

PCR and RFLP Analysis for Identification and Typing of *Helicobacter pylori* Strains from Gastric Biopsy Specimens

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Abstract

Helicobacter pylori is the causative agent of chronic gastritis and peptic ulcer diseases and is also a risk factor for gastric cancer. Since culture of *Helicobacter pylori* is relatively insensitive and cumbersome, PCR-based molecular detection and typing of this organism is gaining importance for strain differentiation. The aim of present study was to apply molecular methods for detection and genotyping of *H. pylori* directly on gastrointestinal biopsy. For this purpose 79 samples were tested by bacteriological methods including Gram stain, urease test and culture in a selective medium. Genomic DNA was directly extracted from gastric biopsy specimens followed by PCR amplification using *ureC* primers. PCR products from positive samples were purified and digested using three different restriction endonucleases, AluI, MboI and CfoI. The numbers of positive samples detected by bacteriological methods were 71, 40 and 73 using Gram stain, culture and urease test respectively. Through PCR assay 64 samples were positive with sensitivity of about 92 and specificity of 100%. The specificity and sensitivity of the PCR assay was higher than that of all bacteriological methods applied. RFLP performed on the positive samples and five patterns for AluI, five patterns for MboI and four patterns for CfoI detected. The patterns were classified based on the number of produced bands in gel electrophoresis. From our study it is concluded that the PCR-based RFLP technique would be an useful approach for identification and genotyping of specific *H. pylori* strains directly in gastric biopsy specimens without culture.

Keywords: *Helicobacter pylori*; UreC; PCR; RFLP

Introduction

Helicobacter pylori is a gram-negative microaero-

philic organism that colonizes human gastric mucosa [13]. It has been proved that *H. pylori* is an etiologic agent of chronic gastritis and peptic ulcer diseases and

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is also a risk factor for gastric cancer [15]. The World Health Organization has classified *H. pylori* as a class I carcinogen [6]. Epidemiological studies have shown that *H. pylori* infection occurs world wide at a high prevalence rate [7].

Recurrence of infection after apparent eradication has also been reported and is associated with recurrence of ulcers. However, it is unclear whether the recurrence of ulcers following *H. pylori* eradication therapy is due to recrudescence of the previous infection or to exogenous re-infection by another strain [17]. An accurate method for the detection and differentiation of *H. pylori* strains in patients both before and after therapy is therefore of great importance for diagnosis, monitoring of treatment, and reduction of the long-term consequences of continued but undetected disease [2].

Attempts to differentiate *H. pylori* strains have been made using a variety of conventional typing schemes, like haemagglutination, biotyping, cytotoxin activity, plasmid profiles and immunoblotting. However, each of these methods due to some inherent difficulties did not gain widespread use for precise differentiation of *H. pylori* strains [2,8,11].

Several nucleic acid techniques, such as restriction endonuclease analysis of genomic DNA and southern blot hybridization with rRNA gene probes have been applied to type *H. pylori* clinical isolates from different patients. The patterns obtained by these methods were complex and difficult to interpret, particularly for large scale analysis of clinical isolates [5,14,16]. Recently, PCR-based restriction fragment length polymorphism (RFLP) analysis and randomly amplified polymorphic DNA (RAPD) methods have been developed for typing of *H. pylori* clinical isolates. PCR based RFLP schemes have been used to analyze *H. pylori* genes, which encode urease and its accessory proteins, including *UreA*, *UreB*, *UreC* and *UreD* [1,5,9].

The published works have shown that PCR-based RFLP typing is a rapid, sensitive method and capable of discriminating among clinical isolates, but the studies were limited to *H. pylori* isolates cultured from gastric biopsy specimens [13].

In this study, we report a PCR-based RFLP analysis to differentiate *H. pylori* strains directly from gastric biopsy specimens without any attempt to culture the bacteria, which is a time consuming and labor intensive task.

Materials and Methods

Bacterial Strains

The type strain ATCC 26694 (kindly donated by Dr.

Leo. Smmeet) was used as positive control in this study. *Proteus mirabilis* and *Klebsiella pneumoniae* (clinical isolate) as an urease positive and *E. coli* (clinical isolate) as an urease negative micro organism, were used as negative control to test the specificity of PCR assay.

Gastric Biopsy Specimens

Gastric biopsy specimens were obtained from 79 patients, 36 women and 43 men with age range 16 to 80 years (mean age 42.2 years), who underwent endoscopy for upper gastrointestinal complains at the Alzahra hospital center. Two gastric biopsy specimens were collected from similar location in the antrum and fundus of the stomach from each patient. One piece of each specimen was examined by bacteriologic tests (Gram stain, urease test and culture). Another biopsy specimen was preserved immediately at -70°C for molecular analysis.

For bacteriological tests, the gastric biopsy sample was homogenized and one part cultured on BHI agar containing antibiotic supplement (Merck) with 5% pack red blood cell (obtained from Blood Transfusion Organization) plus 7% fetal calf serum (Biogen). Plates were incubated at 37°C in microaerophilic atmosphere with 10% CO_2 , 5% O_2 and 85% N_2 . Plates were opened after 5 days. Organisms were identified as *H. pylori* based on colony morphology, Gram stain, and positive catalase and rapid urease tests.

Processing of the Samples for PCR Assay

The frozen biopsy specimen was thawed, crushed and then DNA was extracted using high pure DNA extraction kit (Roche, Germany) according to the manufacturer instruction. Same kit was used for extraction of genomic DNA from standard strains and negative controls. The extracted DNA then quantified through measurement of its OD_{260} by Biophotometer (Eppendorf, Germany).

Primers

UreC (*glmM*) gene in *H. pylori*, which encodes a phosphoglucoseamine mutase [12], was amplified using forward (5'-CCC TCA GAC CAG TCC CAA AAA) and reverse (5'-AAG AAG TCA AAA ACG CCC CAA AAC) primers. Amplification was performed in a 50 μl reaction mixture volume containing 100 pM of each primer, 200 μM each dATP, dCTP, dTTP and dGTP, 10 mM Tris-HCl, pH 8.3, 50 mM KOH, 2 mM MgCl_2 , 2.5

U Amplitaq Gold (Perkin Elmar, USA) and 0.2 µg of template DNA sample. Thirty cycles of amplification were performed in a thermal cycler (Omnigen, UK). Each cycle consisted of 45 seconds denaturation step at 94°C, 30 sec annealing step at 59°C and 90 sec extension step at 72°C followed by final extension step for 10 min at 72°C to ensure full extension of the partially polymerized products.

Subsequently PCR products were loaded on 1.5% agarose gel, containing 0.5 µg ethidium bromide/ml, electrophoresed for 60 min at 100 V, and then visualized under UV light using gel documentation system.

Restriction Digestion of Amplified DNA

The amplified products were purified using PCR products purification kit (Roche, Germany) according to the user manual. Fifteen µl of purified PCR products were digested with 10 U of CfoI, MboI and AluI restriction enzymes separately in an appropriate buffer solution recommended by the manufacturer for at least 3 h at 37°C.

Ten µl of digested products analyzed by electrophoresis on 2.5% agarose gels (Gibco), and the corresponding bands were examined by phototransilluminator and compared with a standard 100 bp ladder (XIII Roche) for size estimation. The number of bands produced by each restriction enzymes used grouped together by using the first letter of enzyme name followed by a number which was indicative of the corresponding fragments produced after digestion.

Results

Sixty four out of 79 cases were positive by PCR assay at sensitivity of 91.4% and specificity of 100%. 73 cases were positive using rapid urease test and sensitivity and specificity of 100% and 66.7% were achieved respectively. 71 cases detected with Gram stain with sensitivity of 88.9% and specificity of 100% and 40 cases were positive on culture at sensitivity of 57.1% and specificity of 100%.

PCR amplification of *H. pylori* type strain 26694 genomic DNA with *ureC* primers produced a 417 bp fragment. The gastric biopsy specimens DNA also yielded a comparable products same as that of *H. pylori* type strains 26694 (Fig. 1). DNA from urease positive species not related to *H. pylori* (*Klebsiella pneumoniae* and *Proteus mirabilis*) and urease negative species (*E. coli*) subjected to PCR amplification using the same primer sets and PCR conditions. No amplified products were detected in any of above mentioned cases.



Figure 1. PCR amplification products of H-pylori ureC gene. Lanes: 1-11 biopsy samples, 12 positive control, 13 negative control and M, molecular weight marker (XIII Roche).

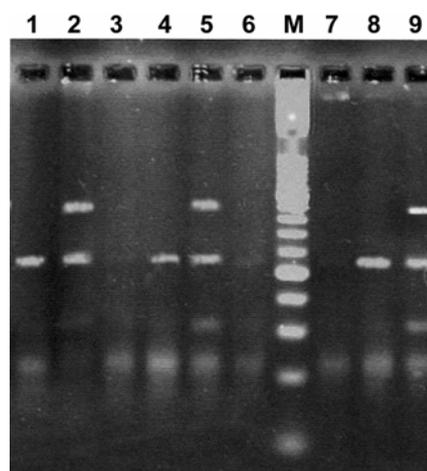


Figure 2. PCR-based RFLP patterns from the gastric biopsy specimens digested with two different restriction enzymes. Lanes: 1-6 RFLP patterns obtained through application of AluI, M, molecular weight marker (100 bp ladder) and 7-9 RFLP patterns for MboI.

RFLP Analysis

The products of 60 PCR Positive biopsy specimens were digested with three restriction enzymes, AluI, MboI and CfoI separately. Five patterns for MboI, five patterns for AluI and 4 restriction patterns for CfoI were observed. The patterns were classified based on the number of bands detectable by gel electrophoresis (Fig. 2).

Two samples totally failed to digest, but for the rest of 58 PCR products, which were digested successfully, 24 various patterns obtained. Eight samples showed

A₁M₁C₁ patterns, six samples A₂M₂C₂, for two patterns of A₃M₄C₂ and A₂M₃C₂ each one included five samples, four samples A₃M₂C₂ and there samples A₃M₃C₂.

Those RFLP products that were not matched with any of the above categories classified as distinct RFLP patterns. 29 samples were included in this group.

Discussion

In recent years, many investigators using molecular techniques have revealed that *H. pylori* possess a remarkable degree of genetic diversity, which is closely related with its epidemiological and pathological characteristics and dynamic of transmission [13]. The PCR-based RFLP analysis has been widely accepted for typing and differentiation of *H. pylori* strains from clinical specimens [13,19]. This method has been used to analyze conserved *H. pylori* genes, especially those encoding urease structural and accessories proteins [9,10,16,18].

In our study, a PCR assay using primer pairs derived from the *ureC* gene employed to amplify a 417 bp fragment from 64 gastric biopsy specimens. Other urease positive bacterial species such as *Klebsiella pneumoniae* and *Proteus mirabilis* genomes were not amplified by this PCR assay. We developed a successful PCR based RFLP assay for differentiation of *H. pylori* strains directly from gastric biopsy specimens without culture. By using this method we have detected five, five, and four distinct patterns by application of three different restriction endonuclease enzymes, CfoI, MboI and AluI, respectively on 58 biopsy specimens.

In one of patient with chronic gastritis, in both obtained specimens, one from antrum and the other from fundus. In both the bacteriological tests comprising of Gram stain, rapid urease and culture were positive. However in the bacterial culture two different colonies were observed and the RFLP patterns demonstrated two distinct patterns indicating the presence of two different strain or subtype.

It has been previously reported that two strains of *H. pylori* have been found in the stomach of some patients, or even three distinct strains reported to be found in some patient's clinical isolates [4,10,16]. Examination of large numbers of isolates from individual patients will permit a better quantitation of the extent of multiple infections.

Although several PCR-based RFLP protocols have been developed for the differentiation of *H. pylori* strains, most of the studies have been carried out on *H. pylori* isolates from gastric biopsy specimens. Culture of *H. pylori* is time consuming and not very sensitive. Our PCR-based RFLP protocol provides a means for direct

detection and differentiation of *H. pylori* strains in gastric biopsy specimens without culture. Clayton, *et al.* [5] also applied the PCR-RFLP analysis method directly on biopsy samples and stated that it is a reliable method for epidemiological investigation on the transmission of this pathogen. This shows that PCR-based RFLP assay maybe useful as a primary approach for identification of specific *H. pylori* strains in gastric biopsy specimens without culturing. Several studies have confirmed that PCR-based RFLP analysis of the *ureC* gene can differentiate *H. pylori* clinical isolates. Fujimoto *et al.* [10] have shown that digestion of 820-bp PCR amplified portion of *H. pylori ureC* gene by the restriction enzymes HhaI, MboI and MseI resulted in 10, 10, and 11 different patterns, respectively. The 25 *H. pylori* clinical isolates, however, could be grouped in to 25 distinct RFLP patterns when the three restriction enzymes were combined.

Chuanfu *et al.* [4] have also shown that digestion of a 1,179 bp PCR amplified fragment of *ureC* gene of *H. pylori* digested by the same restriction endonuclease generated 11, 10, and 6 digestion patterns respectively.

From our study it is concluded that, the PCR-based RFLP technique is a useful approach for identification of specific *H. pylori* strains in gastric biopsy specimens without any need of time taking and labor-intensive culture method.

It is also helpful for epidemiological studies. In addition, by using this method we can determine reinfection, infection with new strain, recrudescence or drug resistance in *H. pylori* infected patients.

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