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Development and Application of a Novel Peptide Nucleic Acid Probe for the Specific Detection of *Helicobacter pylori* in Gastric Biopsy Specimens▽†

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In this work, a fluorescence in situ hybridization (FISH) method for the rapid detection of *Helicobacter pylori* using a novel peptide nucleic acid (PNA) probe is reported. Laboratory testing with several different bacterial species, including other *Helicobacter* spp., has shown that this probe is highly specific for *H. pylori* strains. In addition, the PNA FISH method has been successfully adapted for detection of the pathogen in paraffin-embedded gastric biopsy specimens.

Helicobacter pylori is an important human pathogen that causes chronic gastritis and is associated with the development of peptic ulcer disease, atrophic gastritis, and gastric cancer (27). Infection with *H. pylori* can be diagnosed either by noninvasive testing or by invasive techniques that require an upper endoscopy with collection of gastric biopsy specimens (11). Noninvasive tests are the most usual methods for routine *H. pylori* detection, but they fail to provide complementary information on the location of *H. pylori* in the stomach and on the histopathological lesions underlying the presence of the bacteria. Therefore, there are situations where invasive tests should be performed to provide a more complete diagnosis. Gastric biopsy specimens obtained by upper endoscopy can be analyzed for the presence of the bacterium by culture or by other molecular methods. In recent years, molecular methods for the identification of several bacteria, including random amplified polymorphic DNA, PCR, and fluorescence in situ hybridization (FISH), have been preferred over the more time-consuming culture methods (8, 21, 28).

FISH is arguably the most common method used for the detection and localization of a microorganism or particular groups of microorganisms within a sample (28). It detects nucleic acid sequences by means of a fluorescently labeled probe that hybridizes specifically to its complementary target sequence within the intact cell (15). So far, FISH methods have been based on the use of conventional DNA oligonucleotide probes containing around 20 bases. More recently, peptide nucleic acid (PNA) probes have been developed and optimized for bacterial detection. PNA molecules are DNA mimics in which the negatively charged sugar-phosphate backbone is replaced by an achiral, neutral polyamide backbone formed by repetitive units of *N*-(2-aminoethyl)glycine (17, 18). PNA can hybridize with complementary nucleic acid targets according to the Watson-Crick base-pairing rules (7). Compared with traditional DNA probes and due to the uncharged backbone, PNA probes have superior hybridization characteristics, exhibiting rapid and stronger binding to complementary targets and an absence of electrostatic repulsion (20, 25). The optimum length for a PNA probe is 15 bp.

Several PNA probes have been designed and optimized for different organisms including *Campylobacter* spp., *Candida albicans*, *Mycobacterium avium*, and *Legionella pneumophila* (12, 13, 30). We have previously developed a PNA probe targeting the 16S rRNA of *H. pylori* (sequence, 5′-TAATCAGCACTC TAGCAA-3′) that was shown to be very specific (5). However, due to the extensive genetic diversity observed within *H. pylori*, the high specificity of the probe was counterbalanced by a lack of sensitivity.

Design of the PNA oligonucleotide probe. To identify potentially useful oligonucleotides, the freely available Primrose program was used (http://www.cf.ac.uk/biosi/research/biosoft/Primrose/index.html) together with the 16S rRNA databases of Ribosomal Database Project II (RDP-II), version 8.1 (http://rdp8.cme.msu.edu/html/) (3, 6). In accordance with the Primrose program instructions, the selection of oligonucleotides was based on the 16S rRNA comparison of six randomly chosen *H. pylori* strains. To avoid missing possible sequences of interest, several sets of six random *H. pylori* strains were tested. Based on the large number of 15-bp sequences obtained that could match all targets, additional criteria for the selection of the PNA FISH probe were used. These included no self-complementary structures within the probe and high specificity and sensitivity for *H. pylori*. Once the probe sequence was selected, a search was made at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/) to further confirm the specificity of the probe (14). Afterwards, the desired sequence was synthesized (ATDBio, Southampton, United Kingdom), and the N terminus of the oligomer was connected to Alexa Fluor 546 via a double AEEA linker.

According to the criteria mentioned above, we have chosen

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TABLE 1. Predicted specificities and sensitivities of the probes for *H. pylori* detection[a]

Probe[b]	Type	Specificity (%)[c]	Sensitivity (%)[d]	Fluorescence (%)	Reference(s)
HP16S-1	DNA	82	82	41–60	19
Hpy-1	DNA	86	95	0–5	5a, 8a, 23, 23a
Hprobe	PNA	100	25	6–20	4, 25a
Hpy769	PNA	85	89	6–20	This work

[a] Estimation of binding affinity through fluorescence intensity was based on the work of Fuchs et al. (10).

[b] Sequences are as follows: HP16S-1, 5′-GGA GTA TCT GGT ATT AAT CAT CG-3′; Hpy-1, 5′-CAC ACC TGA CTG ACT ATC CCG-3′; Hprobe, 5′-TAATCAGCACTCTAGCAA-3′. The sequence of Hpy769 is given in the text.

[c] Calculated as (number of *H. pylori* strains detected by the probe)/(total number of bacterial strains detected by the probe) × 100.

[d] Calculated as (number of *H. pylori* strains detected by the probe)/(total number of *H. pylori* strains in the databases) × 100.

the following PNA oligomer sequence: 5′-GAGACTAAGCC CTCC-3′. The probe was designated Hpy769 due to the starting position of the target sequence in the 16S rRNA of *H. pylori* strain NCTC 11637. Searches showed that the Hpy769 probe differed by at least 1 bp from the 16S rRNA sequences of bacteria other than *H. pylori*, except for a few uncultured *Helicobacter* species and one strain of *Helicobacter acinonychis.* More importantly, evaluation with the NCBI BLAST program showed that this probe is identical to 89% of all *H. pylori* sequences, which is a significant improvement over the 25% identity obtained for the PNA probe that has already been published (4) and represents a value comparable to those of other DNA probes used for *H. pylori* detection (19, 23) (Table 1). For the estimation of specificity and sensitivity, only sequences considered to have high quality and more than 1,200 bp by the RDP-II program were selected. The *H. pylori* sequences were further assessed for the existence of chimeras by using Mallard software, version 1.02 (2). At the end, 57 sequences were selected as being trustworthy. This is still a relatively low number for assessing sensitivity and specificity, especially if we bear in mind that many strains also have the bias of being from similar locations and consequently possess similar 16S rRNA sequences. As such, it is likely that these values will change as more sequences are deposited.

Another advantage of the probe is that, as shown in Fig. SA1 (provided as supplemental material), the target sequence is located in a higher-affinity binding area than that for Hpy-1 according to the study of Fuchs et al. (10). Even if both probes were DNA based, this should result in a brighter signal and easier visualization under the microscope for Hpy769. Because the latter is PNA based and hybridizations can therefore be performed under low-salt conditions that promote the destabilization of the secondary structure of the 16S rRNA (25), enhanced signal intensity is expected.

Optimization of the hybridization conditions of the probe. The hybridization method was based on the procedure reported in Azevedo et al. (4) with some modifications. We started by testing different hybridization temperatures, between 50°C and 68°C, and the best hybridization results were obtained at 59°C (data not shown); however, in certain random samples, it was noticed that hybridization was not as bright as expected, which made detection difficult. This problem was solved by altering the fixation procedure to include a step of paraformaldehyde immersion followed by ethanol. The reason why this problem has not appeared for the Hprobe has not been investigated, but it might be related to altered probe characteristics due to the binding of the different type of fluorochrome (Alexa Fluor 546) used in this work.

For the final procedure, smears of *H. pylori* NCTC 11637 prepared by standard methods were immersed in 4% (wt/vol) paraformaldehyde followed by 50% (vol/vol) ethanol for 10 min each and allowed to air dry. The smears were then covered with 30 μl of hybridization solution (24) and a coverslip, placed in moist chambers, and incubated for 90 min at 59°C. Following hybridization, coverslips were removed and slides were submerged in a prewarmed (59°C) washing solution containing 5 mM Tris base (Sigma), 15 mM NaCl (Sigma), and 1% (vol/vol) Triton X (pH 10; Sigma). Washing was performed at 59°C for 30 min, and the slides were allowed to air dry.

The smears were mounted with 1 drop of nonfluorescent immersion oil (Merck) and covered with coverslips. Slides were stored in the dark for a maximum of 48 h before microscopy. Microscopy was conducted using a Zeiss Axioplan (Oberkochen, Germany) and an Olympus BX51 (Perafita, Portugal) epifluorescence microscope equipped with one filter sensitive to the signaling molecule of the PNA probe. Filters that were not able to detect the probe were used as negative controls. For each experiment, a negative control was performed where all the steps described here were carried out but no probe was added to the hybridization solution.

Specificity and sensitivity of the probe. To test the specificity and sensitivity of the probe, several *H. pylori* strains, *Helicobacter* spp., and other bacteria were tested (Table 2). All *H. pylori* strains were maintained on Columbia agar (Oxoid, Basingstoke, United Kingdom) supplemented with 5% (vol/vol) defibrinated horse blood (Biomérieux, Marcy l'Etoile, France). Plates were incubated at 37°C in a CO_2 incubator (HERAcell 150; Thermo Electron Corporation, Waltham, MA) set to 10% CO_2 and 5% O_2, and single colonies were streaked onto fresh plates every 2 or 3 days. All other *Helicobacter* and *Campylobacter* species were grown on *Campylobacter* selective agar (Sigma) supplemented with 5% (vol/vol) defibrinated sheep blood (Probiológica, Sintra, Portugal) and were maintained under conditions similar to those for *H. pylori*, except for *Campylobacter* spp., which were incubated at 42°C; other bacteria used in this study were grown on R2A agar at room temperature (20 to 25°C) for 3 days.

As shown in Table 2, Hpy769 hybridizes with all *H. pylori* strains, whereas no hybridization was observed for the other bacterial species used. It is interesting that, despite the predicted 89% sensitivity, the probe was able to detect all the *H. pylori* strains used in this study. Positive detection of culture collections was already expected, since the 16S rRNA sequences deposited in the RDP-II data bank were known to be complementary to our probe. It could be expected that some clinical isolates would not be detected by our probe. This was not the case, but it is worth mentioning that all clinical isolates were obtained from the same institution (the strain collection of the National Institute of Health in Lisbon, Portugal) and from individuals within the same geographical region, which might imply that 16S rRNA sequences that are conserved between strains are more likely to occur.

TABLE 2. Results of the *H. pylori* probe specificity test

Organism	FISH outcome
H. pylori	
NCTC 11367^T	+
ATCC 700392^T	+
ATCC 700824^T	+
1342[a]	+
1198[a]	+
1320[a]	+
957[a]	+
968[a]	+
1330[a]	+
ATCC 49503^T	+
H. cinaedi	
33221-1.2[b]	−
33052-1.3[b]	−
H. mustelae	
2G1[b]	−
2H1[b]	−
H. felis 2I4[b]	−
H. muridarum 2A5[b]	−
H. mustelae CIP 103769^{Tc}	−
H. canadensis CCUG 47163^{Tc}	−
H. pametensis CIP 104249[c]	−
H. pullorum CCUG 33837^{Tc}	−
H. salomonis CIP 105607[c]	−
H. hepaticus CIP 104100[c]	−
H. cinaedi CIP 105369[c]	−
H. canis CIP 104753[c]	−
Sphingomonas capsulata	−
Staphylococcus sp.	−
Acinetobacter calcoaceticus	−
Campylobacter sp. strain 1	−
Campylobacter sp. strain 2	−
Campylobacter sp. strain 3	−

[a] Clinical isolate kindly provided by Maria Lurdes Monteiro.
[b] Isolate kindly provided by Jay Solnick.
[c] Isolate kindly provided by Francis Mégraud.

All hybridized *H. pylori* strains emitted bright red fluorescence, and the three different morphological types of the bacterium (spiral, U-shape, and coccoid) (1) could be clearly observed (Fig. 1). There was no cross-hybridization to the rRNAs of other bacteria used in this study, and thus Hpy769-labeled *H. pylori* cells could easily be distinguished from non-*H. pylori* strains. This was the first FISH probe targeting *H. pylori* that was tested against such a large number of closely related species. This is particularly important because it has been reported that existing DNA probes are at times unable to discriminate between sequences with only one base pair mismatch (30).

The identification of coccoid forms by this method assumes particular importance because it has recently been shown that the stringent response in *H. pylori* induces, besides the morphological conversion into a coccoid shape, a decrease in total RNA production (16, 29). Nevertheless, this method appears to be sensitive enough to detect these lower numbers of rRNA copies per cell.

Hybridization in gastric biopsy specimens. After designing the probe and optimizing the FISH procedure, we applied the method to 15 histological slides of formalin-fixed, paraffin-embedded gastric biopsy specimens from five patients, four infected with *H. pylori* and one uninfected. Three-micrometer-thick histological slides were deparaffinized and rehydrated in xylol and ethanol. Slides were immersed twice in xylol for 15 min each time and once in decreasing concentrations of ethanol (100%, 95%, 80%, 70%, and 50%) for 5 min in each concentration and were finally washed with distilled water for 10 min. Histological slides were then allowed to air dry.

As shown in Fig. 2, the presence of *H. pylori* could easily be detected using the new PNA probe, though some experience at microcopy is required. After applying the PNA FISH method, we counterstained the sample with the Giemsa staining method to confirm our results (Fig. 3). Briefly, histological slides of gastric biopsy specimens were stained with 2% (vol/vol) Giemsa solution for 30 min and washed in distilled water.

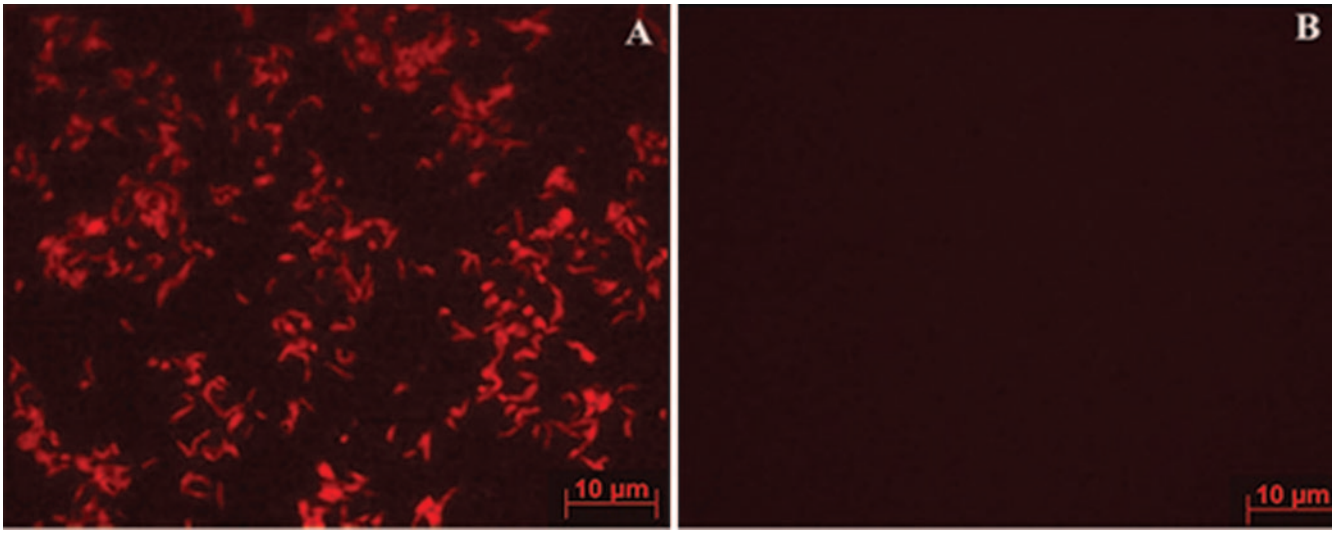

FIG. 1. (A) Detection of *H. pylori* using the red fluorescent probe Hpy769 in a smear of pure culture of *H. pylori* NCTC 11637. Notice the presence of all three morphological types. (B) Lack of signal with Hpy769 in a smear of pure culture of *Helicobacter muridarum* 2A5. The experiments were performed in parallel, and images were obtained with equal exposure times.

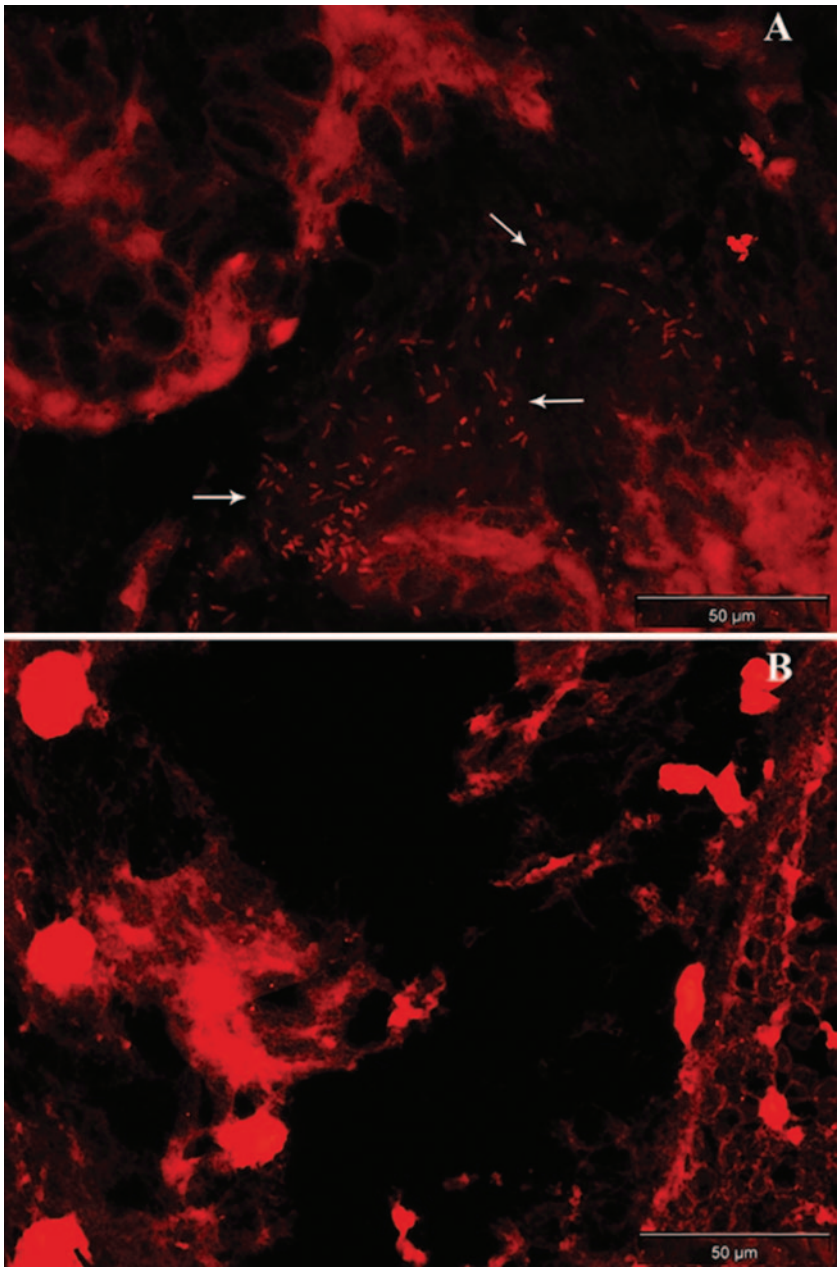

FIG. 2. (A) Detection of *H. pylori* (arrows) using the red fluorescent probe Hpy769 on a histological slide of a gastric biopsy specimen from an infected patient. (B) Results for Hpy769 with a gastric biopsy specimen from a noninfected patient. The experiments were performed in parallel, and images were obtained with equal exposure times.

Slides were then immersed in 95% (vol/vol) alcohol followed by absolute alcohol.

Additional experiments have proven that PNA FISH is also able to be easily adapted to membrane filters and solid surfaces of different metallic and polymeric materials (data not shown).

Conclusions. As has been shown, the Hpy769 probe presented an improvement over the previous PNA probe (4) in the detection of the bacteria and is at least as specific as DNA probes reported in the literature for *H. pylori* detection. Even though its sensitivity is slightly lower than that of probe Hpy-1 based on current data, only when more strains from a range of patients from diverse geographical locations are sequenced will it be possible to have a more correct idea of the exact values. Nevertheless, the higher suitability of PNA probes for adaptation to multiplex experiments (i.e., hybridization of several probes at the same time) implies that future improvements of the method can be more easily undertaken. In fact, Hpy769 was one of the first probes with PNA chemistry coupled with a fluorophore from the Alexa Fluor family. One of the advantages of these dyes over other fluorochromes that are used routinely is that they have a very narrow emission band, allowing better discrimination of different dyes under the microscope in multiplex experiments.

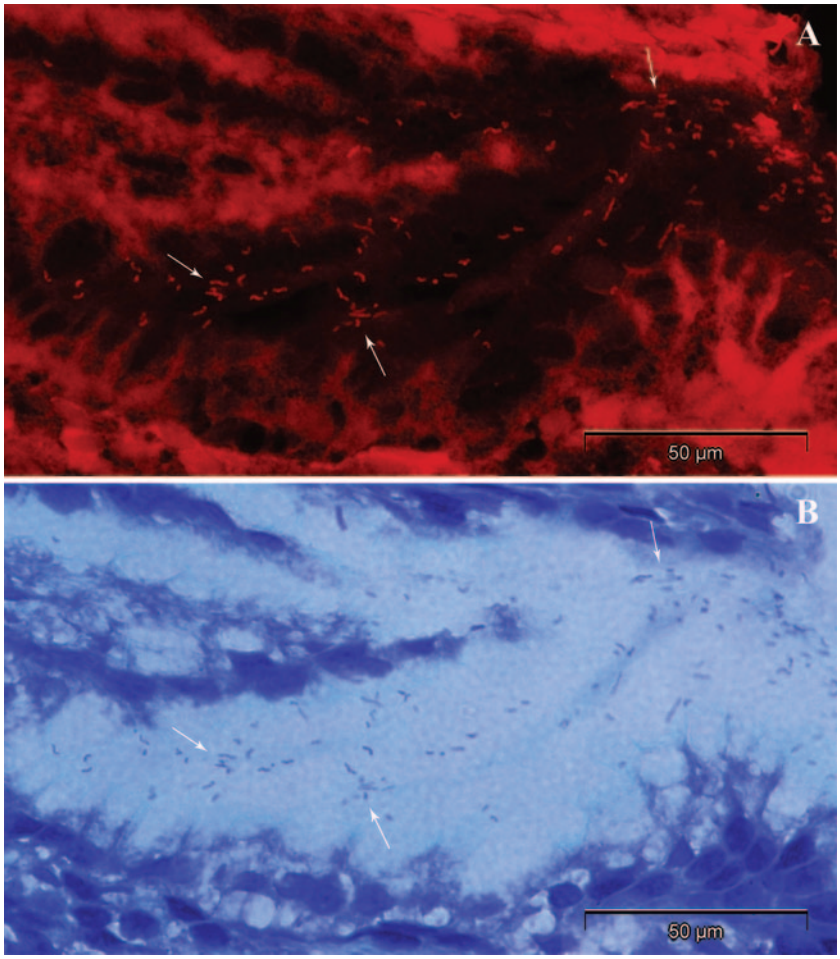

FIG. 3. Detection of *H. pylori* (arrows) on a histological slide of a gastric biopsy specimen by using the red fluorescent probe Hpy769 (A) and counterstaining with Giemsa stain (B).

The PNA FISH procedure using the Hpy769 probe has been shown to be a very sensitive and specific method for the detection of *H. pylori* in a variety of samples, such as smears and gastric biopsy specimens. In addition, the method is easy to implement without the requirement for special equipment or facilities, apart from an epifluorescence microscope.

The detection of the bacteria in biopsy specimens is very useful because by this technique we are able not only to detect all bacteria but also to pinpoint their exact locations in the gastric tissue and even to identify their morphology, which is not possible by standard techniques such as culture methods or even PCR. In the future, the PNA FISH procedure can be adapted to identify *H.* pylori in locations other than the stomach, detect antibiotic-resistant strains (22), and study possible interactions between different *H. pylori* strains colonizing the same individual or between *H. pylori* and other *Helicobacter* spp. that are known to inhabit the human gastrointestinal tract (9, 26).

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