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Application of fluorescent *in situ* hybridization (FISH) for the detection of *Helicobacter pylori*

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: Peptic ulceration following infection of the stomach with *H. pylori* is a common disease. Accurate and rapid detection of the bacteria can lead to the implementation of appropriate treatment and recovery. Chronic infection of the gastric milieu with *H. pylori* may lead to gastric carcinoma. Routine detection of this bacterium in peptic ulcer is based on the urease test and culture of peptic biopsies. Unfortunately, the sensitivity and specificity of both tests are not satisfying. Molecular techniques have been successfully applied for the rapid and accurate detection of bacterial agents in clinical samples. This study was undertaken to evaluate the sensitivity and specificity of fluorescent *in situ* hybridization (FISH) in the detection of *H. pylori* in patients suffering from dyspepsia.

Material/Methods: One hundred gastric biopsy samples taken by endoscopy from the antrum and corpus of the stomach were tested by FISH and compared with the conventional culture method complemented by biochemical tests.

Results: FISH detected *H. pylori* in 48 clinical samples, while the conventional method detected 42 samples. The sensitivity and specificity of FISH for the detection of *H. pylori* were calculated as 98% and 100%, respectively.

Conclusions: The findings of this study suggest that FISH is a highly suitable and rapid method for diagnosing *H. pylori*. Especially when the samples are taken from the antrum and the corpus of the stomach, this technique potentially can be applied routinely for the detection of this bacterium in clinical samples.

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

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BACKGROUND

The colonization of *H. pylori* in the lumen of the human stomach is related to several gastrointestinal diseases such as gastritis, duodenal and peptic ulcers, and cancer and lymphoma of the stomach [1]. Therefore, early detection and treatment of infection with this bacterium plays a significant role in recovery from these diseases [1]. There are several laboratory methods for detecting *H. pylori* infection, including culture, serological, urease, and histological tests. At present, a suitable method is culturing and the isolation of *H. pylori*; however, this is a time-consuming process, requiring 4 to 7 days for incubation, and demands experienced technicians [2,3]. In contrast, serological tests for *H. pylori* infection are rapid, but these tests suffer from cross-reaction between this bacterium and *Campylobacter* species [4]. In addition, since serological tests determine the presence of antibacterial antibodies in the samples, active *H. pylori* infections cannot be distinguished from past infections. The urease test of gastric biopsies is a rapid test for the detection of *H. pylori*, but this test cannot detect the infection with a sufficient degree of certainty [2] and is considered as an auxiliary test. Thus, novel laboratory methods that can detect *H. pylori* infection as rapidly as possible and with acceptable sensitivity and specificity are urgently needed.

Recently, fluorescent *in situ* hybridization (FISH) has been successfully applied for the detection of various bacteria. In this method, a fluorescence-labeled oligonucleotide probe specifically targets and hybridizes special sequences of ribosomal RNA (rRNA), so that the whole bacteria in the specimens can be visualized directly by a fluorescence microscope. Indeed, FISH is a molecular technique that permits us to detect the morphology of the bacteria [5–7]. The advantage of FISH over PCR is that the extraction of DNA from the bacteria is omitted in FISH [3]. Furthermore, there is no need for prior culturing of the samples. Several reports show that FISH has already been successfully applied in the detection of *E. coli* [8], *P. aeruginosa* and *H. influenza* [9], *Streptococci* [9–11], *Legionella* [12], and *Chlamydia* [13]. However, there are discrepancies in the results reported on the sensitivity and specificity of FISH for the detection of *H. pylori*. Moreover, the samples collected in previous studies were taken from the antrum of the stomach. The aim of this study was therefore to detect *H. pylori* in gastric biopsies taken from the antrum and the corpus of dyspeptic patients by FISH for evaluation of its sensitivity and specificity in comparison with the conventional culture method.

MATERIAL AND METHODS

Collection of gastric biopsies

One hundred gastric biopsy specimens from male and female patients aged between 17 and 79 suffering from gastritis, atrophic gastritis, or gastric ulcers or suspected of having malignant gastric ulcer were collected before the patients received antibiotics. The patients were examined for their routine endoscopy. Endoscopy was used for the collection of the biopsies in the hospital by three gastroenterologists. Biopsies collected from the antrum and corpus regions of the stomach were put into thioglycollate medium (Merck, Germany) containing 0.16% agar (Bio Merieux, France) and transferred to the laboratory in a cold box. These specimens were used for the culture and isolation of *H. pylori* [2]. Two biopsy samples were taken from each region of the stomach of each patient. One was used for culturing and the other for FISH. The sample for FISH (without prior culturing) was immersed in 10% formalin and later embedded in paraffin for the preparation of 4-µm tissue sections. In order to enhance the attachment of tissues, the slides were incubated at 55°C overnight. To deparaffinize the specimens, the formalin-fixed and paraffin-embedded gastric biopsy slide samples 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 [14]. The slides were then ready for FISH.

The FISH procedure

A specific oligonucleotide probe for *H. pylori* (Hpy probe), synthesized and labeled by TIB MOLBIOL (Germany) with the sequence 5'-CAC ACC TGA CTG ACT ATC CCG-3', was used. This probe specifically targets and hybridizes 16S rRNA of the *H. pylori* [3,7]. The 5' end of the probe was labeled by FLOUS [5(6)-carboxyfluorescin-N-hydroxyl succinimide-ester], which emits green light after excitation by UV light [9,15]. For hybridization of the samples, each slide of tissue sections was covered with 40 µl of hybridization buffer (0.9 M NaCl, 20 mM Tris-HCl, pH 8, 0.01% SDS, and 20% formamide) containing 5 ng/µl of the Hpy probe. Each field of the glass slide containing control bacteria was covered with 10 µl of the probe-containing hybridization buffer. Then the slides were put separately in a moisture chamber and incubated at 46°C for 90 minutes for hybridization [3,7].

Stringent washing step was carried out in a washing buffer (20 mM Tris-HCl, pH 8, 0.01% SDS, 225 mM NaCl) at 48°C for 15 minutes [3,9]. The slides were then stained with 1 µg/ml DAPI (4',6-diamidine-2'phenylindole dihydrochloride) for 5 minutes [16]. DAPI stains non-specifically the DNA of any cell, including bacteria blue [5,10]. Finally the slides were washed with PBS, left to air-dry, covered with mounting media (DAKO, Denmark) [17], and visualized with a Nikon E400 epi-fluorescence microscope (Japan). This microscope was equipped with LP and BP filters B-2A, G-2A, V-2A, and UV-2A for the detection of blue-, green-, and red-labeled probes. For our study we used filters detecting blue and green light. The pictures were taken with a Leica epi-fluorescence microscope equipped with a standard filter set (Leica, Germany) that was also equipped with a digital camera connected to a computer system.

Culturing the gastric biopsies and isolation of *H. pylori*

Biopsy samples transported to the laboratory were cultured within less than three hours. To culture the samples, the biopsies were put in a sterile Petri dish and sectioned further with a sterile scalpel and homogenized in a drop of sterile transport medium [3]. The samples were then inoculated on selective Brucella agar (Merck, Germany) and Colombia agar (Difco, USA). Both media were supplemented with 10% sheep blood, 10 µg/ml vancomycine, 2.5 unit/ml polymyxin B, 5 µg/ml trimethoprim, and 5 µg/ml amphotericine B [2]. Immediately after inoculation, the plates were incubated at 37°C for 10 days in a moisture jar together with a GENbox microaer sachet (Biomerieux, France) to gener-

Table 1. Comparison of FISH and the culture method on biopsy samples taken from both the antrum and corpus of the stomachs of 100 dyspeptic patients.

Group	Number of samples	Results	
		Culture	FISH
1	41	+	+
2	51	–	–
3	7	–	+
4	1	+	–

Total number =100.

ate a microaerophilic condition. The incubated plates were examined from the third day of incubation to check the growth of *H. pylori*. The appearance of small, round, transparent colonies on the agar were suggestive of the growth of *H. pylori*. Following Gram staining, bacterial morphology was examined with a light microscope. As a complementary confirmation, rapid urease, catalase, and oxidase tests were performed on the grown colonies. *H. pylori* was positive in these three tests. In addition, sensitivity to 30 µg nalidixic acid and cephalothin was performed, by which resistance to the former and sensitivity to the latter were confirmatory of *H. pylori*. This bacterium is resistant to 30 µg disks of nalidixic acid and sensitive to 30-µg disks of cephalothin [2,3].

Fixation of bacterial reference strains and preparation of control slides

In this study, *H. pylori* (ATCC 43504 strain) was used as the positive control and *Campylobacter jejuni* (ATCC 33560 strain) as the negative control for FISH. The strains were cultured on Schaedler agar (Becton Dickinson, Germany) enriched with 5% sheep blood. Several colonies of grown bacteria were picked from the plate and suspended in 500 µl PBS, pH 7.4. The bacterial suspensions were centrifuged at 8000 rpm for 5 minutes (PBS washing). Following centrifugation, the bacterial pellets were re-dissolved in 200 µl PBS, 3 volumes of 4% paraformaldehyde were added to the suspension, and the whole was incubated at 4°C for 1 hour. After incubation, the mixture was recentrifuged, the bacterial cells were washed with PBS, and the pellet was resuspended in 100 µl PBS and an equal volume of absolute ethanol (Merck, Germany). Fixed cells were kept at –20°C [18].

For the preparation of the control slides, 10 µl of each fixed reference strain was put on two different fields of a six-field glass slide (Marienfeld, Bad-Mergentheim, Germany) which was left at room temperature to air-dry [9]. The dehydration steps were carried out by putting the slides in a serial dilution of ethanol (50%, 80%, and absolute ethanol) successively for three minutes [7]. The control slides were kept at –20°C until needed. The control slides underwent the FISH procedure similar to that described above.

Results

Table 1 shows a summary of the samples visualized by FISH and those obtained following culturing of the specimens. Forty-one of the samples were positive according to FISH (showing green fluorescent signals) (Figure 1), the culture method, and the biochemical and disk tests of the isolated bacteria. Fifty-one samples were negative for *H. pylori* according to both FISH and culture results. Seven gastric biopsy samples were FISH positive but culture negative. In one sample, the culture was positive for *H. pylori* but negative according to FISH. None of the negative control bacteria emitted green fluorescent signals (Figure 2). According to our study, the sensitivity and specificity of FISH for the detection of *H. pylori* were 98% and 100%, respectively.

Discussion

The demand for precise and rapid detection of infectious and heritable diseases has made the application of molecular techniques indispensable in the diagnostic laboratory. Among the molecular tests, fluorescent *in situ* hybridization (FISH) is increasingly applied for these purposes. For example, the FISH technique was used by Truong and colleagues to study chromosome 3 arm imbalances quantitatively by FISH [19]. In another report, subtelomeric rearrangements were studied by Bocian in families with idiopathic mental retardation by the same method [20]. Attempts are made to apply FISH in the detection of bacteria, including clinically important pathogens such as *H. pylori*. In this study, the sensitivity and specificity of FISH were evaluated in the detection of *H. pylori* in biopsies taken from the antrum and corpus of the stomachs of dyspeptic patients. The results showed that the sensitivity and specificity of FISH for detecting this bacterium were 98% and 100%, respectively. Therefore, this method is highly suitable for the detection of *H. pylori* in comparison with the culture technique.

In seven biopsy samples, *H. pylori* was detected by FISH, while culture of the samples taken from the same patients were negative for this bacterium. There are three reasons which suggest that the culture-negative results were not due to false positivity of the FISH method, and in such cases the results of cultures should be considered as false negative and trust should be given to the results of FISH [3,7]. Firstly, one of the disadvantages of the culture method in detecting *H. pylori* is that this bacterium is very fastidious and rapidly loses its viability during transport to the laboratory, while such delay for transfer of the samples to the laboratory has no effect on FISH results and the presence of even a small amount of rRNA in this germ is sufficient for FISH to detect the bacteria [3].

Secondly, it has already been proven that the morphology of *H. pylori* occasionally changes from spiral shaped to coccoid forms. These coccoid forms of bacteria do not grow on the growth media, but they contain a sufficient amount of rRNA to be detected by FISH in the tissues. Coccoid forms of *H. pylori* are found both in biopsies and old cultures of the bacterium [7]. The morphology of four FISH-positive samples of the seven false negative culture specimens were coccoid, which could be the reason of for the false negative results of culture. This strongly suggests that the remaining three culture negative samples were also false negatives, which is more likely as they had lost their viability during transport from the department of endoscopy.

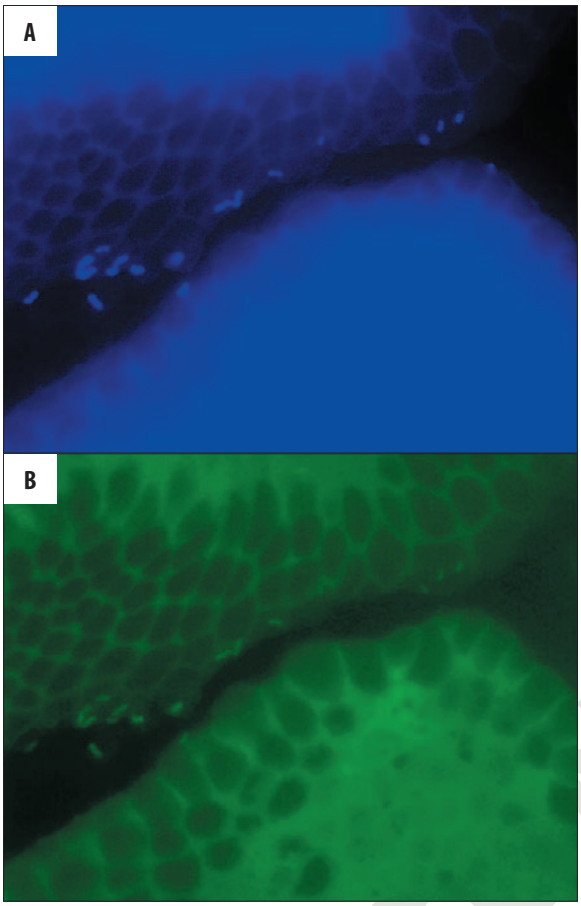

Figure 1. Visualization of *H. pylori* in gastric biopsies of dyspeptic patients using FISH. Panels (**A**) and (**B**) demonstrate the same microscopic field under the relevant filter. Panel (**A**) shows bacteria in blue because of DAPI, panel (**B**) demonstrates bacteria in green, indicating hybridization of Hpy-FLUOS probe with rRNA.

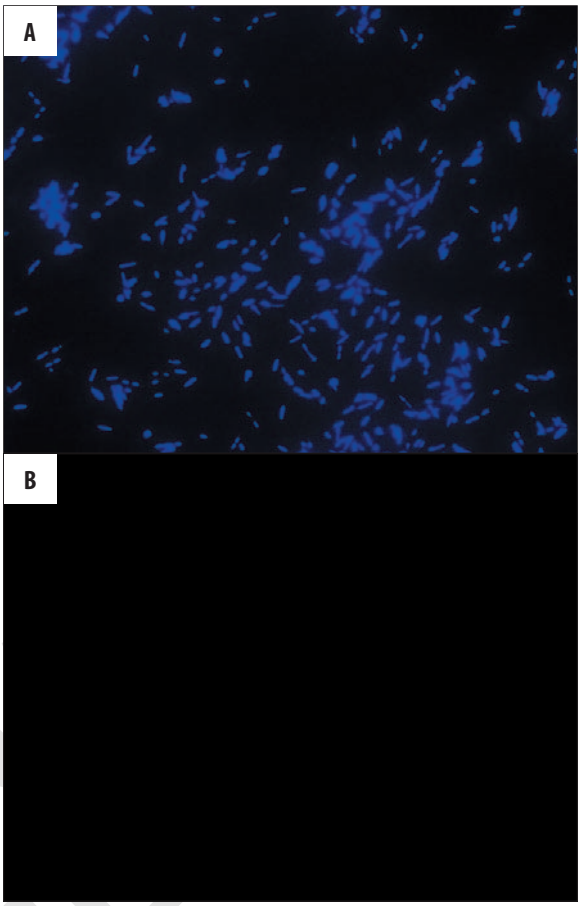

Figure 2. *C. jejuni* as a negative control. Panels (**A**) and (**B**) illustrate the same microscopic field under the relevant filter. Panel (**A**) emitted blue signals because of DAPI, Panel (**B**) with no green signals, indicating failure of hybridization of Hpy-FLUOS with bacterial rRNA.

The final, and perhaps most important, reason is that the Hpy-probe is highly specific and accurate for the detection of *H. pylori* by FISH [7] and could selectively exclude other species of genus *Helicobacter*, members of the *Campylobacter*, and another related bacterial species called *Wolinella succinogenes* [7,14]. *Lactobacillus lactis*, *Streptococcus mutans*, and *Proteus vulgaris* are occasionally isolated from the human stomach. Since it has been shown in a series of experiments that the Hpy-probe does not hybridize to the rRNA of the aforementioned bacteria, this probe is considered to be a highly specific and reliable one for the detection of *H. pylori* and can differentiate this bacterium from other related bacteria [7].

In our study we found a sample that was culture positive but FISH negative, suggesting a false negative of FISH results. Russman and colleagues reported that if the colony-forming unit (CFU) of *H. pylori* in gastric biopsies was less than 10, FISH may fail to detect this bacterium in the biopsies, while culture of the biopsies would show positive [3]. Moreover, it should be mentioned that the distribution of *H. pylori* in the stomach tissue is not even, and perhaps in a FISH-negative/culture-positive sample the collected piece of biopsy for FISH did not harbor the bacterium.

A previous study conducted by Russman and colleagues showed that the sensitivity of FISH in detecting *H. pylori* was 94.4%. This figure was found to be higher in our study (98%). Unlike these researchers, the gastric biopsies we tested for this study were collected from both the antrum and corpus of the stomach, while they collected their biopsies only from the antrum of the stomach. Although the population of *H. pylori* is maximal in the antrum, this bacterium sometimes exists only in the corpus [1]. Thus, the higher sensitivity obtained in our experiment can be justified by the region of collection of the specimens. Although the FISH technique has an inherent limitation in that not only is it not routinely used in many parts of the world, requiring skilled personals and a relatively advanced instrument, but it also needs an invasive method for sampling. However, it is more suitable, rapid and, in the long term, more economical than the culture method. Since the employed technique is not very sophisticated, short-term training of personnel and the provision of an epi-fluorescent microscope can provide the foundation for its wider application as a routine laboratory technique in the future. In addition, as other researchers have reported, clarithromycin-resistant strains of *H. pylori* can be detected via the application of specific probes [3,7,21,22].

CONCLUSIONS

The overall conclusions that can be drawn from these findings suggest that FISH is an accurate, cheap, and rapid method for the detection of *H. pylori* with very acceptable sensitivity and specificity. This method can potentially be used as a routine technique for laboratory diagnosis of this bacterium in clinical samples.

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