Sensitive Detection of *Helicobacter pylori* in Gastric Aspirates by Polymerase Chain Reaction

Rosa URIBE, Toshio FUJIOKA, Akira ITO, Akira NISHIZONO and Masaru NASU
Second Department of Internal Medicine, Microbiology, Oita Medical University
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Key words: *Helicobacter pylori*, gastric juice, DNA extraction, southern hybridization, PCR

Abstract

The detection of *Helicobacter pylori* in gastric aspirate was examined by using the polymerase chain reaction (PCR) method for amplifying a specific fragment of the urease gene A. The ability of PCR to amplify *H. pylori*-specific DNA was analyzed by Southern hybridization with an internal oligonucleotide probe. Twenty-two *H. pylori* strains from clinical isolates and reference strains were studied, and all *H. pylori* strains yielded a 356-bp product that hybridized with the oligonucleotide probe, whereas no amplification was evident with 18 non-*H. pylori* strains. This could detect as little as 50 CFU of *H. pylori* in pure culture and 0.1 pg of purified chromosomal DNA. A total of 50 dyspeptic patients were examined for the presence of *H. pylori* by culture, the rapid urease test and histological examination of antral biopsy samples as well as by PCR in gastric juice aspirate samples. The gold standard for the presence of *H. pylori* was established by minimum concordance of two of three tests performed on biopsy specimens. With this gold standard, 34 of the 50 patients were considered to harbor *H. pylori* infection. PCR correctly identified 32 (94.1%) of these 34 infected patients. PCR had the best combination of sensitivity and specificity in assessing the correct diagnosis of *H. pylori* as compared with those of the rapid urease test and culture. Moreover, we established a fast and simple method for use by improvement of DNA extraction. PCR of the gastric aspirate was shown to be a sensitive and specific procedure which may be an attractive alternative to methods currently used for diagnosis of *H. pylori* infection.

Introduction

*Helicobacter pylori* is a gram-negative spiral bacterium which is regarded as the major etiological agent of human gastritis and peptic ulcers. Moreover, recent data also indicate that *H. pylori* infection is a risk factor for development of gastric carcinoma. Since the discovery of *H. pylori* by Warren and Marshall and its successful isolation by Marshall in 1983, numerous studies have been performed worldwide to develop accurate methods for detection of *H. pylori*. These methods include bacteriological culture, histological examination, the biopsy urease test, the $^{13,14}$C urease breath test and serology. However, none of these tests is considered optimal because of lack of sensitivity, time consumption, or irreproducibility.

Recently, a new molecular biology procedure has been employed for detection of *H. pylori*: PCR, an *in vitro* method of DNA synthesis by which a particular segment of DNA can be specifically
PCR Detection of *H. pylori*

amplified to a level detectable by gel electrophoresis and hybridization. Different PCR protocols have been developed for detection of *H. pylori*\(^{[4]–[12]}\). However, most of them have been used for specimens that required a biopsy\(^{[5]–[8]}\). PCR analysis of gastric aspirates may provide a good alternative to PCR analysis of biopsy specimens since, in contrast to biopsy, gastric aspirates can be collected very easily through a nasogastric tube obviating the need for endoscopy.

PCR detection of *H. pylori* in gastric juice was previously reported\(^{[8]–[11]}\). In some of the studies a very limited number of gastric juice samples were tested\(^{[9]–[12]}\). Other have used time-consuming DNA extraction\(^{[9,11,12]}\) and/or a nested-PCR\(^{[8,11]}\), which make the assay unsuitable for routine clinical laboratory use.

In this study, we evaluated the use of PCR for detection of *H. pylori* in gastric aspirates. Two methods for extraction of DNA from a gastric aspirate sample were compared and one-step PCR was used. PCR results were compared with those of histological examination, bacteriological culture and the rapid urease test to determine whether PCR analysis of gastric aspirates can provide a serious alternative to methods currently used for diagnosis of *H. pylori* infection.

Materials and Methods

Bacterial strains

The organisms used to evaluate the specificity of the PCR method are listed in Table 1. All bacterial strains were isolated and identified by standard microbiologic techniques.

Patients and clinical specimens

Gastric juice sample and corresponding biopsy specimens were obtained from 50 patients with dyspeptic ulcer symptoms referred for upper gastrointestinal endoscopy and biopsy in our hospital during the first half of 1995. Of these patients 33 were males and 17 females, ranging in age from 19 to 78 years. Endoscopic diagnosis included 15 gastric ulcers, 17 duodenal ulcers and 18 cases of

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Source or Strain</th>
<th>No. of Strains</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>H. pylori</em></td>
<td>ATCC 11637</td>
<td>1</td>
</tr>
<tr>
<td><em>H. pylori</em></td>
<td>ATCC 43504</td>
<td>1</td>
</tr>
<tr>
<td><em>H. pylori</em></td>
<td>Clinical isolate</td>
<td>20</td>
</tr>
<tr>
<td><em>H. felis</em></td>
<td>Laboratory strain(^a)</td>
<td>1</td>
</tr>
<tr>
<td><em>Campylobacter jejuni</em></td>
<td>Laboratory strain</td>
<td>2</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>ATCC 27166</td>
<td>1</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>ATCC 39188</td>
<td>1</td>
</tr>
<tr>
<td><em>Enterobacter aerogenes</em></td>
<td>Laboratory strain</td>
<td>2</td>
</tr>
<tr>
<td><em>Enterobacter cloacae</em></td>
<td>Laboratory strain</td>
<td>2</td>
</tr>
<tr>
<td><em>Klebsiella pneumoniae</em></td>
<td>Laboratory strain</td>
<td>1</td>
</tr>
<tr>
<td><em>Morganella morganii</em></td>
<td>Laboratory strain</td>
<td>1</td>
</tr>
<tr>
<td><em>Proteus mirabilis</em></td>
<td>Laboratory strain</td>
<td>1</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>Laboratory strain</td>
<td>1</td>
</tr>
<tr>
<td><em>Pseudomonas cepacia</em></td>
<td>Laboratory strain</td>
<td>1</td>
</tr>
<tr>
<td><em>Serratia marcescens</em></td>
<td>Laboratory strain</td>
<td>1</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>Laboratory strain</td>
<td>2</td>
</tr>
<tr>
<td><em>Staph. coagulase negative</em></td>
<td>Laboratory strain</td>
<td>1</td>
</tr>
</tbody>
</table>

\(^a\)Second Department of Internal Medicine, Oita medical University
atrophic gastritis. The study was fully explained to each patient who was included and their informed consent was obtained either in writing or verbally.

Specimen processing

All patients underwent upper gastrointestinal endoscopy during which a gastric juice sample was collected and immediately neutralized with 1 M NaOH, divided into three aliquots and frozen at −70°C until processed. Three biopsy specimens were taken from the antrum of each patient was tested for *H. pylori* by the rapid urease test\(^{13}\), culture and histological examination. Biopsy specimens were placed into sterile 20% glucose solution and transported to the laboratory within 2 hours. Culture for *H. pylori* was carried out on 70% sheep blood agar plates (Belo-horizonte medium) under microaerobic conditions at 37°C for 4 days. The third biopsy specimen was examined microscopically after hematoxylin and eosin staining and/or Warthin-Starry silver staining for visualization of *H. pylori*.

The gold standard for *H. pylori* status was established by agreement of at least two of the three procedures performed on biopsy specimens mentioned above.

DNA extraction from bacterial strains

Colonies obtained from each bacterial strain on agar were transferred to 400 µl of lysis buffer (50 mM KCl, 10 mM Tris-HCl [pH 7.5], 5 mM MgCl\(_2\), 0.1 mg gelatin per ml, 0.45% NP40, 0.45% Tween 20 and protease K at a final concentration of 100 µg per ml) and incubated for 60 min at 55°C. Nucleic acids were extracted twice with an equal volume of phenol: chloroform and once with chloroform-isoamyl alcohol (24:1, vol/vol). The DNA present in the deproteinized solution was precipitated with ice cold ethanol (100%) at −80°C for 1 hour and then pelleted by centrifugation at 15,000 rpm for 20 min at 0°C. The air-dried pellet was resuspended in distilled water at a final concentration of 1 µg per ml and then was tested for DNA amplification.

DNA extraction from gastric aspirate sample

A 2-ml sample of gastric aspirate obtained from each patients was mixed with 80 µl of 1 M NaOH to neutralize the acidity and centrifugated at 10,000 rpm for 15 min. The supernatant was discarded and the pellet was digested with 350 µl of tissue extraction buffer (50 mM tris HCl [pH 8.0], 0.5% Tween 20, and 200 mg proteinase K per ml). After the mixture was incubated at 55°C for 2 hours, DNA was extracted by phenol-chloroform followed by ethanol precipitation as described above. The air-dried pellet was resuspended in 50 µl of distilled water, 10 µl of which was used for PCR. To investigate whether *H. pylori* could be detected without extensive purification, a second sample of each gastric aspirate was also processed by DNA extraction by boiling as described elsewhere\(^{14}\). All negative samples were examined for PCR inhibition by processing and amplifying the third frozen sample it was after inoculated with 10\(^9\) bacteria from the *H. pylori* culture.

Oligonucleotides

The set of oligonucleotide primers encoding the urease gene A\(^{15}\) reported by Westblom et al\(^{11}\) was selected and used for PCR amplification. The sequences were 5'-CCAAAAGAGTTAAAAACCAA-A-3' and 5'-CCTTCCGTGTAATGTCTTG3'-3', corresponding to nucleotides 13 to 36 and nucleotides 345 to 368 of the urease gene A. An additional 20-mer oligonucleotide, 5'-CGCACTCTTT-TAAAACCAGA-3', corresponding to nucleotides 183 to 202 of the urease gene A was synthesized and used as an internal probe for detection of the amplified product by hybridization.

DNA amplification

Samples (10 µl) were submitted to 45 cycles of amplification in 50-µl volumes containing 1.25 U of Taq polymerase (Takara, Japan), 0.1 mmol of each primer, 0.2 mmol of each four nucleotides, 10 mmol of Tris-HCl (pH 8.4), 50 mmol of KCl and 1.5 mmol of MgCl\(_2\). Reaction mixtures were overlaid with drops of mineral oil to prevent evaporation. After an initial denaturation step of 3 min, the amplification cycle was as follows: denaturation at 95°C for 1 min, annealing at 60°C for 2 min and
primer extension at 72°C for 2 min. The final cycle included extension for a further 5 min to ensure that all amplified DNA was fully double stranded. A positive control (1 ng of \textit{H. pylori} DNA) and a negative control (distilled water) were included in each run of amplification. To prevent contamination of the samples, the following measures were taken. Sample preparation, PCR amplification, and electrophoresis were performed in different rooms. At each step, each sample was uncovered carefully and separately; gloves were changed after the handling of each sample, and DNA solutions were handled with positive-displacement pipettes. A “no DNA” reagent control and one negative sample control were included in each amplification set. The reagent control contained all necessary components for PCR, except template DNA while the negative sample control contained DNA extracted from a gastric aspirate specimen of an \textit{H. pylori}-negative patient.

**Gel electrophoresis and Southern blot hybridization**

Ten microliters of the amplified DNA samples was electrophoresed on 3% agarose gel containing 0.5 μg of ethidium bromide per ml. The samples were considered positive when a band of 356 base pairs could be visualized on the gel. For Southern blot hybridization, the electrophoresed DNA fragments were transferred to a nylon membrane (Hybond-N+) by southern blotting and were fixed to the membrane by UV irradiation. The membranes were prehybridized for 45 min at 42°C in a hybridization solution containing 5 × SSC, 0.1% (w/v) hybridization buffer component (Amersham, England), 0.02% (w/v) SDS and 20-fold diluted liquid block reagent (Amersham, England). After the oligonucleotide probe labelled with FI-DUTP was added to the buffer used in the prehybridization step at a final concentration of 10 ng/ml, the hybridization was performed at 42°C for 4 hours. Filters were then washed twice with 5 × SSC-0.1% (w/v) SDS for 5 min at room temperature and twice with 1 × SSC-0.1% (w/v) SDS for 15 min at 51°C. FI-labelled probes were detected in according to the chemiluminescence detection protocol from Amersham International (ECL, 3′-oligolabelling and detection systems, RPN 2130, 2131).

**Statistical analysis**

The data were analyzed with Epi Info for Windows statistical software (Version 6.0). Calculation was performed along with 95% confidence intervals as suggested by Fleiss for proportions near unity (Fleiss quadratic confidence intervals). To determine whether the tests are statistically different from each other in establishing the correct diagnosis, McNemars test was performed. A p value of < 0.05 was considered statistically significant.

**Results**

**Specificity of PCR**

DNA amplification from all 22 \textit{H. pylori} strains tested by PCR yielded a band of the predicted size while none of the non-\textit{H. pylori} strains tested yielded the 356-bp amplified product (Fig. 1A). The amplified products were verified by Southern blot hybridization (Fig. 1B), with the same results.

**Sensitivity of PCR-hybridization assay**

To determine the minimal detectable concentration of \textit{H. pylori} DNA and the minimal detectable number of \textit{H. pylori} organisms, PCR was conducted on 10 fold serial dilutions of both purified chromosomal DNA and boiled lysates of known numbers (CFU/ml) of bacteria. The 356-bp fragment could be visualized on ethidium bromide-stained gels in reaction mixtures containing 1 pg of total chromosomal DNA (Fig. 2A); the detection level was increased 10-fold in Southern blot hybridization (Fig. 2B) Analysis of PCR products obtained from the serial dilutions of bacteria showed that 100 CFU of \textit{H. pylori} could be detected in ethidium bromide gels (Fig. 3A) and that 50 CFU could detected by Southern blot hybridization (Fig. 3B).
Detection of *H. pylori* in clinical specimens

To determine the feasibility of this PCR assay for detecting *H. pylori* DNA in gastric aspirate specimens, the assay was applied to gastric aspirate samples from 50 patients with dyspeptic ulcer symptoms. Of these patients, 34 (68%) were recorded as *H. pylori* positive by concordance of positivity in at least two of the three tests used to establish the *H. pylori* infection status. PCR correctly detected *H. pylori* in 32 (94.1%) of these 34 patients. When DNA extracted by the boiling procedure was used as template, PCR could detect *H. pylori* in 31 (91.1%) of the 34 positive patients. In only one case, detection was facilitated by purification of DNA. Of the 16 patients considered *H. pylori* negative, all but one were also negative by PCR (Tables 2, 3).

All samples which were initially negative by PCR were also assessed for PCR inhibition by processing and amplifying the remaining frozen aliquot from the same gastric aspirate sample after it was inoculated with $10^9$ bacteria of the *H. pylori* culture. All samples were successfully amplified by the second PCR. The specificity of the PCR bands was verified by Southern blot hybridization with an F1-labelled probe. Hybridization did not detect *H. pylori* in any gastric aspirate samples that did not yield a visible amplified product on the gel, even though hybridization was found to be more efficient than gel electrophoresis for detection of the amplified products (Fig. 2B).

**Comparison of PCR of gastric aspirate with bacterial culture and rapid urease test**

Sensitivity, specificity and predictive values of the PCR, bacterial culture and urease test were
Fig. 3  Sensitivity of the PCR-hybridization assay performed on 10-fold dilutions of a culture of *H. pylori* strain ATCC 11637. (A) Analysis of PCR products by gel electrophoresis: lane 1, $1 \times 10^5$; lane 2, $1 \times 10^4$; lane 3, $1 \times 10^3$; lane 4, $1 \times 10^2$; lane 5, $5 \times 10^1$; lane 6, $1 \times 10^1$; lane 7, $1 \times 10^{-1}$; lane 8, DNA marker. (B) Southern blot analysis after hybridization with the (FI) dUTP labelled internal oligonucleotide probe.

Table 2  Detection of *H. pylori* in gastric aspirate samples by PCR

<table>
<thead>
<tr>
<th><em>H. pylori</em> infection status established by the gold standard&lt;sup&gt;c&lt;/sup&gt;</th>
<th>PCR on gastric aspirates</th>
<th>Purified DNA&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Non-purified DNA&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
<td>%</td>
<td>No.</td>
</tr>
<tr>
<td>Positive n=34</td>
<td>32</td>
<td>94.1</td>
<td>31</td>
</tr>
<tr>
<td>Negative n=16</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

<sup>a</sup> Proteinase K incubation followed by phenol-chloroform extraction and ethanol precipitation

<sup>b</sup> DNA extracted by boiling procedure

<sup>c</sup> Gold standard for *H. pylori* infection status was established by a minimum concordance of two of three methods: bacteriological culture, histological examination, and rapid urease test.

*There was a significant difference between the two gastric aspirate-processing methods for detection of *H. pylori* by PCR.

Calculated in relation to the gold standard and are presented in Table 3. PCR had a sensitivity of 94.1% while the sensitivities of bacterial culture and the rapid urease test were 82.4% and 88.2% respectively. PCR detected the presence of *H. pylori* in gastric aspirates from two patients whose corresponding biopsy was positive by histological examination and the rapid urease test. On the other hand, PCR detected *H. pylori* in four gastric aspirate samples for which the corresponding biopsy specimens were negative by both culture and the rapid urease test and in two which were negative...
Table 3 Sensitivity, specificity and predictive values of diagnostic procedures for H. pylori infection

<table>
<thead>
<tr>
<th>Diagnostic procedure</th>
<th>No. of positive patients/total No. of patients(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sensitivity</td>
</tr>
<tr>
<td>Gastric juice PCR**</td>
<td>32/34(94.1)</td>
</tr>
<tr>
<td>Bacteriological culture***</td>
<td>28/34(82.4)</td>
</tr>
<tr>
<td>Histological examination***</td>
<td>32/34(94.1)</td>
</tr>
<tr>
<td>Urease test(Clo- test)***</td>
<td>30/34(88.2)</td>
</tr>
</tbody>
</table>

*Sensitivity, specificity and predictive values were calculated in relation to the established gold standard.
**PCR performed on purified DNA extracted from gastric aspirate samples
***Culture, histology and Clo- test were conducted on biopsy specimens from antrum and body of the stomach

Discussion

Because of the high prevalence of H. pylori in humans and its important clinical implications in peptic ulcers, atrophic gastritis and gastric cancer, the development of new sensitive and rapid diagnostic procedures for the accurate detection of H. pylori is of great value. In this study, we have evaluated the use of PCR analysis of gastric aspirates for diagnosis of H. pylori infection.

One of the most important aspects of establishing a PCR assay is the selection of the primers. We have selected a set of primers based on the sequence of the H. pylori urease genes, genes whose enzyme products seem to be required for the survival of this bacterium in the acidic gastric environment. Therefore, there is strong selective pressure to maintain the amino acid sequence of this enzyme, resulting in conservation of the DNA sequence among strains. This set of primers, which was first described by Westblom et al., was shown to be very specific for H. pylori detection. We have confirmed this specificity by testing 22 H. pylori strains and 20 non-H. pylori strains of bacteria. Only DNA from H. pylori strains was amplified by PCR and hybridized by the internal oligonucleotide probe.

Our gastric juice-PCR could detect as little as 1 pg of purified H. pylori DNA and 100 CFU of H. pylori, the same level of detection reported by other researchers. By the analysis of PCR amplified products by hybridization with an (FI) dUTP-labelled oligonucleotide probe, we were able to enhance the sensitivity of our PCR to a detection limit of 0.1 pg and 50 CFU of H. pylori.

Using histology as a gold standard, PCR correctly identified 32 to 34 H. pylori-infected patients while the rapid urease test and culture detected 30 and 28 respectively. PCR could detect H. pylori DNA in four gastric aspirates for which the corresponding biopsy specimens were negative by culture and in two that were negative by the rapid urease test. One possible reason for these findings is that PCR can detect the target DNA whether the bacteria are viable or not provided that gross breakdown of nucleic acid has not occurred. In addition, the H. pylori colonization of the gastric mucosa may be patchy, therefore negative biopsies could be obtained from a stomach harboring the organism. Gastric juice PCR may better reflect the H. pylori infection whatever part of the stomach is primary affected. One of the main advantages of gastric juice-PCR is that it obviates gastroduodenoscopy, since a gastric juice aspirate can be obtained with a nasogastric tube.

Because our purpose was to establish a fast and simple method for use in routine diagnosis, we...
have also evaluated the utilization of crude heat-inactivated samples for PCR amplification. The classical proteinase K incubation followed by phenol-chloroform extraction included in most of the PCR protocols is very time consuming and not really applicable to routine clinical laboratory use. In our study, PCR performed on a crude heat-inactivated sample was found to be as sensitive as that performed on purified DNA. In the classical proteinase K procedure incubation requires one night a minimum of from 3 hours. In our method, it takes time of 15 min. The use of non-purified DNA makes the assay shorter and simpler to run and the absence of purified DNA further reduce the risk of contamination. Recently, a rapid and sensitive PCR-based microwell plate assay system for *H. pylori* DNA in gastric juice was reported. Our method makes the assay shorter and simpler than this system. Moreover, we have selected a set of primers based on the urease gene, which was shown to be very specific for *H. pylori* detection.

Summarizing our findings, PCR of a gastric aspirate is a highly specific method for the detection of *H. pylori* infection. The use of crude inactivated samples makes this assay easier and shorter to run without affecting its sensitivity. The need of endoscopy is obviated since gastric juice can be aspirated via a nasogastric tube. Hence, PCR of a gastric aspirate may be an attractive alternative for diagnosis of *H. pylori* infection.

**References**


PCR 法による胃液内 Helicobacter pylori の高感度検出

ウリベ ロサ1) 藤岡 利生1) 伊藤 彰1)
西園 晃2) 那須 勝1)

要 旨
ウレアーゼ A 遺伝子の特異的断片を増幅する PCR 法を用いて、胃液中の H. pylori の検出を試みた。また、PCR 法による H. pylori に特異的な DNA の増幅は、その中間域のサザン法にて確認した。22株の臨床分離株と標準株を対象とし、すべての H. pylori 株において、サザン法にて確認された356bp の産物を得たが、20株の非 H. pylori 株においては増幅されなかった。また今回の方法では、H. pylori 菌体50CFU 又は DNA 0.1pg にて検出可能であった。さらに50例の消化器症状を有する患者に、前庭部生検よりの培養、ラピッドウレアーゼテスト及び組織法と、加えて胃液の

PCR 法によって H. pylori の存在診断を行った。H. pylori の存在は、前出の 3 法のうち少なくとも 2 法の陽性により判定したこの方法により、50例中34例が陽性と判定された。さらに、この34例中32例（94.1%）が PCR 陽性であった。PCR 法は、H. pylori の検出の感度と特異性において、培養とラピッドウレアーゼテストに比べ優れていた。さらに、DNA の抽出法を改良する事により、迅速にかつ簡便な検出法を確立した。今回的研究で、胃液の PCR 法は高感度で特異的な手法として H. pylori 感染の診断において有用な方法である事が実証された。