This Study

This was a prospective, blinded single-center study. A total of 100 patients were approached to participate in the study. However, 3 patients were excluded because they dropped out of the study after endoscopic examination. Thus, 97 patients [45 males (46.4%, mean age ± SD = 58.4 ± 16.5 years), 52 females (53.6%, 58.4 ± 16.4 years)] were included in this study (table 1). Of the 97 (100.0%) patients studied, 56 patients (57.7%) tested negative for *H. pylori* infection by RUT. Of the 41 patients who tested positive for *H. pylori* infection, 15 patients (36.6%) underwent eradication therapy (a combination of proton pump inhibitor, amoxicillin, and clarithromycin). *H. pylori* infection was successfully eradicated in 11 (73.3%) of these patients (table 2).

Table 1. Clinical features of patients

	n	Age ± SD years	RUT positive
Total	97	58.4 ± 16.4	
Male	45	58.4 ± 16.5	
Female	52	58.4 ± 16.4	
Endoscopic diagnosis	97		
Gastric neoplasias			80.0% (4/5)
Gastritis			34.3% (24/70)
Gastric ulcers			61.5% (8/13)
Duodenum ulcers			55.6% (5/9)

Table 2. Pyrosequencing analysis of SNPs

23S rRNA SNPs (n = 35)		
Wild type	65.7%	23/35
A2143G	0%	0/35
A2143C	0%	0/35
A2144G	34.3%	12/35
Successful eradication therapy (n = 15)		
Wild type (n = 10)	90.0%	9/10
A2144G (n = 5)	40.0%	2/5

Valuable Volume of Genomic DNA Recovered from Gastric Washes

To collect genomic DNA recovered from gastric washes, samples from each of the 97 patients were collected during endoscopic examination. The genomic DNA was then extracted using the standard phenol-chloroform method. The total volume of genomic DNA in each patient (73.4 ± 121.4 μg) was analyzed with a NanoDrop ND-1000 spectrophotometer (Nano Drop Technologies, Wilmington, Del., USA). The amount of DNA in the RUT-positive samples was significantly higher than in the negative samples (fig. 1).

Detection of H. pylori *Using RUT and DNA from Gastric Washes*

We compared the detection capabilities between our method (gastric washes) and RUT. RUT is one of the available methods for detecting *H. pylori* infection using an endoscope and is widely accepted as a screening test. The detection rate of *H. pylori* infection, as determined by RUT, was higher in subjects with gastric neoplasms or GI ulcers than in subjects with gastritis (table 1). PCR from gastric washes were used to evaluate the presence of *ureA* (GW-*ureA*), and PCR for *cagA* from gastric washes

Table 3. Detection of *H. pylori* infection

	n	%
H. pylori detection		
RUT (+)	41	42.3
GW-*ureA* (+)	35	
N/A	–	
GW-*ureA* (–)	6	
H. pylori IgG (+)	3	
H. pylori IgG (–)	3	
RUT (–)	56	57.7
GW-*ureA* (+)	9	
H. pylori IgG (+)	4	
H. pylori IgG (–)	5	
GW-*ureA* (–)	47	
N/A	–	
Sensitivity and specificity of gastric wash-based assay (GW-*ureA*)		
Sensitivity		85.4
Specificity		83.9
GW-*cagA*		
Positive in both RUT and GW-*ureA* (+)	35	100

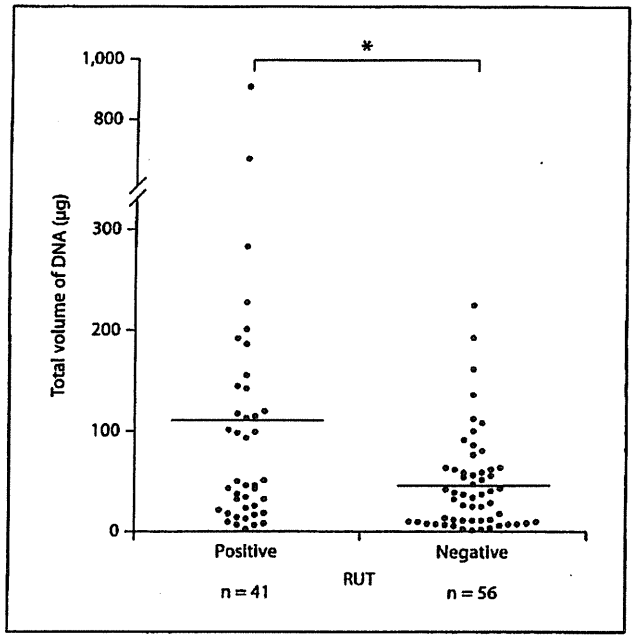

Fig. 1. Comparison of DNA quantity obtained in different categories of samples. RUT positive: genomic DNA from gastric washes obtained in RUT-positive patients; RUT negative: genomic DNA from gastric washes obtained in RUT-negative patients from 97 cancer-free individuals (* p = 0.002). DNA volumes of RUT-positive samples were significantly higher than in negative samples.

were performed to type the *H. pylori* strains. The proportion of concordant tests between RUT and GW-*ureA* was 85.4% (35/41; table 3). The sensitivity of the GW-*ureA* was 85.4% and specificity was 83.9% when DNA was detected by electrophoresis (table 3). Nonconcordant results were found in 15 patients. Six of 41 patients tested negative for *H. pylori* by GW-*ureA*, but positive by RUT. On the other hand, 9 of 56 tested negative for *H. pylori* by RUT, but positive by GW-*ureA* (table 3). We then confirmed *H. pylori* infection using another test (IgG antibodies against *H. pylori* by an indirect ELISA using each patient's serum) in these 15 cases. Based on the results, 5 patients were presumed to be possibly false-positive [RUT (–), HP IgG (–), GW-*ureA* (+)] and 3 patients false-negative [RUT (+), HP IgG (+), GW-*ureA* (–)].

We were able to detect *cagA* and analyze 23S rRNA SNPs in 35 cases that were positive by both RUT and GW-*ureA*. In *cagA* gene analysis using PCR from gastric washes, 35 of 35 patients (100.0%) had a *cagA* gene in *H. pylori* (table 3).

23S rRNA Analysis of H. pylori *DNA Recovered from Gastric Washes*

Mutations in the *H. pylori* 23S rRNA gene were revealed in 12 of 35 (34.3%) gastric washes obtained from endoscopic examination. A mutation was found only in the A2144G region of the 23S rRNA gene in each of the 12 cases (fig. 2, table 2). Fifteen patients, including 5 infected with mutant strains, received the standard *H. pylori* eradication regimen (rabeprazole 20 mg/day, amoxicillin 1,500 mg/day, and clarithromycin 400 mg/day for 7 days). Eradication was achieved in 2 of the 5 (40.0%) patients with a mutant infection and 9 of the 10 (90.0%) patients with a wild-type infection (table 2).

Discussion

H. pylori is a ubiquitous Gram-negative bacterium that grows in the mucus layer that coats the inside of the human stomach, despite the stomach's acidic environment [18]. People who are infected with *H. pylori* almost always develop chronic gastritis [19]. In addition, between 2 and 20% of people infected with *H. pylori* will develop peptic ulcers [20]. Moreover, epidemiology studies have shown that individuals infected with *H. pylori* have an increased risk of gastric adenocarcinoma [21, 22].

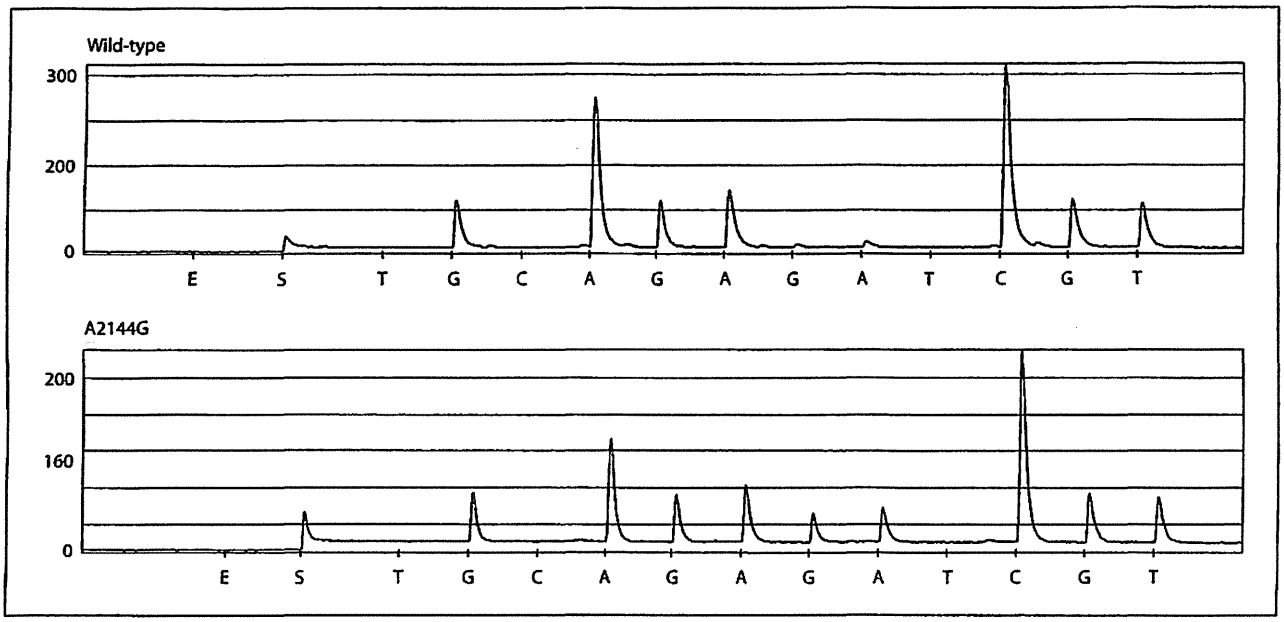

Fig. 2. Pyrogram of *H. pylori* 23S rRNA gene mutation analysis in gastric washes. A mutation was found only in the same region (A2144G) of the 23S rRNA gene in each of the 14 cases (lower part of pyrogram).

A number of less invasive tests have been developed to establish the presence of *H. pylori* infection. However, these tests are only for detection of the presence of *H. pylori*. To assess the outcome of eradication therapy and analyze some strains of *H. pylori*, clinical application of the PCR assay may be a possible method. Additionally, some researchers have already reported the use of a gastric juice-based PCR assay [23–28] to detect *H. pylori* infection [12, 29]. PCR assay on gastric washes (gastric juice and gastric aspirates) have an advantage over conventional methods which require gastric biopsy specimens, such as bacterial culture and RUT. Moreover, Kawamata et el. [30] discovered a more sensitive assay using nested PCR.

In our study, the volume of DNA recovered by means of gastric washing was not only enough to detect *ureA* and *cagA* genes in the PCR-based method, but also enough to analyze 23S rRNA SNPs by pyrosequencing (fig. 2). According to the results of the RUT method, 56 patients (56/97, 57.7%) showed no evidence of *H. pylori* infection, while the remaining 41 patients (41/97, 42.3%) were infected with *H. pylori*. Nonconcordant results were found in 15 patients. Six of 41 were negative for *H. pylori* by GW-*ureA*, but positive by RUT. On the other hand, 9 of 56 were negative for *H. pylori* by RUT, but positive by GW-*ureA* (table 3). To confirm *H. pylori* infection, we carried out another test (IgG antibodies against *H. pylori* by an indirect ELISA using each patient's serum), by which 3 of 6 were presumed to be false-negative and 4 of 9 false-positive (table 3).

Data concerning the presumed false-positive results from GW-*ureA* is likely due to the high sensitivity of the method. Another possible explanation for this discordant result could be the differences in the range of detection since gastric washes cover a larger stomach surface than biopsies. Additionally, since the PCR assay is based on DNA evaluation, contamination of gastric fluids or endoscopic equipment is possible and may result in false-positive results [31]. This suggestion should be ruled out in the present study, though, as scrupulous routine procedures in conformance to guidelines were adopted in the process of disinfecting both the endoscope and the accessories [32].

Therefore, it is possible that in our 3 false-negative cases, the GW-*ureA* PCR assay gave negative results because the *H. pylori* DNA, though present, was degraded in the gastric washes. Additionally, Kawamata et al. [30] mentioned in their publication that the HPU1 primer that they used refers to the targeted region where Clayton et al. [16] found nucleotide variation to be frequent. Although the variation was found on the 5′ side and thus

would not significantly affect PCR amplification compared to variation on the 3′ side, the difference of conservation may still have contributed to the difference of sensitivity between the PCR assay and RUT or IgG antibodies against *H. pylori* [30]. We then analyzed the *cagA* gene of *H. pylori* using samples of gastric washes by using specific primers. We were able to detect *cagA* in 35 cases that were positive by both RUT and GW-*ureA*. In *cagA* gene analysis using PCR from gastric washes, 35 of 35 patients (100.0%) had a *cagA* gene with their *H. pylori* (table 3).

An increasing number of antibiotic-resistant *H. pylori* infections have been reported, and they are known to be correlated with SNPs in multiple alleles of 23S rRNA. According to the recent clinical reports, 27.7–34.5% of the strains were resistant to clarithromycin [8, 33]. Common regions of the mutation are at 2143 or 2144 in domain V of the 23S rRNA gene of *H. pylori*. For genotyping analysis, conventional molecular methods used to evaluate key drug (clarithromycin) resistance includes conventional DNA direct sequencing, allele-specific PCR, PCR-restriction fragment length polymorphism (RFLP) analysis, dot blot/probe hybridization techniques, real-time PCR, and the SNaPshot primer extension method [24, 28, 30, 34–38]. Each of these techniques offers its own advantages and limitations. Among the techniques, RFLP is one of the most commonly used, but is laborious and expensive. Therefore, a cost-effective and high-throughput genotyping method would be ideal for large-scale population-based studies. Pyrosequencing was reported to be more sensitive than RFLP and direct sequencing [38].

The novel 23S rRNA SNP was analyzed from 35 (35/35, 100%) samples of gastric washes by pyrosequencing analysis. Our results showed the same rate of mutation as that of the previous report, where 12 (12/35, 34.3%) of the DNAs recovered from gastric washes showed genetic alteration (SNP) in 23S rRNA (table 2). Moreover, in all of the mutations we saw an adenine (A) to guanine (G) mutation at 2144 of the 23S rRNA gene of *H. pylori* (fig. 2, table 2).

Finally, we compared drug sensitivity (eradication with clarithromycin) with the results of SNP analysis (A2144G mutation) in our study. Although there were a limited number of samples, there seems to be a good correlation with drug sensitivity (table 2). We confirmed that we can use gastric washes, which are generally thrown out as waste during endoscopic examination, in the PCR and pyrosequencing assay. We also identified that it can detect *H. pylori* infection (*ureA*, *cagA* gene) with high sensitivity as in previous reports, allowing us to simultaneously evaluate clarithromycin resistance using gastric washes by high-throughput pyrosequencing analysis.

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Disclosure Statement

There are no conflicts of interest to disclose.

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