Published in final edited form as:

Methods Mol Biol. 2013 ; 943: 279–287. doi:10.1007/978-1-60327-353-4_19.

PCR Detection of *Helicobacter pylori* in Clinical Samples

Emiko Rimbara, Masanori Sasatsu, and David Y. Graham

Abstract

Helicobacter pylori is an important pathogen whose primary niche is the human stomach. *H. pylori* is etiologically associated with gastric inflammation (gastritis), peptic ulcer disease, and gastric cancer. Both noninvasive (e.g., urea breath and stool antigen tests) and invasive (gastric biopsy for histology, culture, or PCR) tests are used for diagnosis. PCR detection of *H. pylori* has been reported using a variety of clinical samples including gastric biopsy, gastric juice, saliva, dental plaque, and stools as well as environmental samples. Whenever possible, noninvasive tests are preferred over invasive tests. *H. pylori* are excreted in the stool. Culture from stool is variable whereas stool antigen testing is widely used. Stool consists of a complicated mixture of commensal bacteria and chemicals and often includes inhibitors of PCR. Nevertheless, simple extraction methods are available to efficiently extract DNA from human stools and nested-PCR targeting the 23S rRNA gene have proven to be highly sensitive for the detection of *H. pylori*. Detection of clarithromycin susceptibility/resistance is important clinically and the mutation of the 23S rRNA gene responsible for resistance can also be detected using stool. This described method can be modified for other clinical samples such as gastric juice or biopsy material.

Keywords

Helicobacter pylori; Stool; Feces; Gastric juice; Nested-PCR; 23 S rRNA

1. Introduction

Helicobacter pylori is a Gram-negative spiral bacterial pathogen whose primary niche is the human stomach. *H. pylori* is etiologically related to gastritis and peptic ulcer disease and gastric cancer. Many diagnostic methods have been developed including the urea breath tests, rapid urease tests, and measurement of anti-*H. pylori* antibody from serum and urine, special histologic staining and immunostaining, and stool antigen testing. Many PCR methods targeting putative *H. pylori* specific genes have also been reported (1–4). For clinical studies, a positive diagnosis requires a positive culture or two positive indirect tests (i.e., histology and rapid urease test). PCR targeting *H. pylori*-specific gene can be one of the choices. Most commonly DNA extracted from gastric biopsies or gastric juice is used for the detection of *H. pylori* by PCR. A number of different commercial kits (e.g., from Qiagen or Promega) are available as well as well-described laboratory methods using proteinase K, CTAB, and phenol–chloroform extraction can be used for extraction of DNA from gastric biopsy material.

PCR-targeting *H. pylori* specific gene from gastric biopsy is a relatively simple procedure. In contrast, stools are complex mixtures that often contain PCR inhibitors and other commensal bacteria such that false-negative and false-positive results are a larger problem. Besides detection of the presence of the infection, PCR may also provide information regarding presence of *H. pylori* virulence factors as well as information regarding

antimicrobial susceptibility/resistance via genotyping PCR and DNA sequencing of PCR products. Because drug-resistance among *H. pylori* has been increasing worldwide and is a major cause of treatment failure, there is an increasing need for pretreatment antimicrobial susceptibility testing (i.e., tailored therapy). PCR-based methods targeting genes related to antibacterial resistance are suitable to both diagnose the presence of the infection and provide information regarding antimicrobial resistance (i.e., detection of clarithromycin resistance caused by the mutation of the position 2,142 and 2,143 of the 23 S rRNA gene) (5, 6).

Here, we describe nested-PCR methods targeting the 23 S rRNA using DNA extracted from stool for detection of *H. pylori* from feces as well as for detection of mutations of the 23 S rRNA gene. The method is based on a published method developed at the Tokyo University of Pharmacy and Life Sciences, Tokyo (7, 8).

2. Materials

2.1. DNA Extraction from Stool (see Note 1)

1. Beads to disrupt cells. Add beads to a 2 mL screw cap tube by adding 250 mg of silica powder (63–210 μm), 32.5 mg of ceramic beads (1–2 mm), and 75 mg of glass beads. The tube is then placed into the cell crusher (see Note 2).
2. Cell crusher: FastPrep FP120 instrument (Qbiogene, Carlsbad, CA, USA) (see Note 2).
3. 0.1 M phosphate buffer (pH 7.0): Mix 168 mL of 0.2 M Na_2HPO_4 and 32 mL of KH_2PO_4 , and the adjust the volume to 200 mL.
4. 7.5 M guanidine solution containing 5% sarcosine (see Note 3): Dissolve 35.82 g guanidine hydrochloride (MW 95.53) in 20 mL sterile ultra pure water, adjust to 50 mL, and add 2.5 g *N*-lauroyl-sarcosine.
5. 3.5 M sodium acetate (pH 5.2): Dissolve 47.6 g $\text{NaOAc}\cdot 3\text{H}_2\text{O}$ (MW 136.08) in 80 mL ultra pure water, adjust to pH 5.2 by glacial acetic acid, and adjust volume to 100 mL.
6. Wizard SV Gel and PCR Clean-up systems (Promega, Madison, WI, USA).

2.2. PCR and Electrophoresis

1. DNA polymerase (see Note 4): Ex Taq polymerase (Takara Biomedicals, Kyoto, Japan) and GoTaq[®] Green Master Mix is used for first PCR second PCR, respectively.
2. dNTP Mixture (Takara Biomedicals, Kyoto, Japan).
3. 10 $\times$ Ex Taq Buffer 20 mM Mg^{2+} plus (Takara Biomedicals, Kyoto, Japan).
4. Bovine Serum Albumin solution (Takara Biomedicals, Kyoto, Japan).

¹For DNA extraction from other clinical samples, such as biopsy, saliva, and plaque, it may be best if the combination of beads is changed. In case of gastric juice: (1) Centrifuge 300 μL of gastric juice and resuspend in 100 μL of sterile ultra pure water. (2) Boil for 3 min. (3) Mix with 100 μL of Membrane binding solution and proceed from step 8 in Subheading 3.1 (9).

²Many kinds of cell crushers are available commercially and could be used. For example, we confirmed that the Mini-BeadBeater-1 (BioSpec Products, Inc., OK) was effective using the following combination of beads: 1,500 mg of Zirconia/silica (0.1 mm), 300 mg of Zirconia/silica (1.0 mm), and 300 mg of Zirconia/silica (2.3 mm).

³If a precipitate appears, it can be dissolved by heating at 50°C or using a microwave.

⁴Since the first PCR is more important procedure than the second PCR, a DNA polymerase with a high amplification efficiency is strongly recommended for the first PCR. ExTaq polymerase can also be used for the second PCR.

5. Primer: The primers Hp23S 1835F (5'-GGTCTCAGCAAAG AGTCCCT-3') and Hp23S 2327R (5'-CCCACCAAGCATT GTCCT-3') are used for first PCR, and the primers Hp23S 1942F AGGATGCGTCAGTCGCAAGAT and Hp23S 2308R CCTGTGGATAACACAGGCCAGT are used for second PCR (see Note 5).
6. Electrophoresis buffer (50× TAE buffer): Dissolve 242 g Tris-HCl in 500 mL H₂O, add 100 mL 0.5 M Na₂ EDTA (pH 8.0) and 57.1 mL glacial acetic acid, and adjust volume to 1 L with H₂O.
7. DNA ladder marker (range between 100 and 1,000 bp).

3. Methods

To detect *H. pylori* DNA from stool by PCR, the extraction step is a critical step to eliminate PCR inhibitors in stool. Moreover, because the number of *H. pylori* cells is less than for many commercial bacteria present in stool, an efficient method to extract DNA from stool is required. There are a number of commercial kits available for DNA extraction from stool, however, we found the method using beads to crush the cells to be efficient and simple and the method requires a smaller amount of sample (50–100 mg) compared to commercial kits which often require more than 250 mg.

To avoid false-positive results a negative control using water as a template should be performed. Duplicate or triplicate reactions are also suggested for increased reliability. To prevent the false negatives, see Notes 7 and ⁸.

3.1. DNA Extraction from Feces (Fig. 1)

1. 50–100 mg of feces is added to the tube containing the beads (see Note 6).
2. Add 980 mL phosphate buffer and 180 mL of the 7.5 M guanidine solution containing 5% sarcosine to tube and mix gently using yellow pipette tip to break up any lumps of stool.
3. Place the tube in the FastPrep FP120 instrument and run it 20 s at 5.5 speed.

⁵The primer targeting other *H. pylori* genes, such as 16S rRNA, *ureA*, or *vacA*, can be applied instead of the 23S rRNA (1–3). Primers for nested-PCR should be designed based on a region in which there is a high homology between *H. pylori* strains and a low homology with other bacteria. As new *Helicobacter* sp. are being identified rapidly, there is always the possibility of false-positive results such that PCR results along should be interpreted with caution when used for treatment decisions. Results of studies based on PCR determination of the presence of *H. pylori* in environmental samples, or clinical samples from sites other than stomach or stool, especially from patients without proven *H. pylori* infections, should be viewed with considerable caution (10). At a minimum one must show that the primer pairs used are specific (e.g., not positive in patients without *H. pylori* infections). Possibly, the use of multiple *H. pylori*-specific virulence genes (e.g., CagA or VacA) (1, 11, 12) would allow more accurate PCR detection in environmental samples.

⁷To avoid the false-positive results, negative control (water) should perform on each PCR step. Positive controls should also be performed; however the DNA template for positive control should be added after finishing all samples and negative controls to avoid contamination. False-negative results are usually related to DNA extraction or the first PCR. If a false negative is suspected, the first PCR step should be performed again and changing the amount of DNA template (see Note 8). Otherwise, restart from the DNA extraction step. False-negative results caused by PCR inhibitors in the DNA samples can be excluded by adding a positive control (with serial dilution) to the DNA samples and comparing the PCR results with the simple PCR using same positive controls. In the case of DNA extracted from biopsies or gastric juice, the first PCR can be deleted and one can perform a single PCR using Ex Taq polymerase and primers Hp23S 1942F and Hp23S 2308R as follows; initial denaturation at 95°C for 2 min, followed by 5 cycles: 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s; then 30 cycles: 94°C for 15 s, 60°C for 15 s, and 72°C for 20 s.

⁸The amount of DNA template can be increased up to 10 µL or decreased to 1 µL depending on the DNA concentration and purity of the sample. Measurement of A₂₆₀/280 might be useful but does not always correspond to the results probably because the proportion of the DNA attributable to *H. pylori* differs between samples. To avoid false-negative results we recommend testing 3, 5, and 10 µL of DNA template.

⁶Stool stored in freezer in long duration (e.g., more than 5 year) may produce false-negative results. Avoid repeating the freeze and thaw of stool. This amount is assumed when the stool is solid. When the stool contains much fiber or much water increase the amount of sample (more than 100 mg) to avoid false-negative results. When the loose stool is used, centrifuge approximately 300–600 µL of the sample at 12,000 × g for 5 min and use the precipitate.

4. Centrifuge the tube at $16,000 \times g$ for 30 s.
5. Transfer the supernatant (Approximately 750 mL) to new 1.5 mL eppendorf tube and add 250 mL of 3.5 M sodium acetate (pH 5.2). Mix the tube gently.
6. Centrifuge 5 min at $16,000 \times g$ and transfer 700 mL of the supernatant to new eppendorf 1.5 mL tubes.
7. Add 700 mL of Membrane binding solution (Promega) and mix by pipetting.
8. Add 700 mL of mixture to SV Minicolumn in a Collection Tube (Promega) and incubate at room temperature for 1 min.
9. Centrifuge at $16,000 \times g$ for 1 min, discard flow through and reinsert Minicolumn into the Collection Tube.
10. Add the remaining 700 mL of the mixture (step 7) to the SV Minicolumn in the Collection Tube and repeat steps 8 and 9.
11. Add 700 μ L of Membrane Wash Solution (ethanol added as per the instructions) to the column and centrifuge at $16,000 \times g$ for 1 min. Discard flowthrough and reinsert the Minicolumn into the Collection Tube.
12. Repeat Step 11 with 500 μ L of the Membrane Wash Solution. Centrifuge at $16,000 \times g$ for 5 min.
13. Empty the Collection Tube and centrifuge the column assembly for 1 min with the microcentrifuge lid open to allow evaporation of any residual ethanol.
14. Transfer the column to a 1.5 mL eppendorf tube and add 58 mL of nuclease-free water to the column. Incubate at room temperature for 2 min and then centrifuge at $16,000 \times g$ for 1 min.
15. Store the DNA solution at -20°C until using for PCR.

3.2. Nested-PCR Targeting the 23 S rRNA Gene of *H. Pylori* (see Note 7)

1. Prepare the first PCR reaction mixture on ice by adding the following quantities of reagents: 50 mL in the order listed: 33.75 mL of sterile ultra pure water, 5.0 mL of $10\times$ Ex Taq Buffer, 4.0 mL of dNTP Mixture (each 2.5 mM), 5.0 mL of Bovine Serum Albumin solution, 1.0 mL of primer Hp23S 1835 F (10 pmol/mL), 1.0 mL of primer Hp23S 2327R (10 pmol/mL), and 0.25 mL TaKaRa Ex Taq (5 U/mL).
2. Aliquot 45 mL of the PCR reaction mixture to each PCR tube and add 5 mL of the DNA template. Mix well (see Note 8).
3. Using thermal cycler, perform PCR: initial denaturation at 95°C for 2 min, followed by 5 cycles: 94°C for 30 s, 57°C for 30 s, and 72°C for 30 s; then 30 cycles: 94°C for 15 s, 57°C for 15 s, and 72°C for 20 s.
4. Prepare the second PCR mixture on ice by adding: 50 μ L in the order listed: 20 μ L of sterile ultra pure water, 25.0 μ L of Master Mix, 1.0 μ L of primer Hp23S 1942 F (10 pmol/ μ L), and 1.0 μ L of primer Hp23S 2308R (10 pmol/ μ L).
5. Aliquot 47 μ L of the PCR reaction mixture to each PCR tube and add 3 μ L of the first PCR product. Mix well.
6. Perform PCR as follows; initial denaturation at 95°C for 2 min, followed by 25 cycles: 94°C for 10 s and 63°C for 20 s.

7. Final PCR products are confirmed using electrophoresis in 2.5% agarose gels stained with ethidium bromide. The PCR product should be 367 bp (see Note 9). An example of the results is shown in Fig. 2.

Acknowledgments

Dr. Rimbara and Dr. Sasatsu are supported by a grant from the High-Tech Research Centre Project for Private Universities provided by the Ministry of Education, Culture, Sports, Science and Technology and by the Matching Fund Subsidy for Private Schools of Japan. This work was supported in part by the Office of Research and Development Medical Research Service Department of Veterans Affairs. Dr. Graham is supported in part by Public Health Service grant DK56338 which funds the Texas Medical Center Digestive Diseases Center and R01 CA116845. We especially thank Drs. Norihisa Noguchi, Prof. Takashi Kawai, and Prof. Shinichi Takahashi for their expertise, advice, and encouragement.

References

1. Park CY, Kwak M, Gutierrez O, Graham DY, Yamaoka Y. Comparison of genotyping *Helicobacter pylori* directly from biopsy specimens and genotyping from bacterial cultures. *J Clin Microbiol.* 2003; 41:3336–3338. [PubMed: 12843087]
2. Smith SI, Oyedele KS, Arigbabu AO, Cantet F, Megraud F, Ojo OO, Uwaifo AO, Otegbayo JA, Ola SO, Coker AO. Comparison of three PCR methods for detection of *Helicobacter pylori* DNA and detection of *cagA* gene in gastric biopsy specimens. *World J Gastroenterol.* 2004; 10:1958–1960. [PubMed: 15222045]
3. Mapstone NP, Lynch DA, Lewis FA, Axon AT, Tompkins DS, Dixon MF, Quirke P. Identification of *Helicobacter pylori* DNA in the mouths and stomachs of patients with gastritis using PCR. *J Clin Pathol.* 1993; 46:540–543. [PubMed: 8331177]
4. Hammar M, Tyszkiewicz T, Wadstrom T, O'Toole PW. Rapid detection of *Helicobacter pylori* in gastric biopsy material by polymerase chain reaction. *J Clin Microbiol.* 1992; 30:54–58. [PubMed: 1370850]
5. Taylor DE, Ge Z, Purych D, Lo T, Hiratsuka K. Cloning and sequence analysis of two copies of a 23 S rRNA gene from *Helicobacter pylori* and association of clarithromycin resistance with 23 S rRNA mutations. *Antimicrob Agents Chemother.* 1997; 41:2621–2628. [PubMed: 9420030]
6. Versalovic J, Shortridge D, Kibler K, Griffy MV, Beyer J, Flamm RK, Tanaka SK, Graham DY, Go MF. Mutations in 23 S rRNA are associated with clarithromycin resistance in *Helicobacter pylori*. *Antimicrob Agents Chemother.* 1996; 40:477–480. [PubMed: 8834903]
7. Noguchi N, Rimbara E, Kato A, Tanaka A, Tokunaga K, Kawai T, Takahashi S, Sasatsu M. Detection of mixed clarithromycin-resistant and -susceptible *Helicobacter pylori* using nested PCR and direct sequencing of DNA extracted from faeces. *J Med Microbiol.* 2007; 56:1174–1180. [PubMed: 17761479]
8. Rimbara E, Noguchi N, Yamaguchi T, Narui K, Kawai T, Sasatsu M. Development of a highly sensitive method for detection of clarithromycin-resistant *Helicobacter pylori* from human feces. *Curr Microbiol.* 2005; 51:1–5. [PubMed: 15971095]
9. Rimbara E, Tamura R, Tanuma M, Noguchi N, Kawai T, Sasatsu M. Evaluation of clarithromycin resistance in *Helicobacter pylori* obtained from culture isolates, gastric juice, and feces. *Helicobacter.* 2009; 14:156–157. [PubMed: 19298344]
10. Sugimoto M, Wu JY, Abudayyeh S, Hoffman J, Brahem H, Al-Khatib K, Yamaoka Y, Graham DY. Unreliability of results of PCR detection of *Helicobacter pylori* in clinical or environmental samples. *J Clin Microbiol.* 2009; 47:738–742. [PubMed: 19129407]
11. Tiwari SK, Khan AA, Ahmed KS, Ali SM, Ahmed I, Habeeb A, Kauser F, Hussain MA, Ahmed N, Habibullah CM. Polymerase chain reaction based analysis of the cytotoxin associated gene pathogenicity island of *Helicobacter pylori* from saliva: an approach for rapid molecular

⁹To ensure that the amplified product is *H. pylori*, DNA sequencing of PCR product is strongly recommended. This also can provide information regarding clarithromycin-resistance caused by the mutation at position 2,142 and 2,143 of the 23S rRNA gene. The mutation is usually the transition from adenine to guanine, but the transversion from adenine to cytosine has also been reported. The primers, Hp23S 1942F and Hp23S 2308R, can be used for DNA sequencing.

- genotyping in relation to disease status. *J Gastroenterol Hepatol*. 2005; 20:1560–1566. [PubMed: 16174074]
12. Sinha SK, Martin B, Gold BD, Song Q, Sargent M, Bernstein CN. The incidence of *Helicobacter pylori* acquisition in children of a Canadian First Nations community and the potential for parent-to-child transmission. *Helicobacter*. 2004; 9:59–68. [PubMed: 15156905]

Fig. 1.
Method for the isolation of DNA from feces.

Fig. 2.

Comparison of methods for the isolation of DNA from feces in *H. pylori* -positive (1 and 2) and -negative (3) samples. (a) Electrophoresis of DNA isolated from feces (1% agarose gel). Lane M1, *Hind*III-digested λ DNA; lanes (a) and (b) DNA isolated from 200 to 50 mg feces, respectively, using a QIAamp DNA Stool Mini kit; lanes (c) DNA isolated from 50 mg feces using the method described in this chapter. (b) Agarose gel (2.5%) electrophoresis of the nested PCR *H. pylori* 23 S rRNA gene amplification product (arrow) from DNA isolated as described in (a). Lane M2, 100 bp DNA ladder reproduced from *see ref. (7)* with permission from The Society for General Microbiology.