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Comparison of fluorescent *in situ* hybridization and histological method for the diagnosis of *Helicobacter pylori* in gastric biopsy samples

Authors' Contribution:
A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

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Summary

Background: The establishment of *Helicobacter pylori* in the stomach and duodenum is associated with gastritis, peptic ulcers, and gastric cancer. Application of suitable methods, including molecular techniques, for an accurate detection of *H. pylori* can lead to the administration of appropriate drugs and successful therapy. In this study, fluorescent *in situ* hybridization (FISH) was compared with histology for the diagnosis of *H. pylori* in gastric biopsy specimens.

Material/Methods: Flourescently labeled oligonucleotdie probes that target ribosomal RNA were utilized in the FISH procedure. Ninety-one gastric biopsy specimens were tested by FISH and by histology using hematoxylin-eosin (H-E) and Geimsa stains. Furthermore, clarithromycin resistance in 39 of the 91 specimens was examined by FISH.

Results: The sensitivity and specificity of FISH for the detection of *H. pylori* were 97.9% and 100%, respectively. Of the 39 samples that were tested for clarithromycin resistance, 19 were FISH positive for *H. pylori*, of which 15 and 4 specimens were infected with clarithromycin-susceptible and clarithromycin-resistant strains, respectively. There were coccoid forms of *H. pylori* in a few of the specimens.

Conclusions: FISH is a highly sensitive and specific technique for the diagnosis of *H. pylori* infection. It can be a method of identification when a patient is infected with coccoid forms of *H. pylori*. The ability of FISH for determination of clarithromycin resistance is a considerable advantage of this method over histology.

key words: **fluorescent *in situ* hybridization • FISH • *Helicobacter pylori* • clarithromycin**

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BACKGROUND

Infection with *H. pylori* is one of the most common diseases in humans and is associated with gastritis, peptic ulcers, lymphoma, and cancer of the stomach [1]. Therefore, rapid diagnosis of this microorganism is needed. There are several methods for detecting *H. pylori* infection. For example, serological tests are rapid, but anti-*H. pylori* antibodies are present three to six months after treatment; therefore, active infections cannot be distinguished from past infections [1,2]. The rapid urease test of gastric biopsies cannot detect infection with a sufficient degree of certainty, so it can be considered as an auxiliary test [2]. The culture and isolation of *H. pylori* is a specific method and can detect antibiotic susceptibility; however, it is a time-consuming process requiring four to seven days of incubation with no high sensitivity [2,3]. Histological examination is a suitable technique with very high sensitivity and specificity for the detection of *H. pylori*, but it cannot determine antimicrobial susceptibility [1].

Fluorescent *in situ* hybridization (FISH) is considered as a rapid and specific detection method in diagnostic microbiology. Fluorescently labeled oligonucleotide probes that target ribosomal RNA are utilized in the FISH technique for direct molecular detection of microorganisms in clinical samples [4–6]. FISH has been successfully applied for the detection several bacteria, such as treponemes [7], *Salmonella* [8], *Streptococci* [9–11], *Legionella* [12], and *Chlamydiae* [13]. In previous studies, the application of FISH for the detection of *H. pylori* and claritromycin-resistant strains in comparison with conventional culture methods were reported by Russmann et al. [3] and also by our research team [14,15]. The aim of this study was to compare FISH with histology for the detection of *H. pylori* in gastric biopsy specimens.

MATERIAL AND METHODS

Preparation of gastric biopsy specimens

Gastric biopsy samples from 91 dyspeptic patients were examined. The specimens were taken before the patients received antibiotics from the antrum and corpus of the stomach during routine endoscopy and put into 10% formalin for fixation. Afterwards the samples were embedded in paraffin, cut into 4-µm-thick sections, and placed on glass slides. To enhance the attachment of tissues, the slides were incubated at 55°C overnight. To deparaffinize the tissue sections, the slides were submerged in hexane (Merck, Germany) twice, each time for 30 minutes. The slides were then submerged in absolute ethanol (Merck, Germany) twice, each time for 30 minutes [16]. The slides were left to air dry and were then ready for examination.

Oligonucleotide probes

Probe Hpy, synthesized and labeled by TIB MOLBIOL (Germany), was used to specifically identify *H. pylori* (Table 1). This probe specifically targets and hybridizes a 16S rRNA position of the *H. pylori* [6]. The 5' end of probe Hpy was labeled with fluorochrome FLOUS [5(6)-carboxyfluorescein-N-hydroxy succinimide-ester], which emits a green signal [9,17].

Probes ClaR1, ClaR2, and ClaR3, which were synthesized and 5' labeled (Metabion, Germany) with fluorochrome Cy3 (red signal), detect 23S rRNA point mutations responsible for clarithromycin resistance and were therefore used to detect clarithromycin-resistant strains of *H. pylori* (Table 1) [6]. There are three described mutations in which the adenine residues at position 2143 and 2144 are replaced by guanine (A2143G and A2144G) or cytosine (A2143C). Probes ClaR1, ClaR2, and ClaR3 detect A2143G, A2144G, and A2143C point mutations, respectively [3,6]. Probe ClaWT (Metabion, Germany), which hybridizes to the 23S rRNA of clarithromycin-sensitive strains (wild type), was unlabeled [6].

FISH

Fifty-two specimens were examined only with probe Hpy. The FISH procedure was performed as previously described [3,6,14,15]. Briefly, for hybridization of the samples, each slide of the tissue sections was covered with 40 µl of hybridization buffer (0.9 M NaCl, 20 mM Tris-HCl, pH 8, 0.01% SDS, 20% formamide) containing 5 ng/µl of probe Hpy [14]. The remaining 39 specimens were tested with a mixture of probes Hpy, ClaR1, ClaR2, ClaR3, and ClaWT; therefore the slides of these specimens were covered with 40 µl of hybridization buffer containing 5 ng/µl of the each of the five probes [15]. Then the slides were put separately into a moisture chamber and incubated at 46°C for 90 minutes for the hybridization step [3,6,14,15]. The classification of the samples into the two groups of 52 and 39 was random.

Stringent washing was carried out in washing buffer (20 mM Tris-HCl, pH 8, 0.01% SDS, 225 mM NaCl) at 48°C for 15 minutes [3,9,14]. The slides were then stained with 1 µg/ml DAPI (4',6-diamidine-2'-phenylindole dihydrochloride) for 5 minutes [18]. DAPI nonspecifically stains the DNA of any cell, including bacteria, blue [5,10]. Finally, the slides were washed with PBS, left to air dry, covered with fluorescent mounting medium (DAKO, Denmark) [14,15,19], and visualized with a Nikon E400 epi-fluorescence microscope (Japan) equipped with different filters.

Histology

All the 91 specimens were investigated for *H. pylori* infection by means of a histological method using hematoxylin-eosin (H-E) and Geimsa stains.

Analysis of test

The sensitivity and specificity of FISH were calculated with the formulas $a/(a + c) \times 100$ and $d/(b + d) \times 100$, respectively, where a = true positive, b = false positive, c = false negative, and d = true negative.

RESULTS

Ninety-one gastric biopsy samples were examined by FISH (using probe Hpy-FLOUS) and histology for the detection of *H. pylori* (Table 2). Forty-six of the samples were *H. pylori* positive according to FISH (showing a green fluorescent signal) and the histological method. Forty-three samples were negative for *H. pylori* according to both FISH and histology. One specimen was histology positive but FISH negative. In

Table1. Oligonucleotide probes for detection of H.pylori and clarithromycin resistance.

Probe	Sequence(5′-3′)	Target site
Hpy	CAC ACC TGA CTG ACT ATC CCG	16S rRNA
ClaR1	CGG GGT CTT CCC GTC TT	23S rRNA(mutant)
ClaR2	CGG GGT CTC TCC GTC TT	23S rRNA(mutant)
ClaR3	CGG GGT CTT GCC GTC TT	23S rRNA(mutant)
ClaWT	CGG GGT CTT TCC GTC TT	23S rRNA(wild)

Table2. Examination of 91 gastric biopsy samples by FISH(using probe Hpy) and histology for detection of *H. pylori*.

Number of specimens	Results	
	FISH	Histology
46	Positive	Positive
43	Negative	Negative
1	Negative	Positive
1	Positive	Negative

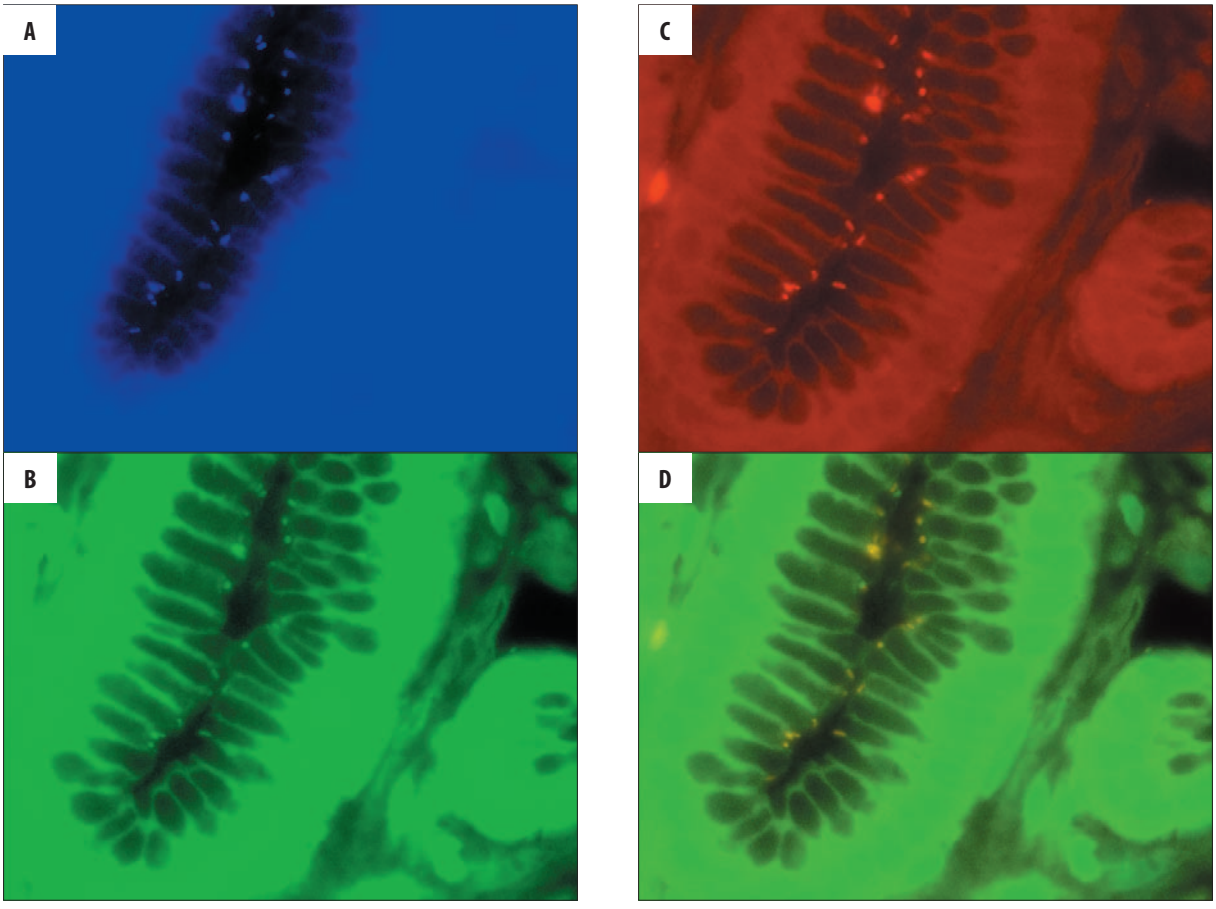

Figure 1. Visualization of *H. pylori* in gastric biopsy specimen by FISH. Panels **A**, **B**, **C**, and **D** demonstrate the same microscopic field under the relevant filter. (**A**) shows bacteria in blue because of staining DNA with DAPI. (**B**) demonstrate bacteria in green, indicating hybridization with probe Hpy-FLOUS. (**C**) shows red signal, indicating hybridization with probe ClaR1-Cy3, ClaR2-Cy3, or ClaR3-Cy3; therefore the strain is clarithromycin resistant. (**D**) the combination of red and green of a resistant strain represent a yellow or orange signal. The specimen contains a number of coccoid forms.

one sample, *H. pylori* was detected by FISH but not by histology. According to this study, the sensitivity and specificity of FISH for the detection of *H. pylori* were 97.9% and 100%, respectively. The 95% confidence interval for sensitivity was 93.9-100%.

Thirty-nine of the 91 specimens were also studied by FISH to detect clarithromycin-susceptible and -resistant strains of *H. pylori*. The susceptible strains were hybridized with probes Hpy and ClaWT and thus were observed with a green signal. However, the resistant strains were hybridized with probes Hpy and ClaR1, ClaR2, or ClaR3 and therefore emitted both green and red fluorescent signals (Figure 1). Of the 39 samples that were tested for clarithromycin resistance, 19 were FISH positive for *H. pylori* (19 samples of a total 47 FISH-positive specimens), of which 15 samples were infected with clarithromycin-susceptible and 4 with clarithromycin-resistant strains. There were coccoid forms of *H. pylori* in a few of the specimens.

Discussion

Molecular techniques such as FISH are increasingly applied for precise and rapid detection of heritable and infectious diseases. For example, quantitative study of chromosome 3 arm imbalance by FISH was reported [20]. Subtelomeric rearrangements in families with idiopathic mental retardation were investigated by the FISH technique [21]. Application of FISH to identify pathogenic bacteria has been among the most successful studies during these years. In our investigation, comparison of FISH and histology for the detection of *H. pylori* in gastric biopsy specimens was studied. The sensitivity and specificity of FISH were 97.9% and 100%, respectively. FISH is therefore a suitable method for detecting *H. pylori*.

In one specimen, *H. pylori* was detected by histology but not by FISH, which is the false negative result of FISH (Table 2). This specimen emitted a high background fluorescence, which could be the cause of masking the specific fluorescent signal. Sometimes a little paraffin residual in tissue sections exhibits a fluorescent signal. Background fluorescence is a limitation of FISH which was also reported in other specimens, such as sputum and various tissues that contain elastin, collagen, or blood cells [9,22].

There is another interpretation for the aforementioned histology-positive FISH-negative sample in that it may be a false-positive result of histology. In addition to *H. pylori*, *Helicobacter heilmannii* may occasionally be present in the human stomach. *H. heilmannii* is much less commonly observed in the stomach than *H. pylori* and occurs in perhaps 1% of persons [1]. Although *H. heilmannii* usually has a longer size and more pronounced helical morphology than *H. pylori*, these distinctions may not be absolute and these bacteria are sometimes observed similar together *in vivo* [23]. Moreover, isolation of C*ampylobacter jejuni* from the gastric biopsy of a patient with gastric ulcer has been reported [23]. Therefore it is probable that the mentioned specimen in our study contained microorganisms such as *H. heilmannii* or *C. jejuni* which were observed to be similar to *H. pylori* on the histological slide. However, probe Hpy is highly specific for the detection of *H. pylori* by FISH and could differentiate this bacterium from *H. heilmannii* and *C. jejuni*. Trebesius and colleagues have shown that probe Hpy does not hybridize to the rRNA of *H. heilmannii*, *C. jejuni*, and other closely related bacterial species [6].

One specimen was FISH positive but histology negative (Table 2). The following reason suggests which this FISH result is not false positive, but it could be a false-negative result of histology. A few *H. pylori* (mild infection) were seen on the FISH slide of this sample, most of which were of coccoid form. Transformation of spiral *H. pylori* to coccoid, non-cultivable forms occasionally occurs *in vitro* and *in vivo*. It has been shown that coccoid forms of *H. pylori* contain a sufficient amount of ribosomal RNA to be detected specifically by FISH [6]. However, since the histological method relies on typical morphology (curved and spiral forms), it could be possible that mild *H. pylori* infection with the presence of mostly coccoid forms was missed in histological examination. Keeping in mind that, firstly, FISH detects coccoid forms accurately and, secondly, the establishment of coccoid forms would be a cause of drug resistance, therapy failure, and recurrent infections with *H. pylori* [6], this technique clearly has an advantage.

In this study, 39 gastric biopsy samples were investigated by FISH to detect clarithromycin-susceptible and -resistant strains of *H. pylori* (see results). Clarithromycin is one of the most important drugs for the eradication of *H. pylori* infection. However, *H. pylori* is gradually acquiring resistance to this antibiotic [1,3,6]. Therefore, determination of clarithromycin resistance is essential for successful therapy. Histological methods do not allow resistance determination, so the ability of FISH to detect clarithromycin resistance is the major advantage of this technique over histology. This is especially important for coccoid forms since they are non-cultivable [6]; thus it is not possible to determine resistance by routine culture and antibiogram.

As shown in panel C of Figure 1, the resistant strains emitted a focal and strong red signal. Cy3 is a cyanine dye and is superior to the classical dyes because it provides brighter staining and is very stable to photobleaching.

Conclusions

In conclusion, although background fluorescence of tissue sections is seldom a limitation of FISH, as a whole it is a highly sensitive and specific molecular technique for the diagnosis of *H. pylori* infection. FISH can be a method of identification when a patient is infected with coccoid forms, which is an advantage of this technique. The ability of FISH to detect clarithromycin-resistant and -susceptible strains is a considerable advantage of this method compared with histology.

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