

Assessment of a novel method to detect clarithromycin-resistant *Helicobacter pylori* using a stool antigen test reagent

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Abstract

Background

The resistance rate of *Helicobacter pylori* to clarithromycin (CAM) is high among the infected children in Japan; therefore, a new method for detecting CAM-resistant *H. pylori* using a less invasive technique is strongly desired. We aimed to confirm the clinical usefulness of our newly developed Nested polymerase chain reaction-QProbe (quenching probe) (Nested PCR-QP) method using stool specimens.

Methods

The first was an evaluation of our method using the residual solution of the *H. pylori* stool antigen test for adolescents, and the second was an evaluation of our method using culture testing for adults.

Results

Of the 57 middle school students with *H. pylori*, the Nested PCR-QP test results of 53 (90.3%) could be analyzed; 28 students were found to have CAM resistance mutations. The results indicating genetic mutation in 28 and no mutation in 23 students were consistent with those of PCR-direct sequencing. In the 23 adults who were diagnosed with *H. pylori* infection using the rapid urease test and culture testing, it was possible to use Nested PCR-QP for analyzing all 21 adults who tested positive in the stool *H. pylori* antigen test. The results obtained for all the 21 adults were consistent with those obtained via the drug susceptibility test.

Conclusions

Our novel method could be useful for non-invasively detecting CAM resistance mutations in *H. pylori*. This may help select an eradication drug to reduce eradication failure rates against *H. pylori*.

Background

Helicobacter pylori (*H. pylori*) infects the gastric mucosa, where it causes chronic inflammation, which, in turn, increases the risk of gastric cancer [1–4]. It is known that *H. pylori* eradication reduces the risk of gastric cancer [3, 5–8]. It is also believed that *H. pylori* eradication is more effective in reducing the risk of gastric cancer in younger individuals than in elderly individuals who have suffered from chronic gastritis for long duration [9–14].

While the age-standardized incidence and mortality rates of gastric cancer are decreasing among both men and women, gastric cancer has the highest incidence in men and the third-highest incidence in women among all cancers. Furthermore, gastric cancer mortality is the second-highest in men and third-highest in women among all cancer mortalities [15]. Recently, the number of municipalities in Japan that perform *H. pylori* testing and eradication in middle school students for preventing gastric cancer has been increasing [16, 17]. In 2016, Saga Prefecture became one of the first prefectures in Japan to begin testing for *H. pylori* in all 3rd-year middle school students in the prefecture and performing eradication therapy for those who tested positive [18]. This effort is part of the “*A Helicobacter pylori* screening and treatment program to eliminate gastric cancer among junior high school students in Saga Prefecture.” While the *H. pylori* tests being conducted throughout Japan differ across municipalities, many regions utilize the urine *H. pylori* antibody test as the primary test and then utilize either the urea breath test or the stool *H. pylori* antigen test as the secondary test [19–21].

Eradication therapy is performed for those who test positive in the secondary test. As the targeted patients are children, eradication drug selection is extremely important from the perspectives of safety and efficacy. The conventional treatment for *H. pylori* infection is clarithromycin (CAM) administration [22, 23]. As *H. pylori* CAM resistance rate is high among young people in Japan [24, 25], CAM resistance must be assessed via drug sensitivity testing prior to the selection of an effective and safe eradication drug. However, as the drug susceptibility test used on strains cultured from gastric biopsy tissue involves a highly invasive test sample collection procedure, it places a heavy burden on patients. This makes it difficult to perform the testing in children, particularly in those who are asymptomatic. It is known that the major cause of CAM resistance in *H. pylori* is 23S rRNA genetic mutations (A2142C, A2142G, A2143G) [26]. In recent years, there have been reports on a genetic test for detecting *H. pylori* CAM resistance utilizing a non-invasive stool test. The sensitivity of such tests, however, remains insufficient (60–80%), and the specificity has not been fully evaluated [27–29].

Therefore, we developed the Nested polymerase chain reaction-QProbe (quenching probe) (Nested PCR-QP) method to detect 23S rRNA genetic mutations that are associated with CAM resistance with a high degree of sensitivity using the fluid remaining after the stool antigen test. The objective of this study was to confirm the clinical usefulness of the novel Nested PCR-QP method that utilizes stool specimens.

Methods

To confirm the clinical usefulness of Nested PCR-QP using stool specimens, we designed two clinical studies. The first was a method using the residual solution of the *H. pylori* stool antigen test for adolescents, as it was difficult to perform esophagogastroduodenoscopy (EGD) in adolescents, and the second was a method using culture testing for adults.

1. Nested PCR-QP detection sensitivity and correlation with eradication therapy

The subjects were middle school students in Saga Prefecture who underwent *H. pylori* testing between August and December 2017 under our program to eliminate gastric cancer. Consent to participate in the study was obtained from 71 middle school students. These 71 students who tested positive in the urine *H. pylori* antibody test (RAPIRAN®; Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan) as the primary test underwent stool *H. pylori* antigen testing (Testmate Rapid Pylori Antigen®; Wakamoto Pharmaceutical Co., Ltd. Tokyo, Japan) as the secondary test. Among these students, the secondary test results were positive for 57 and negative for 14. Thus, the 57 patients who tested positive in the secondary test were judged to be infected with *H. pylori*. We then performed Nested PCR-QP measurements using the leftover specimens from the stool antigen tests that the 57 students testing positive had undergone. DNA sequencing analysis was performed for all 57 samples by carrying out Nested PCR-QP using PCR-direct sequencing. Students who tested positive in the stool antigen test underwent a 7-day treatment comprising 20 mg vonoprazan (VPZ, Takeda Pharmaceutical Co., Ltd. Tokyo, Japan), 750 mg amoxicillin (AMPC), and 200 mg clarithromycin twice a day, regardless of the results regarding CAM resistance mutations. Confirmation of the eradication therapy was conducted using the urea breath test (UBIT® tablet, 100 mg and POC One®; Otsuka Electronics Co., Ltd. Hirakata, Japan).

2. Nested PCR-QP in relation to culture test and drug susceptibility test

Considering that EGD was not eagerly performed in asymptomatic children, we planned another study on adults at the Imamura Hospital (Tosu, Japan). The subjects were 23 adults who were diagnosed with *H. pylori* infection on the basis of the results of rapid urease test (RUT; Helicocheck®; Institute of Immunology, Co., Ltd., Tochigi, Japan) and culture testing performed at the hospital, where they had undergone EGD between February 2018 and September

2018. The culture testing and drug susceptibility testing (H. pylori drug sensitivity test: H. pylori MIC measurement) was performed by BML, Inc. We utilized the breakpoints recommended by the Japan Society of Chemotherapy for CAM and amoxicillin [30] and the breakpoints listed in the EUCAST Trial breakpoint table v.8.1 for metronidazole (MNZ) [31]. Stool specimens were used in the stool H. pylori antigen test, and the remnant solution of those who tested positive was used to perform Nested PCR-QP measurements.

3. Nested Pcr-qp

Nested PCR-QP is a novel genetic analysis method that analyses 23S rRNA genetic mutations (A2142C, A2142G, and A2143G) that are associated with CAM resistance in H. pylori. The basic components are the Nested PCR and QProbe [32]. The sample used in Nested PCR-QP is stool H. pylori antigen test remnant solution. DNA is extracted from the Nested PCR-QP samples measuring 100 µL using QIAamp® DNA Mini kit(QIAGEN GmbH, Hilden, Germany)to obtain 150 µL of DNA solution. This DNA solution is the 1st PCR template for Nested PCR-QP.

The 1st PCR primers are Hp23S 1835F and Hp23S 2327R in accordance with the method described by Noguchi et al [33]. The 1st PCR utilizes a 1 µL template created using a T100 Thermal Cycler (Bio-Rad Laboratories Inc., Hercules, CA, USA) and is performed using Ampdirect plus BIOTAQ HS DNA Polymerase (Shimadzu corp., Kyoto, Japan). The PCR reaction protocol was as follows: After primer annealing for 10 min at 95°C, 36 cycles were performed at 94°C for 30 s, 50°C for 60 s, and 72°C for 60 s.

The 1st PCR reaction mixture diluted 20-fold in sterilized water was used in the 2nd PCR template; the 2nd PCR primer was the newly designed HP F1(5'-CCAGAGATTCAGTGAAATTGTAGTGGAGGTG-3') and HP R3(5'-GGCTCCATAAGAGCCAAAGCCCTTAC-3'), and the probe utilized was the newly designed HP QP1(5'-CCGCGGCAAGACGGAAAGAC-BODIPY-3'). The 2nd PCR and melting curve analysis were performed using a 5 µL template from LightCycler Nano (Roche Diagnostics K.K., Tokyo, Japan) and KOD DNA Polymerase (Toyobo Co., Ltd, Osaka, Japan). The PCR reaction protocol was as follows: After primer annealing for 2 min at 98°C, 65 cycles were performed at 95°C for 5 s and 55°C for 15 s. Melting curve analysis was performed as follows: After the 2nd PCR, warming was performed from 40°C to 80°C at a rate of 0.09°C/s, and measurements were obtained using fluorescent light with a wavelength of 510–528 nm. Melting curve analysis using fluorescence quenching allowed us to determine that there were no mutations when the fluorescence quenching inflection point was between 67°C and 71°C (wild type) but there were mutations between 59°C and 63°C (A2142G, A2143G) and between 63°C and 67°C (A2142C).

4. Statistical Analysis

The effectiveness of eradication therapy was compared between the Nested PCR-QP mutation group and the non-mutation group using chi-square test. Statistical significance was set at a P-value of < 0.05. Statistical analyses of the data were performed using JMP Pro13 (SAS Institute Inc., Cary, NC, USA).

Results

1. Assessment of CAM resistance genetic mutations using Nested PCR-QP

The results of stool H. pylori antigen testing and Nested PCR-QP performed in the 71 students who tested positive in the urine H. pylori test, which was used as the primary test for H. pylori, are shown in Table 1. Of the 57 students who tested positive in the stool H. pylori antigen test, Nested PCR-QP analysis was possible for 53 students (93.0%). Twenty-eight (52.8%) of these 53 students were found to have CAM resistance mutations: 1 student had A2142C and

27 students had A2143G. None of the students had the A2142G mutation. Twenty-five students did not have any CAM resistance mutation. These results were consistent with those of PCR-direct sequencing.

Table 1
Determination of CAM resistance mutation using
Nested PCR-QP

Fecal antigen test	Nested PCR-QP	(n = 71)
Positive	Mutation	28
	Wild type	25
	Not detected	4
Negative	Not tested	14
PCR: polymerase chain reaction, QP: Q probe		

Investigation of the eradication therapy results in 41 students for whom it was possible to conduct follow-up examinations indicated that CAM resistance mutations affected eradication therapy. The eradication therapy success rate in the non-mutation group was 94.4% (17/18), whereas in the CAM resistance mutation group, it was 82.6% (19/23; P = 0.25; Table 2). The selection of the eradication therapy drugs did not change according to CAM resistance mutations.

Table 2
Effect of CAM resistance mutation on eradication therapy

		Eradication therapy		P-value*
		Success (n = 36)	Failure (n = 5)	
Nested PCR-QP	Mutation (n = 23)	19	4	0.25
	Wild type (n = 18)	17	1	
PCR: polymerase chain reaction, QP: Q probe*Fisher's exact test				

2. Nested PCR-QP in relation to culture test and drug sensitivity test

The results of culture and drug susceptibility testing as well as stool H. pylori antigen test and Nested PCR-QP performed in 23 adults with H. pylori infection confirmed using RUT and culture testing are shown in Table 3. The results of drug susceptibility testing indicated that the CAM resistance rate was 30.4% (7/23), AMPC resistance rate was 4.3% (1/23), and MNZ resistance rate was 34.8% (8/23). The sensitivity of the stool H. pylori antigen test was 91.3% (21/23). No CAM resistance mutation was detected in Nested PCR-QP for two adults with a negative stool H. pylori antigen test result.

Table 3
Comparison of fecal antigen test and Nested PCR-QP to culture test results

	Sex	Age (y/o)	RUT	Culture test								FAT	Nested PCR -QP
Result	Drug susceptibility test												
	CAM		AMPC		MNZ								
	MIC	Result	MIC	Result	MIC	Result							
	(µg/mL)		(µg/mL)		(µg/mL)								
Case 1	M	55	+	3 +	<0.015	S	< 0.015	S	8	S	+	W	
Case 2	F	63	+	3 +	<0.015	S	< 0.015	S	16	R	+	W	
Case 3	M	63	+	3 +	<0.015	S	< 0.015	S	8	S	+	W	
Case 4	F	67	+	3 +	0.03	S	< 0.015	S	8	S	+	W	
Case 5	M	25	+	3 +	4	R	< 0.015	S	> 16	R	+	M	
Case 6	M	30	+	2 +	0.03	S	< 0.015	S	16	S	-	Not tested	
Case 7	F	61	+	3 +	4	R	< 0.015	S	> 16	R	+	M	
Case 8	F	83	+	3 +	<0.015	S	< 0.015	S	8	S	+	W	
Case 9	M	53	+	3 +	4	R	< 0.015	S	8	S	+	M	
Case 10	F	49	+	3 +	<0.015	S	< 0.015	S	16	R	+	W	
Case 11	F	63	+	3 +	<0.015	S	< 0.015	S	8	S	+	W	
Case 12	M	55	+	3 +	0.03	S	< 0.015	S	16	R	+	W	
Case 13	M	40	+	3 +	2	R	0.03	S	> 16	R	+	M	
Case 14	F	57	+	3 +	2	R	0.06	R	8	S	+	M	
Case 15	F	68	+	3 +	<0.015	S	< 0.015	S	8	S	+	W	

RUT: Rapid urease test, FAT: Fecal antigen test, CAM: Clarithromycin, AMPC: Amoxicillin, MNZ: Metronidazole, MIC: Minimum inhibitory concentration, S: Sensitive, R: Resistant, W: Wild type, M: Mutation

	Sex	Age (y/o)	RUT	Culture test				FAT				Nested PCR -QP
Case 16	M	50	+	3 +	0.06	S	< 0.015	S	16	R	+	W
Case 17	M	63	+	3 +	<0.015	S	< 0.015	S	8	S	+	W
Case 18	F	37	+	3 +	<0.015	S	< 0.015	S	8	S	+	W
Case 19	M	32	+	3 +	<0.015	S	< 0.015	S	8	S	+	W
Case 20	M	53	+	3 +	<0.015	S	< 0.015	S	8	S	+	W
Case 21	F	45	+	3 +	<0.015	S	< 0.015	S	4	S	–	Not tested
Case 22	M	45	+	3 +	2	R	< 0.015	S	16	R	+	M
Case 23	F	47	+	3 +	4	R	< 0.015	S	8	S	+	M
RUT: Rapid urease test, FAT: Fecal antigen test, CAM: Clarithromycin, AMPC: Amoxicillin, MNZ: Metronidazole, MIC: Minimum inhibitory concentration, S: Sensitive, R: Resistant, W: Wild type, M: Mutation												

The results of correlation testing between Nested PCR-QP and drug susceptibility testing are shown in Table 4. Of the 23 adults who underwent the tests, it was possible to use Nested PCR-QP to analyze 23S rRNA genetic mutations in all 21 adults who tested positive in the stool *H. pylori* antigen test. The CAM resistance mutation results for all these 21 adults were consistent with the results of drug susceptibility testing in culture tests.

Table 4
Coincidence rate of Nested PCR-QP for drug susceptibility test for CAM

Nested PCR-QP				
		Mutation (n = 7)	Wild type (n = 14)	Not tested (n = 2)
DST for CAM	Resistant (n = 7)	7	0	0
	Sensitive (n = 16)	0	14	2
PCR: polymerase chain reaction, QP: Q probe, DST: drug susceptibility test, CAM: clarithromycin				

Discussion

The novel Nested PCR-QP method developed by us utilizes the remnant solution of the stool *H. pylori* antigen test, making it possible to simultaneously identify the presence of the *H. pylori* gene and CAM resistance mutations without the need to perform EGD. Thus, it may be a useful aid for *H. pylori* eradication drug selection.

Studies on *H. pylori* eradication therapy have indicated that the recent CAM resistance rates are between 30% and 40% and that these rate have increased to between 40% and 50%, particularly among young people in Japan [24, 25].

Although an eradication therapy regimen is generally selected after determining whether the patient has CAM resistance using enrichment culture or sensitivity testing, these require tissue samples to be obtained endoscopically for use in culture and sensitivity testing. Many young individuals are asymptomatic in spite of *H. pylori* infection [34], and highly invasive tests such as EGD place a significant burden on the patient. Therefore, a non-invasive test that can be used in younger patients and that allows making a definitive diagnosis of *H. pylori* infection while simultaneously determining whether the patient is CAM resistant is highly recommended as this would increase the efficiency of eradication therapy. Furthermore, a novel genetic diagnostic test that utilizes the remnant solution (reagent) of patients who have tested positive in the non-invasive stool *H. pylori* antigen test and that is able to detect the presence of a CAM resistance mutation could significantly impact this field of medicine.

The novel Nested PCR-QP method developed by us could help identify positive results for 53 subjects (93%) out of 57 middle school students in Saga Prefecture who tested positive in the stool *H. pylori* antigen test despite the insufficient sensitivity and specificity of the conventional *H. pylori* genetic test that utilizes stool samples [27–29]. Furthermore, the use of the QProbe method facilitated the identification of a CAM resistance mutation in 28 subjects and identified the lack of such a mutation in 25 subjects (wild type). These results were consistent with the analytical results of PCR-direct sequencing. Our study including 23 adult subjects who were diagnosed with *H. pylori* infection using RUT and culture testing showed that when the remnant solution of the 21 subjects who tested positive in the stool *H. pylori* antigen test was used in Nested PCR-QP, all 21 subjects tested positive. Of these 21 subjects, 7 were found to have a CAM resistance mutation and 14 were found to have no mutation (wild type). These results were consistent with the results of drug sensitivity testing using stomach biopsy samples in all cases.

Furthermore, in subjects undergoing eradication therapy (VPZ, AMPC, CAM) who could be followed up, it was found that while the success rate for those with no CAM resistance mutation was 94.4% (17/18), the success rate for those with such a mutation was 82.6% (19/23). While this is not a statistically significant result, it demonstrates that there is a tendency for higher eradication therapy failure rates among those with a CAM resistance mutation. The use of our novel Nested PCR-QP method has the potential to improve the success rates of *H. pylori* eradication therapy.

This study has several limitations. First is the fact that we were unable to investigate the influence of the ingestion of gastric antacids such as proton pump inhibitors. Although we were able to evaluate that there was no difference in drug susceptibility test results among drugs used to eradicate *H. pylori*, we were unable to assess the influence of *H. pylori* genotype differences.

Conclusion

The novel Nested PCR-QP method is able to simultaneously identify the presence of *H. pylori* genes and the presence of CAM resistance mutations, without the need to perform EGD, using the remnant solution from stool *H. pylori* antigen tests. This could become a useful aid in the selection of an eradication drug aimed at reducing *H. pylori* eradication failure rates and further improve gastric cancer prevention.

Declarations

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Study concept and design: Toshihiko Kakiuchi and Muneaki Matsuo

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Obtained funding: Toshihiko Kakiuchi

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Study supervision: Muneaki Matsuo

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

The institutional review boards of the Saga University Hospital (approval number 2017-04-06, 2017-12-10) and the Imamura Hospital (approval number 2018-01) approved the present study. This study is registered in the clinical trials registry (UMIN000030632; <https://upload.umin.ac.jp/cgi-open-bin/ctr/ctr.cgi?function=brows&action=brows&recptno=R000034977&type=summary&language=J>) of the University Hospital Medical Information Network (UMIN). First case registered on 27 December 2017. Written informed consent was obtained from all participants and their parents or guardians. We had access to the study data and had reviewed and approved the final manuscript.

Consent for publication

Not applicable.

Competing interests

All authors declare that they have no competing interests.

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