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Rapid and Easy Detection of *Helicobacter pylori* by in situ Hybridization

Various in situ hybridization (ISH) methods have been used to identify *Helicobacter pylori*, a causative organism responsible for chronic gastritis and peptic ulcer disease, but they were hard to perform and time consuming. To detect *H. pylori* in a rapid and easily reproducible way, we developed synthetic biotinylated oligonucleotide probes which complement rRNA of *H. pylori*. Formalin-fixed and paraffin-embedded tissues from 50 gastric biopsy specimens were examined. Using a serologic test and histochemical stain (Warthin-Starry silver stain and/or Giemsa stain) as a standard, 40 of them were confirmed to be *H. pylori*-positive. Our ISH was quickly carried out within one hr and results were compared with those obtained from immunohistochemical stain. The ISH produced a positive reaction in 38 of 40 cases (95%). All *H. pylori*-negative cases failed to demonstrate a positive signal. The ISH has a sensitivity comparable to those of conventional histochemical and immunohistochemical stain, and has high specificity. In conclusion, ISH with a biotinylated oligonucleotide probe provides a useful diagnostic method for detecting *H. pylori* effectively in routinely processed tissue sections.

Key Words : *Helicobacter pylori; In situ hybridization; RNA, ribosomal; Biopsy*

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INTRODUCTION

Organisms in the genus *Helicobacter* are helical, curved, unbranched, flagellated gram-negative rods 0.3 to 1.0 μm wide and 1.5 to 5.0 μm long (1, 2). Most human *Helicobater* infections are caused by *Helicobacter pylori*. This important pathogenic bacteria has received much attention as a causative agent of chronic gastritis (3), peptic ulcer (4), and gastric cancer (5).

For the detection of *H. pylori*, various biopsy based methods have been used. The gold standard test for the diagnosis of *H. pylori* infection has been either histology or a combination of histology and culture. According to the development of new staining methods (6, 7) for simultaneous visualization of *H. pylori* and gastric morphologic features, recognizing infections in a histologic section is usually simple. However, there are problems in the histopathologic diagnosis; contamination of bacteria from the mouth, change of *H. pylori* morphology to the coccoid form due to antimicrobials (8) and growth of other intragastric bacteria in patients with achlorhydria associated gastric disorder (9). In these states, it is not easy to identify *H. pylori* accurately by using histochemical stain only in biopsied tissue sections.

In situ hybridization has the potential to overcome these problems. It allows identification of infectious organism and additional morphologic information. Numerous in situ hybridization techniques have been developed. Utilization of double-stranded DNA probes of various length from whole genomic DNA to about 100 bp segment of 16S ribosomal RNA (rRNA) sequences revealed valuable results for the detection of *H. pylori* (10-12) in tissue sections. However, these in situ hybridization procedures are harder to perform and more time consuming than routine histochemical stain. In this study, we tried to develop a synthetic single-stranded oligonucleotide probe for in situ hybridization to detect *H. pylori* easily and rapidly within one hour and to compare the sensitivity of in situ hybridization with that of immunohistochemistry.

MATERIALS AND METHODS

Tissue specimen

Antral mucosal biopsy specimens from fifty patients with gastric disorder were collected endoscopically. The biopsy cases were obtained from Chonnam University Hospital in Korea from 1995 to 1996. The biopsy specimens consisted

of 10 gastric ulcer, 36 gatritis (including 10 *H. pylori* negative cases) and 4 gastric cancer. The biopsy specimens were routinely fixed in 10% neutral buffered formalin and embedded in paraffin. For routine histopathologic observation, a hematoxylin-eosin stain was done.

For a negative control, ten *H. pylori* negative gastritis cases were used. To confirm the specificity of the probe, experimental negative control specimens were made by injecting fresh pork with other bacterial organisms, *Streptococcus pneumoniae, Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa* and *Proteus vulgaris* (all clinical isolates). Briefly, microorganisms cultured on agar plates were harvested in sterile saline and injected into fresh pork pieces in order to copy actual tissue specimens. They were immediately fixed in 10% neutral buffered formalin for 48 hours and embedded in paraffin.

Five μm sections were cut from the paraffin blocks, placed on positively charged Probe-On Plus glass slides (Fisher scientific, Pittsburgh, U.S.A.), and air dried.

Criteria of *H. pylori* infection

To identify *H. pylori* in the 50 cases, serological test (ELISA, BIO-RAD GAP method), histochemical stain (Warthin-Starry silver stain and Giemsa stain), immunohistochemical stain (ABC method) and in situ hybridization assay were performed.

For the serological diagnosis of *H. pylori* infection, the assessment of specific IgG level in serum was performed. The cut-off titer was set at 15 unit/mL. In histochemical stain, the grading score (13) was used to assess the presence of *H. pylori*. Grade 1 to 3 were considered positive.

In this study, the absence or presence of *H. pylori* was confirmed by combining a serological test and histochemical stain. To be considered a *H. pylori*-infected case, specimens were required to have positive results in serological test and in microscopic detection of organisms on histochemical stain (Warthin-Starry silver stain and/or Giemsa stain). All biopsy specimens were reviewed by a single pathologist who was blinded to the study.

Immunohistochemical stain

Immunohistochemical stain was performed with the avidin-biotin complex method using anti-*H. pylori* antibody (Signet).

Probe design

Two *H. pylori* specific oligonucleotide probes were designed and commercially synthesized. Twenty-two and 23 base nucleotide sequences complementing the following nucleic acid sequences, *H. pylori* 5S rRNA (Hp5S-1 probe), and 16S rRNA (Hp16S-1 probe), were selected. Using Genbank analysis (BLAST search) (14), these two probes were shown to have 100% homology to *H. pylori* nucleic acid only. A previously known probe (15, 16) complementing a portion of *H. pylori* 16S rRNA (Hp16S-2 probe) was also used as a third probe. A known 22 base oligonucleotide probe (17) for *Pneumocystis carinii* (Pc probe) was also used as a negative control probe. The detailed sequences of the four oligonucleotide probes are described in Table 1.

All probes were labelled with biotin moieties at the 3' termini (Research Genetics, Huntville, U.S.A.). The probes received lyophilized, were reconstituted to 1 μg/μL in 1 mM Tris-EDTA buffer, pH 7.5 and stored in 10 μL aliquots

Table 1. Sequences of oligonucleotide probes for the detection of *H. pylori*

Probe	Nucleotide sequence	Position	Length
Hp5S-1	5' >CCGACCTACATTCCCACTCTTGAAA<3'	5S rRNA	25-mer
Hp16S-1	5' >GGAGTATCTGGTATTAATCATCG<3'	16S rRNA	23-mer
Hp16S-2	5' >GGACATAGGCTGATCTCTTAGC<3'	16S rRNA	22-mer
Pc*	5' >TCTCTGAGGTATGGCCGTAACT<3'	5S rRNA	22-mer

* Pc : negative-control probe complementing *P. carinii* 5S rRNA

Table 2. Procedure of in situ hybridization for the detection of *H. pylori*

Reagent	Time (minute)*	Temperature (℃)
AutoDewaxer (RG)[F]	1 (2 times)	110
AutoDewaxer (RG)	1 (2 times)	RT[R]
Absolute alcohol	0.1 (6 times)	RT
Pepsin (RG)	4.5	110
Probe		
heating	1	110
cooling	0.4	RT
heating	2	105
cooling	1	RT
heating	0.5	105
cooling	1	RT
heating	0.5	95
cooling	2	RT
heating	0.5	85
cooling	3	RT
heating	0.5	85
cooling	4	RT
2X SSC	0.1 (3 times)	RT
Streptavidine-alkaline phosphatase (DAKO)[D]	8	45
Enhancer (RG)	0.1	RT
Fast Red TR and Naphthol AS-MX phosphate(RG)	10 (2 times)	45
Distilled water	0.1	RT
Hematoxylin (RG)	4	RT
Distilled water	0.1 (2 times)	RT

[F]RG : Research Genetics, Huntville, U.S.A. [R]RT: Room Temperature
[D]DAKO : DAKO, Carpinteria, U.S.A. *Total time = 58.2 minutes

at -20℃ prior to use. For in situ hybridization, the probes were diluted to various concentration from 0.1 to 10 ng/μL with a non-formamide based Probe diluent (Research Genetics, Huntville, U.S.A.).

In situ hybridization

All in situ hybridization assays were carried out using manual capillary action technology on the Microprobe staining system (Fisher Scientific, Pittsburgh, U.S.A.) with the modified one-hour method (17-18, 20). The procedure is summarized in Table 2.

RESULTS

Serological test and Histochemical stain

The specific IgG titer of 50 subjected sera was in the range of 7 to 162 unit/mL, mean 34.2±18.6 unit/mL. Warthin-Starry silver-stained and Giemsa-stained sections disclosed organisms in 40 cases, and whose IgG titers were all above 15 unit/mL. Therefore, 40 of the 50 cases met the *H. pylori* positive criteria (combination of serologic test and histochemical stain) for this study. On the Warthin-Starry silver staining, *H. pylori* were detected as spiral-shaped rods, black in the mucus layer of surface epithelium and in the superficial portions of glands (Fig. 1).

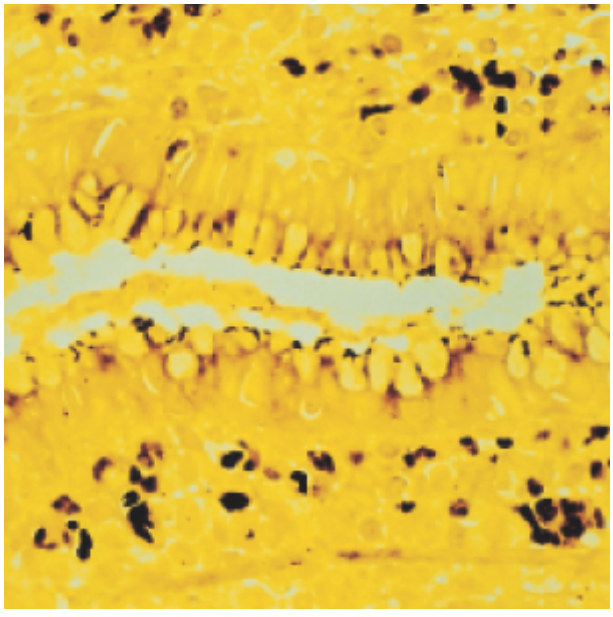

Fig. 1. Some *H. pylori* were identified as black-stained rods in the mucous layer of surface epithelium. (Warthin-Starry silver stain, × 200)

Hybridization condition

Several preliminary experiments were performed to determine the adequate condition for optimal signal. To decrease the nonspecific binding observed in mucus and mucus-secreting cells, several modifications were done to pretreatment and hybridization conditions. Reaction time of pepsin treatment affected staining intensity and tissue destruction. Digestion times ranging from 1 to 10 min were tried. Four and a half to five min with pepsin solution (2 mg/mL) were found to give an optimal signal with minimal background and good preservation of morphology. Concentration of probes also made a difference in the staining results. Although a weak positive reaction was observed at probe concentration of 0.1 ng/μL, the optimal concentration for most specimens was 1 to 2 ng/μL.

Application to clinical specimens

At the probe concentration of 1 ng/μL, the in situ hybridization assay for 16S rRNA of *H. pylori* produced a positive reaction in 38 of 40 cases (95%) each, using the Hp16S-1 and Hp16S-2 probes. In the case of Hp5S-1 probe, positive staining was only observed in six of 40 cases (15%) (Table 3). The positive signals were predominantly observed in the bacterial organisms along the luminal aspect of surface epithelial cells and in the pits (Fig. 2, 3). No signals were detected in the epithelium and lamina propria. Immunohistochemical stain (Fig. 4) showed positive reaction in 36 of 40 cases (90%). When compared to in situ hybridization assay, there was agreement in 36 cases (90%; 35 positive, 1 negative). Three cases were shown negative by immunohistochemical stain, but positive by in situ hybridization. One case was negative by in situ hybridization, but positive by immunohistochemical stain (Table 4).

Table 3. Reactivity of four different oligonucleotide probes for *H. pylori* ribosomal RNA

Probe	Positive (%)	Negative (%)
Hp5S-1	6 (15)	34 (85)
Hp16S-1	38 (95)	2 (5)
Hp16S-2	38 (95)	2 (5)
Pc*	0 (0)	40 (100)

* Pc : negative-control probe complementing *P. carinii* 5S rRNA

Table 4. Number of cases with positive and negative *in situ* hybridization stains for the detection of *H. pylori* compared to immunohistochemical stain

Immunohistochemical stain	In situ hybridization	
	Positive (%)	Negative (%)
Positive	35 (87.5)	1 (2.5)
Negative	3 (7.5)	1 (2.5)

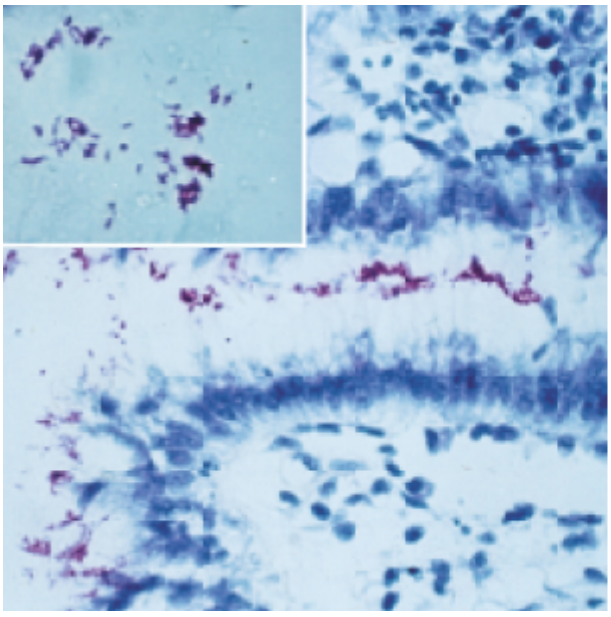

Fig. 2. In situ hybridization with Hp16S-1 oligonucleotide probe for the detection of *H. pylori*. Positive signals were predominantly observed in the bacterial organisms along the luminal aspect of surface epithelial cells (Hematoxylin counter stain, × 200; inset, × 400).

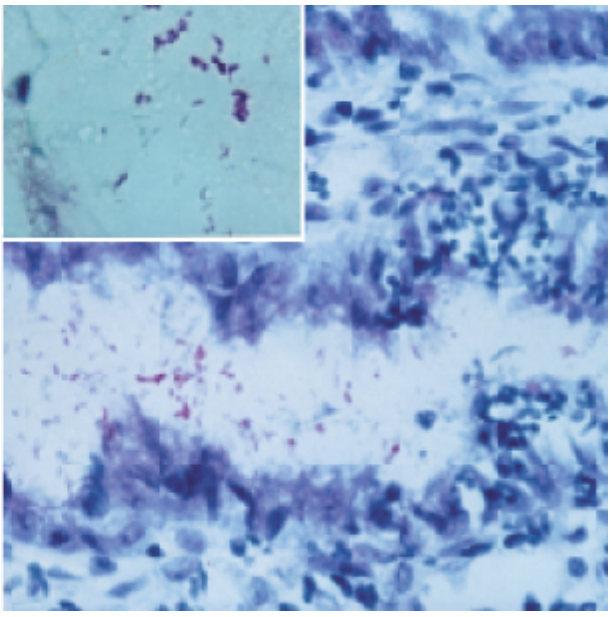

Fig. 3. in situ hybridization with Hp16S-2 oligonucleotide probe for the detection of *H. pylori*. Numerous red-colored rods were scattered in the lumen of gastric gland (Hematoxylin counter stain, × 200; inset, × 400).

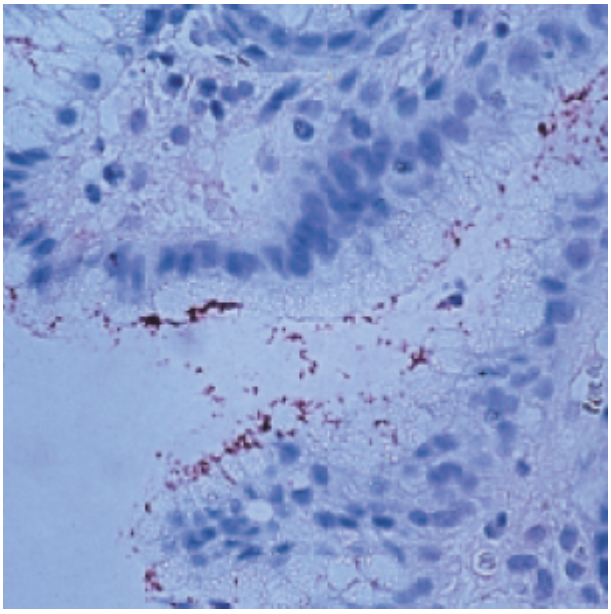

Fig. 4. Many *H. pylori* were stained with reddish-brown color along the luminal aspect of surface epithelial cells. (Immunohistochemical stain, × 200)

Specificity of probes

Ten *Helicobacer*-negative specimens failed to demonstrate a positive signal by in situ hybridization with *H. pylori* specific probes. When sections of meat injected with cultured bacteria were hybridized with *H. pylori* specific probes, no positive reactions were observed in both Gram-positive and Gram-negative bacilli. No signals of *H. pylori* was visible when sections were hybridized with the negative control probe (Pc probe) and hybridization mixture without any probe.

DISCUSSION

In situ hybridization is an effective technique for the diagnosis of infectious agents in tissues sections. In situ hybridization used for rRNA sequences may be beneficial for organisms that are not easily cultured, and for organisms that are seen with routine histochemical stains but their cultures are negative or not performed. Ribosomal RNA is present in cells to perform protein synthesis. Because of its abundance and being highly conserved, rRNA is an optimal target for in situ hybridization. Some probes complementary to specific rRNA sequences have been used to detect various infectious agents, including protozoa (17, 19), fungus (20, 21) and bacteria (15, 22). Therefore, our oligonucleotide probes were made specifically for 5S rRNA (Hp5S-1 probe) and 16S rRNA (Hp16S-1 probe) of *H. pylori*. A previously known probe (Hp16S-2 probe) (15, 16) was also complementary to rRNA.

When the 3 probes were applied to clinical specimens at

the probe concentration of 1 ng/μL, there was a difference in the sensitivity. The two probes complementary to 16S rRNA of *H. pylori* had good detection rate (95%), but Hp5S-1 probe did not. We tried other probes specific to 5S rRNA of *H. pylori*. They did not have good results, either (data not shown). At high concentration (>5 ng/μL) of Hp5S-1 probe, *H. pylori* showed faint positive signals, but nonspecific signals were detected in the gastric epithelium and lamina propria also. Therefore, it is suggested that 5S rRNA portion might not be a good target for the *H. pylori*-detecting oligonucleotide probe.

When the detection rate was compared, sensitivity of in situ hybridization (95%) was higher than immunohistochemistry (90%), but lower than histochemistry (100%). So, commonly used histochemical stains nicely demonstrate organisms. It is already reported that the Giemsa stain has a 98% sensitivity for the diagnosis of *H. pylori* infection (1). However, histopathologic diagnosis alone has problems in various situations such as contamination of bacteria from the mouth, change of *H. pylori* morphology (8), and growth of various intragastric bacteria in patients with achlorhydria (9). Although a large proportion of the gastric disorders have been attributed to infection by *H. pylori*, some patients suffering from these symptoms showed culture negative and antibody negative for *H. pylori*. Hansing et al. (23) reported that an uncharacterized organism was proposed as the causative agent of gastric inflammation in some patients (8.5%) who underwent biopsy. In these conditions, histochemical stains are no species-specific diagnostic methods to identify *H. pylori* in tissue sections. However, in situ hybridization method has the potential to overcome these problems while still using conventionally processed biopsy material.

It is reported that bacterial nucleic acids have limited accessibility due to the bacterial cell wall; cell walls of Gram-positive microbes seem to be less permeable than Gram-negative organism (22). The enzymatic predigestion step with pepsin appeared to be necessary for a good hybridization reaction. Pepsin treatment improved probe penetration by digesting the proteins of the bacterial cell wall. Pepsin predigestion could be effectively performed under high temperature condition for a short duration. Another obstacle is the nonspecific binding of biotin with the bacterial cell wall (24). The specificity of biotinylated probe was tested by (a) in situ hybridization with a negative control probe (biotin-labelled Pc probe) specific to 5S rRNA of *P. carinii*, (b) in situ hybridization with a hybridization mixture without any probe. There were no visible labelling in the *H. pylori* and gastric mucosa. The specificity of probes was also tested using sections containing other various bacterial organisms (two Gram-positives and three Gram-negatives). Both bacterial organisms were negative by in situ hybridization using the three different probes. Thus, it is proved that biotin-labelled oligonuclotide probes have high specificity.

Several molecular biological techniques have been described in the identification of *H. pylori*, including hybridization (25) with radioactively and non-radioactively labelled genomic and cloned DNA probes, in situ hybridization (10-12, 15, 26), and PCR (27). In situ hybridization offers the advantage of preserving tissue morphology and being sensitive enough to permit detection of even a small number of organisms. The first in situ hybridization technique for detecting *H. pylori* was introduced by van den Berg et al. (10). They used the whole genomic DNA of *H. pylori* labelled with biotin as their probe. Total hybridization time was 10 hr at 37℃. Bashir et al. (11) used a biotinylated 109 base pair probe. This probe is more specific and shows no cross reaction with gastric epithelial cells and the closely related organisms, but it must be incubated overnight at 37℃. Leimola-Virtanen et al. (26) used a commercial probe to detect *H. pylori* in biopsied tissue of oral mucosal ulcer. Overnight hybridization was needed, too. Karttunen et al. (12) used digoxigenin labelled PCR product as a probe. Their probe had 520 base pair segment of 16S rRNA from *H. pylori*. The specificity of the probe was tested using sections containing other bacteria. Their probe should be hybridized overnight at 37℃.

Previous in situ hybridization assays with various probes provide sensitive and specific results for the detection of *H. pylori*. However, these techniques needed a lot of time (10 hr to overnight hybridization in a humidified chamber), and were harder to perform than a routine histochemical stain. Because these hybidization methods do not provide a rapid and cost-effective method for routine diagnosis of *H pylori* infection, they may be valuable only in selected problem situations or unusual locations such as the oral mucosa (26).

Rapid and cost-effective identification of infectious pathogens in routinely processed tissue is of great importance in diagnostic molecular pathology. Recently, Barrett et al. (15) have developed a rapid in situ hybridization assay with biotinylated oligonucleotide probe. Total reaction time was reduced to about two hours. In this study, by using biotinylated oligonucleotide probes and increasing pepsin-digestion time to 4.5 min, we could successfully reduced hybridization time to less than 20 min and perform all procedure within 60 min. Since oligonucleotide probes used in this study were very short segments of DNA (less than 25 bases), they could readily permeate tissues, even those fixed with aldehyde fixatives. Pepsin pretreatment improved probe penetration also by digesting the proteins of the bacterial cell wall. So, our procedure is no longer hard to perform nor time consuming.

In conclusion, in situ hybridization with biotinylated oligonucleotide probes revealed good reactivity in the detection of *H. pylori*. This method is rapid and cost-effective also. Thus, this one-hour in situ hybridization assay is expected to be widely applicable to the detection of *H. pylori* in the formalin-fixed paraffin-embedded tissue sections.

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