Detection of a *Helicobacter pylori* Gene Marker in Gastric Biopsy Samples by Non-radioactive *in situ* Hybridization

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The presence of *H. pylori* was examined by non-radioactive *in situ* hybridization using a digoxigenin-labelled DNA probe and a synthetic *H. pylori* urease gene-specific thymine-thymine dimerized oligonucleotide probe. *H. pylori* was detected in 20/33 gastric biopsy specimens, and was localized in gastric epithelia and neutrophils. No non-

specific DNA signal was detected in colonic mucosa, esophageal mucosa, skin and liver specimens. The DNA probe did not cross-hybridize with DNA from other bacteria. As this method is specific and not time-consuming, it may be useful for revealing the location of *H. pylori*.

Key words: *Helicobacter pylori*, Gastric biopsy, ISH

I. Introduction

Helicobacter pylori (*H. pylori*) is a gram-negative, spiral-shaped organism colonizing the gastric mucosa of humans and is associated with both antral gastritis and duodenal ulcer [1, 8, 12, 18]. Epidemiological data suggest a relationship between early infection with *H. pylori* and an increased risk of developing gastric adenocarcinoma in later life [21, 22].

H. pylori is only found in association with gastric mucus-secreting cells *in vivo*. The organism lies within and beneath the gastric mucus layer, and can also be attached to the surface of gastric epithelial cells [14]. It is not found in either the duodenum or the esophagus except at sites of gastric metaplasia, suggesting a particular tropism of the organism for the gastric mucosal surface in humans [29].

Many diagnostic tests have been developed for detecting infection with this organism [3, 5, 9, 10, 13, 19, 24]. These include the ¹⁴C-urea breath test, serodiagnostic tests, morphological examination, culture of *H. pylori*, ELISA, rapid urease test and polymerase chain reaction (PCR). However, each test has both advantages and disadvantages; some are costly, time-consuming and nonspecific. A reliable and rapid means of detecting *H.*

pylori in tissue samples would therefore be useful for the clinical management of patients with severe symptoms facilitating the prompt introduction of antibiotic therapy.

The present experiments were designed to detect *H. pylori* by *in situ* DNA hybridization, which is a specific and inexpensive procedure, and takes 60 hr to complete. This method of detection would enable retrospective and prospective analyses of clinical samples to be done, thus helping to elucidate the role of this organism in gastroduodenal diseases.

II. Materials and Methods

Clinical samples

Thirty-three human gastric biopsy specimens, two colonic biopsy specimens (ulcerative colitis), two esophageal mucosa biopsy specimens, two liver tissue samples and two skin biopsy specimens were obtained from the Department of Pathology, Saitama Medical Center.

DNA probes

Two kinds of probes were used for *in situ* detection of a *H. pylori* DNA-DNA probe from the genome of the organism and a synthetic oligonucleotide specific for the *H. pylori* urease gene [4]. Bacterial genomic DNA was extracted as described previously from cultured *H. pylori*. The purified genomic DNA was treated with *EcoRI*, and DNA fragments 0.5–1.0 kb in length were used as a probe. A digoxigenin (DIG)-labelled DNA probe was kindly sup-

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plied by KREATECH Biotechnology BV, Amsterdam, Netherlands [28]. The non-specific DNA probe (pBR322) was used as a negative control.

For thymine-thymine dimer *in situ* hybridization, the oligonucleotide was synthesized on an Applied Biosystems synthesizer (391-EP) by the automated phosphoramidite coupling method and purified as described by the manufacturer.

Thymine-thymine was introduced at the 5' and 3' ends to obtain a thymine-thymine dimerized DNA probe after UV irradiation [16, 25]. The oligonucleotide used as a probe for the detection of *H. pylori* was derived from the sequenced urease gene and did not cross-hybridize with urease genes from other species theoretically [4]. The anti-sense probe was 5'-TT700TACACAGAGCTAAA-GAGCGTGGTTTTCATGGCGCTAAAAGCGATGACA-A749CTT-3'. The sense probe as a negative control was 5'-TTGTTGTCATCGCTTTTAGCGCCATGAAAACCAC-GCTCTTTAGCTCTGTGTATT-3'.

In situ hybridization

Two kinds of non-radioactive *in situ* hybridization were used: that using the DIG-labelled probe (a) and that using the thymine-thymine (T-T) dimerized probe (b).

(a): The hybridization using the DIG-labelled probe was carried out according to the manufacturer's instruction sheet (KREATECH Biotechnology BV). After 4–6- μ m paraffin sections had been deparaffinized in xylene, they were hydrated and then incubated in distilled water containing 2.5 mg/ml pepsin (Sigma Chemical Co., St. Louis, MO, USA) and 0.01 N HCl for 30 min at 37°C. Probe and target DNA were denatured simultaneously by placing the slides for 5 min on a 90°C hot plate. The hybridization was performed in a hybridization mixture (2xSSC, 50% formamide, 10% dextran sulfate, 10% Denhardt solution, 0.5% Tween 20, 250 μ g/ml salmon sperm DNA and the DIG-labelled probe) by incubating the slides overnight in a humidified chamber at 37°C. After a 10 min-blocking, alkaline phosphatase-conjugated anti-digoxigenin antibody (1 : 500 diluted) was added to the slides for 30 min. Thereafter, nitroblue tetrazolium (NBT) solution was added, and incubation was carried out for 60 min in the dark. The sections were counterstained with fast red and mounted with glycerol gelatin for microscopical evaluation.

(b): T-T dimer was introduced into the synthetic probe by UV irradiation as described in detail previously [17, 25]. The anti-sense synthetic probe was irradiated at a rate of 5 J/m²/s for 23.3 min (7000 J/m²). The hybridization was carried out with 1 μ g/ml synthetic probe at 37°C overnight as described elsewhere [16, 17]. Then the sections were reacted with peroxidase-labelled anti-T-T monoclonal antibody (10 μ g/ml) for 1 hr. Thereafter, the sections were visualized with diaminobenzidine and H₂O₂ for microscopical examination.

Culture

The biopsy samples collected at Saitama Medical Center were inoculated onto selective agar plates containing horse blood, vancomycin (10 μ g/ml), colomycin (10 U/ml), trimethoprim (5 μ g/ml) and amphotericin B (2 μ g/ml), and incubated at 37°C under microaerophilic conditions for up to 7 days. The organisms were identified as *H. pylori* on the basis of morphology in Gram stains and by the oxidase, catalase, and rapid urease tests [7].

III. Results

Detection of H. pylori by in situ hybridization

H. pylori was detected in 20/33 cases by *in situ* hybridization using the DIG-labelled DNA probe. *H. pylori* was localized along and within the gastric epithelia (Fig. 1A). Interestingly, *H. pylori* was also recognized in neutrophils (Fig. 1A). However, the organism was not detected in ulcerative colitis specimens, esophageal mucosa, cutaneous tissue and liver tissue. These findings were further confirmed by *in situ* hybridization using the thymine-thymine dimerized synthetic probe (Fig. 1C). On the other hand, the specimens did not stain in the absence of the probe and/or the primary antibody (negative control). The non-specific DNA probe (pBR322) or sense oligodeoxynucleotide did not hybridize with *H. pylori* DNA in the positive gastric specimens (negative control).

Culture of H. pylori

H. pylori was detected in 16/33 gastric biopsy specimens (Table 1).

IV. Discussion

We have described a new technique for detection of *H. pylori*. A rapid, sensitive, and specific test for *H. pylori* would be of great value because of the clinical importance of this pathogen and the large number of laboratory identifications being undertaken routinely throughout the world on a daily basis. The aims of this study were to develop a simple method for the localization and specific identification of *H. pylori* present in clinical biopsy samples. Under stringent conditions, our method did not detect DNA from other bacteria and did not produce cross-reactions with clinical samples other than gastric biopsy specimens.

A wide variety of diagnostic tests have been developed for detecting infection with *H. pylori* [3, 5, 9, 10, 13, 19, 24]. However, all have certain advantages and disadvantages. The ¹⁴C-urea breath test is a reliable noninvasive assay, but it is costly and time-consuming and involves exposure to radioactivity. Serodiagnostic tests have been developed, but these do not allow differentiation of a current active *H. pylori* infection from a previous one. The urease test is a rapid method for detecting *H. pylori* in biopsy specimens, but false-positive and false-negative results have been reported [3, 11]. Microscopic analysis

Fig. 1. Detection of *Helicobacter pylori* DNA by *in situ* hybridization. **A:** Gastric mucosa (No. 16) with immunostaining products of *Helicobacter pylori* DNA. The signals are recognizable in the glandular epithelia and neutrophils (→). ×200. Counterstaining with fast red. **B:** Gastric mucosa (No. 16) when pBR322 DNA fragments were used as a negative control. ×200. Counterstaining with fast red. **C:** *Helicobacter pylori* DNA in gastric mucosa (No. 18) detected by T-T dimer *in situ* hybridization. The immunostaining products are recognizable along the surface of epithelial cells (→). ×200. **D:** Gastric mucosa (No. 18) when a sense *Helicobacter pylori* DNA-specific oligodeoxynucleotide was used as a negative control. ×200. **E:** Gastric mucosa (No. 18) stained with hematoxylin-eosin. *Helicobacter pylori* is recognizable in the glandular epithelia (→). ×400.

Table 1. Detection of *Helicobacter pylori* by *in situ* DNA hybridization and the culture method

Sample No.	Patient		Clinical Diagnosis	ISH*	Culture†
	Sex	Age			
1	M	59	gastric ulcer	—	+
2	M	73	colonic polyp, chronic gastritis	+	—
3	M	52	chronic gastritis	—	—
4	M	54	chronic gastritis	—	—
5	M	65	gastric erosion	—	—
6	F	78	chronic gastritis	—	—
7	F	73	chronic gastritis	—	—
8	F	72	chronic gastritis	—	+
9	F	42	renal failure, chronic gastritis	+	+
10	F	51	chronic gastritis	+	+
11	F	43	chronic gastritis	+	—
12	F	54	chronic gastriti	+	+
13	M	49	chronic gastritis	—	—
14	M	69	renal failure, chronic gastritis	+	+
15	F	53	renal failure, chronic gastritis	+	—
16	M	43	chronic gastritis	+	+
17	F	57	renal failure, chronic gastritis	—	+
18	M	58	renal failure, chronic gastritis	+	+
19	F	57	gastric ulcer	—	+
20	M	49	chronic gastritis	—	—
21	M	45	gastric ulcer	—	—
22	M	45	chronic gastritis	+	+
23	M	62	chronic gastritis	+	—
24	M	59	gastric polyp	+	—
25	F	44	chronic gastritis	+	+
26	M	75	chronic gastritis	+	+
27	F	78	chronic gastritis	+	—
28	F	65	gastric ulcer	+	+
29	F	71	chronic gastritis	—	—
30	F	67	chronic gastritis	+	—
31	F	63	gastric erosion	+	—
32	M	51	gastric ulcer	+	+
33	F	51	gastric ulcer	+	+

* The positivity of *in situ* hybridization (ISH) was determined as follows: + in immunostaining products were recognized, and — if no products were recognized.

† The presence of *Helicobacter pylori* was determined by culture using Skirrow medium: + if bacterial colonies were observed, and — if no colony was observed.

of stained biopsy smears is a nonspecific test and has a low sensitivity for detection of this organism. Culture of *H. pylori*, which is a fastidious and slow growing organism, is both difficult and time-consuming, requiring 5 to 7 days of incubation [12, 15]. The detection of *H. pylori* by PCR facilitates both retrospective and prospective analyses of clinical samples, but does not localize *H. pylori* in tissues [5]. The detection efficiency of PCR is low in paraffin-embedded clinical samples, since the DNA obtained from such samples is usually of poor quality because of fixation effects [23]. A nested primer amplification approach, which has been reported for *M. leprae* detection by PCR, may be more useful. On the other hand, our method

seems to have more advantages than the above. It takes only 1 to 2 days to get results [6], and it localizes *H. pylori* in tissues. Since the precise location of *H. pylori* can be determined in clinical tissues, it is possible to examine the involvement of this organism in the pathogenesis of active gastric ulcer and gastric cancer in humans. Furthermore, it may provide a rapid means of detecting viable but non-culturable *H. pylori*.

A discrepancy in the detection rate between *in situ* hybridization (20/33) and the culture method (16/33) was seen. This may reflect the differences between the methods: the former detects the presence of *H. pylori* on the surface of gastric epithelia, whereas the latter detects

H. pylori in tissue cross-sections. Alternatively, this may also reflect the difference of location where the gastric biopsy was taken. The two gastric biopsies were taken at the sites which were in close proximity, but not taken from the same locations.

H. pylori is usually detected along and within the gastric epithelia [6]. It has been reported that the attachment of *H. pylori* to human gastric epithelia is mediated by a blood group antigen (Le^b) [2]. Interestingly, *H. pylori* was detected in neutrophils which were demonstrated morphologically and by peroxidase reactivity. This is not surprising because it has been demonstrated that *H. pylori* is present in the lysosomes of neutrophils [26, 27]. It indicates that *H. pylori* is phagocytosed by activated neutrophils.

V. References

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