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Validation of a Fluorescence *In Situ* Hybridization Method Using Peptide Nucleic Acid Probes for Detection of *Helicobacter pylori* Clarithromycin Resistance in Gastric Biopsy Specimens

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Here, we evaluated a previously established peptide nucleic acid-fluorescence *in situ* hybridization (PNA-FISH) method as a new diagnostic test for *Helicobacter pylori* clarithromycin resistance detection in paraffin-embedded gastric biopsy specimens. Both a retrospective study and a prospective cohort study were conducted to evaluate the specificity and sensitivity of a PNA-FISH method to determine *H. pylori* clarithromycin resistance. In the retrospective study ($n = 30$ patients), full agreement between PNA-FISH and PCR-sequencing was observed. Compared to the reference method (culture followed by Etest), the specificity and sensitivity of PNA-FISH were 90.9% (95% confidence interval [CI], 57.1% to 99.5%) and 84.2% (95% CI, 59.5% to 95.8%), respectively. In the prospective cohort ($n = 93$ patients), 21 cases were positive by culture. For the patients harboring clarithromycin-resistant *H. pylori*, the method showed sensitivity of 80.0% (95% CI, 29.9% to 98.9%) and specificity of 93.8% (95% CI, 67.7% to 99.7%). These values likely represent underestimations, as some of the discrepant results corresponded to patients infected by more than one strain. PNA-FISH appears to be a simple, quick, and accurate method for detecting *H. pylori* clarithromycin resistance in paraffin-embedded biopsy specimens. It is also the only one of the methods assessed here that allows direct and specific visualization of this microorganism within the biopsy specimens, a characteristic that allowed the observation that cells of different *H. pylori* strains can subsist in very close proximity in the stomach.

Helicobacter pylori is a Gram-negative bacterium that colonizes the human stomach mucosa and plays a major role in several gastric diseases such as peptic ulcer disease, gastric mucosa-associated lymphoid tissue (MALT) lymphoma, and gastric carcinoma (1–3). Typically, treatment of *H. pylori* infection necessitates the administration of a proton pump inhibitor with two antibiotics, clarithromycin and amoxicillin or metronidazole, for a period of 7 to 14 days, according to the guidelines of the Maastricht Consensus in Europe and the American Gastroenterological Association in the United States (4, 5). The excessive and indiscriminate use of clarithromycin is leading to an increase of *H. pylori* resistance to this antibiotic, reducing the treatment success (6–9). The prevalence of primary clarithromycin resistance reaches 30% in southern European countries and in the United States is around 10% to 15% (7, 9).

Although several resistance mechanisms can occur, clarithromycin resistance in *H. pylori* is almost always associated with point mutations in the peptidyltransferase region encoded by the V domain of the *H. pylori* 23S rRNA gene (10–12). The three most prevalent point mutations are the transitions A2142G and A2143G and the transversion A2142C (7, 10, 13, 14). Other point mutations in this gene may also be associated with clarithromycin resistance, but either these are less prevalent or a causal link between them and resistance has yet to be definitely proven (15–18).

The agar dilution method is the reference method proposed by the Clinical and Laboratory Standards Institute (CLSI), formerly the National Committee for Clinical Laboratory Standards, to be used for *H. pylori* antibiotic susceptibility testing (19, 20). Alternatively, clinical laboratories use the Etest method as it is considered less technically demanding (21, 22). However, the Etest and the agar dilution method are more tedious and time-consuming to perform than molecular methods such as PCR or fluorescence *in situ* hybridization (FISH) (23–25). PCR-based methods have been used as a suitable alternative (18, 25–27) but can be affected by DNA contamination or degradation (12, 28). FISH is typically based on fluorescently labeled DNA probes that hybridize with specific rRNA sequences of microorganisms (23, 29). Limitations of this method include the degradation of the probe by proteases and nucleases present in the sample, poor permeability of the microbial cell wall for the probes, and low accessibility of the probe to the target region of the rRNA due to the ribosomal secondary structure. To overcome these limitations, peptide nucleic acid (PNA) probes are being used instead of the typical DNA molecules (28). PNA molecules are synthetic DNA mimics with a neutrally charged chemical backbone that confers higher affinity for DNA

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or RNA complementary sequences (30, 31). PNA probes are usually smaller (approximately 13 to 18 bp) than DNA probes (>18 bp), increasing their ability to penetrate the bacterial cell wall, and are more resistant to nucleases and proteases (30).

PNA-FISH methods have been previously developed by our group for the detection of *H. pylori* in gastric biopsy specimens and determination of clarithromycin susceptibility (32). However, only a preliminary assessment was performed regarding the sensitivity and specificity of the methods applied to gastric biopsy specimens. In the present work, we validated a PNA-FISH assay for *H. pylori* and clarithromycin susceptibility determination in paraffin-embedded gastric biopsy specimens, using culture and Etest as reference methods and a PCR-based method in retrospective and prospective studies.

MATERIALS AND METHODS

Study settings. Both a retrospective study and a prospective cohort study were conducted to evaluate the specificity and sensitivity of a PNA-FISH method to determine *H. pylori* clarithromycin resistance (33). PNA-FISH results were compared to those of other commonly used methods, namely, *in vitro* susceptibility testing (Etest) following culture and PCR-sequencing, to assess resistance status. The three methods were performed in different Portuguese institutions by different laboratory technicians, who were unaware of the results provided by the other methods. PNA-FISH was performed at the Centre of Biological Engineering at the University of Minho (CEB), *H. pylori* culture and Etest were performed at the Instituto Nacional de Saúde Dr. Ricardo Jorge in Lisbon (INSARJ), and PCR-sequencing was carried out at the Instituto de Patologia e Imunologia Molecular da Universidade do Porto (IPATIMUP). This study was previously approved by the ethics committee of the Portuguese Institute of Oncology (IPO) in Porto, and informed consent was obtained from all patients.

Retrospective study. Thirty paraffin-embedded gastric biopsy specimens from 24 patients (20 cases with a single biopsy specimen, 2 cases with 2 biopsy specimens, and 2 cases with 3 biopsy specimens) collected between 2003 and 2009 at Endoclab (Porto, Portugal) were used. The selected biopsy specimens were all *H. pylori* positive and had a known clarithromycin resistance status determined by Etest. The same biopsy specimens were then analyzed by PNA-FISH and PCR-sequencing (see descriptions of methods below).

Prospective study. Ninety-three patients who submitted to an upper gastrointestinal endoscopy with biopsy collection at IPO during 2010 participated in this study. There was no previous information about *H. pylori* resistance for any of these patients. Thirty-five of them were taking antibiotics or proton pump inhibitors during the last month before biopsy specimen collection. Four biopsy specimens were collected from each patient. Two (one from the antrum and the other from the corpus) were used for histopathological examination at IPO, one from the antrum was used for culture and Etest, and another from the antrum was used for PNA-FISH and PCR-sequencing.

Culture and clarithromycin susceptibility testing. One antral biopsy specimen was placed at 4°C in a transport medium (Portagerm pylori; bioMérieux, Marcy l'Etoile, France). Within less than 24 h, the sample was transported to INSARJ, where *H. pylori* culture and *in vitro* susceptibility testing were performed as previously described (34). Briefly, biopsy specimens were ground with a tissue homogenizer (Ultra Turax; Labo Moderne, Paris, France) and inoculated into a selective medium (bioMérieux, Marcy l'Etoile, France) and a nonselective medium (Biogerm, Maia, Portugal) at 37°C in a microaerobic environment (Anoxomat; MART Microbiology BV, Drachten, The Netherlands) for up to 14 days of incubation. Identification of *H. pylori* was performed according to conventional tests (assays of colony and Gram stain morphology, catalase, oxidase, and hydrolysis of urea).

Susceptibility testing for clarithromycin was performed by the Etest (bioMérieux, Marcy l'Etoile, France). In brief, a bacterial suspension with opacity equivalent to the McFarland 3 standard was inoculated by swabbing on plates containing Mueller-Hinton agar medium (Becton Dickinson, Madrid, Spain) and 10% (vol/vol) horse blood (Probiológica, Belas, Portugal). Plates were incubated at 37°C under microaerobic conditions and analyzed after 72 h of incubation. The MIC breakpoint used for clarithromycin was ≥1 μg/ml (35).

Handling of paraffin-embedded gastric biopsy specimens. Twenty-four-hour formalin-fixed gastric biopsy specimens were paraffin embedded and subsequently analyzed by PCR-sequencing and PNA-FISH. Biopsy specimens were processed overnight with subsequent immersion in solutions of 4% buffered formaldehyde (Prolabo, VWR, Portugal) twice for 1 h each time, 80% ethanol (Proclínica, Odivelas, Portugal) for 1 h, 96% ethanol for 1 h, 96% ethanol for 1 h 30 min, 99% ethanol twice for 1 h 30 min each time, 96% ethanol for 1 h 30 min, xylol (Sigma-Aldrich, Sintra, Portugal) twice for 1 h 30 min each time, and paraffin (Thermo Shandon, United Kingdom) at 60°C twice for 1 h 30 min each time. Subsequently, the material was included in paraffin blocks and identified with the number of the examination. Cuts of 3-μm- and 10-μm-thick sections of paraffin-embedded blocks were obtained from each biopsy specimen, changing the blade after each cut to avoid cross-contamination.

PCR-sequencing. Total DNA was extracted from 10-μm-thick cuts of paraffin-embedded biopsy samples after digestion with proteinase K for at least 12 h at 55°C. Proteinase K was inactivated by incubation at 95°C for 10 min. Ten microliters of the lysates was used for PCR of the human housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH), to monitor the DNA quality, as previously described (36). A 1-μl volume of the lysate was also used for PCR amplification of the *H. pylori* 23S rRNA gene using previously described primers (27). PCR products were sequenced using BigDye Terminator v3.1 cycle sequencing kits (Applied Biosystems, Foster City, CA) and run in an ABI Prism 3130 DNA automated sequencer (Applied Biosystems). The genotype of *H. pylori* strains found in patients 1 to 8 of the prospective study (see Table 2) was also assessed by GenoType HelicoDR (Hain Lifescience, Germany) according to the manufacturer's instructions and a protocol previously described (6). The GenoType HelicoDR is a CE-marked kit that was developed to detect the same point mutations that were assessed in this study by PNA-FISH (6).

PNA-FISH. Three-micrometer-thick paraffin cuts, adhered to microscopy slides, were deparaffinized and rehydrated in xylol and ethanol based on a previously described protocol (32, 37). Sections were immersed in xylol (Fisher Chemical, Leicestershire, United Kingdom) three times (the first time for 15 min and then twice for 10 min each time), absolute ethanol (Panreac, Barcelona, Spain) (twice for 7 min 30 s each time), and decreasing concentrations of ethanol (95% twice for 7 min 30 s each time; 80% for 10 min; 70% for 10 min; and 50% twice for 15 min each time). Finally, sections were immersed in 1% (vol/vol) Triton X-100 (Sigma-Aldrich, Sintra, Portugal) solution for 20 min at 63°C. Histological slides were then allowed to air dry.

The probes previously developed by our group to assess *H. pylori* clarithromycin resistance were shortened from 15 bp to 13 bp (for Hp1, 5′-GTC TCT CCG TCT T-3′; for Hp2, 5′-GTC TTC CCG TCT T-3′; for Hp3, 5′-GTC TTG CCG TCT T-3′; and for Hpwt, 5′-GGT CTT TCC GTC T-3′) in order to carry out hybridization assays at a lower temperature but with the same specificity performance (data not shown). Probes detecting mutations that conferred clarithromycin resistance were labeled with Alexa Fluor 488 (emission in the green wavelength), whereas the probe targeting susceptible strains was labeled with Alexa Fluor 594 (emission in the red wavelength). Approximately 20 μl of hybridization solution was added to the histological slides, which were then covered with coverslips and incubated for 1 h at 63°C. The hybridization solution contained a 200 nM concentration of the probe mixture, 10% (wt/vol) dextran sulfate, 10 mM NaCl, 30% (vol/vol) formamide, 0.1% (wt/vol) sodium pyrophosphate, 0.2% (wt/vol) polyvinylpyrrolidone, 0.2% (wt/vol) Ficoll, 5 mM disodium EDTA, 0.1% (vol/vol) Triton X-100, and 50

mM Tris-HCl (all from Sigma-Aldrich, Sintra, Portugal, except the disodium EDTA, which was from Pronalab, Lisbon, Portugal). Subsequently, the slides were transferred to a Coplin jar containing prewarmed (63°C) washing solution that consisted of 5 mM Tris base, 15 mM NaCl, and 1% (vol/vol) Triton X-100 (all from Sigma-Aldrich, Sintra, Portugal), where the coverslips were carefully removed. The washing step was carried out for 30 min at 63°C. The slides were allowed to air dry and mounted with one drop of mounting oil and covered with a coverslip before microscopy observation. The completion of the whole procedure took approximately 3 h.

Microscopic visualization. Histological slides of the PNA-FISH procedure were observed using an epifluorescence microscope (BX51 Olympus, Hamburg, Germany) equipped with a charge-coupled-device (CCD) camera (DP71; Olympus) with filters adapted to the Alexa Fluor 488 (bandpass [BP] 470- to 490-nm exciter filter, farb teiler [FT] 500-nm splitter, longpass [LP] 516-nm filter) and 594 (BP 530- to 550-nm exciter filter, FT 570-nm splitter, LP 591-nm filter) signaling molecules attached to the probes. The filters that were not sensitive to the reporter molecules were used as negative controls. Visualization of samples was carried out within less than 48 h after the experimental PNA-FISH procedure.

Validity indexes. Statistical validity parameters such as sensitivity, specificity, likelihood ratios, and respective 95% confidence intervals (CI) were determined as previously described (38, 39), using the VassarStats: Website for Statistical Computation (http://vassarstats.net).

RESULTS

PNA-FISH optimization. The four PNA probes were first tested separately to ensure that the shortening of the sequences would not affect the individual performance of each probe. Because the purpose of this study was not to discriminate individually each point mutation associated with clarithromycin resistance but rather to discriminate between susceptible and resistant *H. pylori* strains, a mixture of the four PNA probes was used instead for the rest of the work. Both the individual probes and their mixture were highly sensitive and specific to detect *H. pylori* of a known status in gastric biopsy specimens (Fig. 1A to D). Furthermore, the presence of mixed infections with cells of susceptible and resistant *H. pylori* strains located less than 5 μm apart within the same biopsy specimen was also detectable by the PNA probes (Fig. 1E and F).

Retrospective study. Of the 30 histological samples, 26 showed total agreement between the Etest, PNA-FISH, and PCR-sequencing (86.7% concordance; 95% CI, 68.4% to 95.6%). Of these, 10 samples were considered to have susceptible *H. pylori* strains and 16 to have resistant strains (see Table S1 in the supplemental material). Overall, the PNA-FISH method was in full agreement with PCR-sequencing (Table 1; see also Tables S1 and S3 in the supplemental material), but because the culture and Etest are considered to be the reference methods, PNA-FISH showed specificity and sensitivity of 90.9% (95% CI, 57.1% to 99.5%) and 84.2% (95% CI, 59.5% to 95.8%), respectively. Three samples presented a susceptible genotype but a resistant phenotype, whereas in one sample a resistant genotype and a susceptible phenotype were observed. In the particular case of patient 6, in two distinct biopsy specimens, two different *H. pylori* strains (one resistant and the other susceptible) were detected (see Table S1 in the supplemental material).

Prospective study. Of a total of 93 patients, the biopsy specimens of 21 were positive for *H. pylori* by culture and hence were included for the sensitivity and specificity determinations (Table 1; see also Tables S2 and S3 in the supplemental material). The biopsy specimens of 5 patients presented a resistant phenotype, and those of the other 16 presented a susceptible phenotype. Besides PNA-FISH, other molecular methods, namely, GenoType HelicoDR for patients 1 to 8 and PCR-sequencing for patients 9 to 21, were also tested. The latter two methods were used interchangeably because, as indicated by Cambau et al. (6), the concordance between GenoType HelicoDR results and those of genotyping is 99.5%.

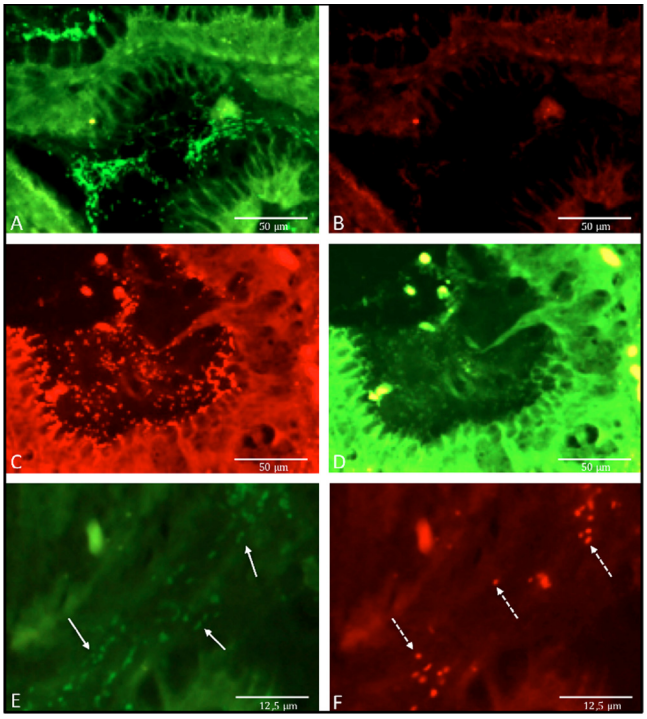

FIG 1 Detection of *H. pylori* strains in gastric biopsy histological slides by PNA-FISH. (A) Visualization of a resistant strain in the green channel. (B) Visualization of the same microscopic field in the red channel, where no *H. pylori*-sensitive strains are present. (C) Visualization of a susceptible strain in the red channel. (D) Visualization of the microscopic field presented in panel C in the green channel. The very faint cells that are picked up in the green channel are the result of bleed through, but the signal intensity can be easily differentiated from that of a true positive signal. (E and F) Presence of a mixed infection within the same biopsy specimen. (E) *H. pylori* cells that contain the point mutations associated with clarithromycin resistance and that can be seen only in the green channel (solid arrows). (F) *H. pylori* cells in which no point mutations are present (wild type) and that can be seen only in the red channel (dashed arrows). As can be observed in panels E and F, cells of different *H. pylori* strains can subsist in very close proximity in the stomach (in locations less than 5 μm apart). They also appear interspersed in the biopsy specimen and are not segregated according to species. Images were obtained with equal exposure times. (Original magnification, ×600).

Regarding the *H. pylori* strains with a resistant phenotype, one of them (corresponding to patient 19) could not be confirmed by PNA-FISH, since no bacterial cells could be visualized in the biopsy sample used to perform this method. For patients 18 and 21, the PNA-FISH method detected both genotypes (resistant and susceptible). When this occurred, the patient was considered to be resistant to clarithromycin, as it was considered that the presence of both genotypes would lead the clinical physician to use a therapy not involving clarithromycin. Therefore, PNA-FISH showed a sensitivity of 80.0% (95% CI, 29.9% to 98.9%) and a specificity of 93.8% (95% CI, 67.7% to 99.7%) for the detection of resistant strains. The positive-likelihood ratio was 12.9, indicating a strong

TABLE 1 Summary of the results obtained for the clarithromycin-resistant samples in the retrospective and prospective studies

Method and result[a]	No. of samples with indicated result	
	Etest positive	Etest negative
Retrospective study		
PNA-FISH-positive result	16	1
PNA-FISH-negative result	3	10
Positive result by other molecular method	16	1
Negative result by other molecular method	3	10
Prospective study		
PNA-FISH-positive result	4	1
PNA-FISH-negative result	1	15
Positive result by other molecular method	4	0
Negative result by other molecular method	1	16

[a] Other molecular method, GenoType HelicoDR or PCR-sequencing.

TABLE 2 Validation indexes obtained for the prospective study comparing the PNA-FISH- and PCR-based methods to the *in vitro* susceptibility Etest for the resistance results

Parameter	PNA-FISH value(s)	Value(s) determined by other molecular method[a]
Sensitivity	80% (95% CI, 29.9%–98.9%)	80% (95% CI, 29.9%–98.9%)
Specificity	93.8% (95% CI, 67.7%–96.7%)	100% (95% CI, 75.9%–100%)
Likelihood ratio positive	12.9	Infinite
Likelihood ratio negative	0.21	0.2

[a] Other molecular method, GenoType HelicoDR or PCR-Sequencing.

association between a PNA-FISH-positive result and the probability of the sample having a resistant *H. pylori* strain (38, 39) (Table 2). Regarding the biopsy specimens harboring *H. pylori* with a susceptible phenotype, PNA-FISH detected both susceptible and resistant genotypes for patient 13 (Fig. 1E and F), whereas the other two methods detected the only clarithromycin-susceptible *H. pylori* strain.

Among the 72 patients that were *H. pylori* negative by culture, in 12 cases the housekeeping gene GAPDH could not be amplified, suggesting either that the DNA was degraded or that there were PCR inhibitors, and these cases were not further assessed by PCR-sequencing. In the remaining 60 cases, 36 (60%) were also negative by both PNA-FISH and PCR-sequencing (Fig. 2). Fourteen (23%) cases were positive by both PNA-FISH and PCR-sequencing (13 susceptible and 1 resistant). In 10 patients, discrepant results were obtained with the molecular methods: in 8 (14%) biopsy samples, *H. pylori* was detected by PNA-FISH but no positive result was obtained by PCR, whereas 2 (3%) patients tested positive by PCR but were negative by PNA-FISH. In these cases, the use of nonspecific dyes in the biopsy specimens indicated that no bacterial cells were present in the tissue sections used for PNA-FISH.

DISCUSSION

New methods capable of detecting *H. pylori* clarithromycin susceptibility profile in a rapid and simple way became an emerging concern due to increased therapy failure of *H. pylori*. Here, we evaluated a new diagnostic method, based on PNA-FISH, that is able to provide results in a very short period of time, meaning that a diagnosis can be obtained in less than 24 h. For that, assays comparing the PNA-FISH technique with the standard methods (culture and Etest) and other molecular methods (PCR-sequencing, GenoType HelicoDR) were performed in retrospective and prospective studies.

Regarding the retrospective study, the few discrepancies between the molecular methods and the Etest in the results obtained may have two possible explanations. The first one is related to the existence of mechanisms of resistance apart from those associated with the three point mutations assessed in this study (15, 40, 41). In fact, the 86.7% concordance obtained between PNA-FISH and the Etest is in agreement with what has been observed in other studies, where the three point mutations assessed here were present in 84% to 90% of clarithromycin-resistant *H. pylori* strains (7, 14). The other explanation is the possibility of the presence of two or more *H. pylori* strains in the same patient (i.e., a mixed infection). Recent studies seem to support the view that mixed infections in the stomach environment are much more common than previously thought (13, 22, 42–44), an observation that is also supported by this study (see below). The fact that the biopsy specimen used for culture was not the same one used for the molecular methods only increases the chances that different strains were evaluated by different methods.

In one case, two genotypes were identified in two different biopsy specimens by both PNA-FISH and PCR-sequencing, whereas the culture method identified only the resistant strain. Because the culture used a different biopsy specimen, it is possible that only one of the strains was present in there. An alternative would be the overgrowth of the resistant strain in the culture plate, inhibiting the growth and recovery of the susceptible strain. In any case, the fact that the two molecular methods were in agreement strongly points to the presence of a mixed infection in that patient. Furthermore, that patient sample would be considered resistant to clarithromycin by all three methods.

In general, the results obtained for the prospective study were not very different from those obtained for the retrospective study, and many of the arguments used above to explain discrepant results are also applicable to the prospective study. One of the observations that occurred only in the prospective study was the

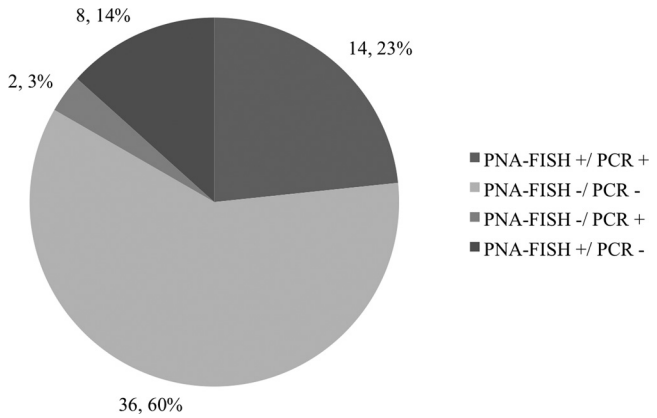

FIG 2 Correspondence between the molecular results (PCR-sequencing and PNA-FISH) for biopsy specimens that were negative by culture in the prospective study. The figure key presents the percentage of cases associated with each correspondence, as well as the number of cases.

TABLE 3 Predictive values of PNA-FISH for different rates of clarithromycin resistance[a]

% clarithromycin resistance (reference)	PNA-FISH predictive value (95% CI)	
	Positive	Negative
7 (50)	0.49 (0.17–0.81)	0.98 (0.93–1)
12 (51)	0.64 (0.37–0.91)	0.97 (0.90–1)
21(14)	0.77 (0.56–0.98)	0.95 (0.84–1)
24[b]	0.80 (0.60–1.00)	0.94 (0.83–1)
28 (52)	0.83 (0.65–1.00)	0.92 (0.80–1)
37 (53)	0.88 (0.73–1.00)	0.89 (0.74–1)
45 (54)	0.91 (0.78–1.00)	0.85 (0.68–1)
57[c]	0.94 (0.83–1.00)	0.78 (0.57–0.99)

[a] Negative predictive values and positive predictive values were calculated as previously described (49).
[b] Data are from this work (prospective study).
[c] Data are from this work (retrospective study).

simultaneous presence of resistant and sensitive strains in a single biopsy specimen, as assessed by PNA-FISH. This observation highlights and reinforces the idea that mixed infections are more common than previously considered. In this specific case, different *H. pylori* strains appear interspersed in the same biopsy specimen and do not seem to occupy specific niches. In fact, cells belonging to different *H. pylori* strains are found less than 5 μm apart but not in direct contact. PNA-FISH is the only method that is able to provide this type of information, and in the future use of the method might be invaluable to bring further information on the distribution and localization of different *H. pylori* strains.

Overall, the validity index values for resistance obtained for the *H. pylori*-positive patients of the prospective study support the idea that the PNA-FISH and PCR-based methods are accurate means of detecting and delineating clarithromycin-resistance status (Table 2). However, PCR-based methods require DNA isolation and amplification, which implies that these methods are more technically demanding and take longer to be accomplished (12). Interestingly, for PNA-FISH a variation of predictive values is expected to occur in accordance with variable rates of resistance to clarithromycin (Table 3). In spite of the likely underestimation of the specificity and sensitivity of PNA-FISH, due to the presence of two or more strains in some patients, high positive predictive values are expected for a prevalence level ranging between 20% and 30%, which are typical resistance rates in the more developed countries (9, 45, 46).

Patient specimens that were *H. pylori* negative by culture were positive by one or both molecular methods. It is well known that *H. pylori* is a fastidious bacterium that needs to be handled very carefully in order to be recovered in culture medium (6, 12, 47). To ensure the best possible conditions to promote *H. pylori* growth, all biopsy samples were processed in less than 24 h and were transported in a specific medium under refrigerated conditions. Nonetheless, it appears likely that *H. pylori* was present in some samples but remained uncultured, at least for those cases where the molecular methods were in agreement ($n = 14$). This might, for instance, be related to the amount of viable *H. pylori* present in the samples (12, 42). Another possible explanation is the uptake of antimicrobials and proton pump inhibitors by patients shortly before the endoscopy, a condition that would cause the microorganism to become unculturable (48). Here, 9 of the patient samples that were *H. pylori* negative by culture and positive by at least one molecular method were in this situation.

For the cases where discrepancies between molecular methods occurred, 8 samples were *H. pylori* positive by PNA-FISH but negative by PCR. However, 5 of these patients were infected with very low concentrations of *H. pylori* (only a few bacterial cells per biopsy sample were detected by PNA-FISH). This indicates that the limit of detection of PCR might be higher than that of PNA-FISH. Another possible explanation is the occurrence of false positives in the results determined by the PNA-FISH method (32). In fact, the probe targeting susceptible strains is fully complementary for a significant number of species belonging to the *Proteobacteria* group and as such is able to detect these bacterial species. However, when *H. pylori* is absent, the majority of the stomach microbiome is constituted by Gram-positive bacteria (43); as such, the possibility of false positives occurring is quite low. Another possible explanation is the presence of *H. pylori* only in the tissue section that was used for PNA-FISH and not in that used for PCR. The presence of false PCR positives or the heterogeneous distribution of *H. pylori* strains in the biopsy samples might also serve to explain the presence of two patient samples that tested positive by PCR but negative by PNA-FISH.

Conclusions. It is well established that clarithromycin resistance reduces the eradication rates of *H. pylori* gastric infections, since this antibiotic is one of the drugs most widely used in treatment regimens. Until now, susceptibility tests have mostly been based on fastidious and time-consuming culture methods. The requirements for more prompt and accurate methods led us to develop a PNA-FISH method to detect the three most prevalent point mutations associated with clarithromycin resistance. PNA-FISH proved to be a quick technique that can detect with high accuracy *H. pylori* strains and discriminate with high specificity both susceptible and resistant genotypes on gastric biopsy specimens simultaneously with histology tests. The regular application of this technique can lead in the future to a more rational antimicrobial therapy use and hence improve the patient's chances of a more effective *H. pylori* eradication.

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L.C., M.J.V., and N.F.A. are inventors with a patent application (WO 2011/030319) describing the four PNA probes reported here. All of the other authors of this article are aware of the patent and agreed with its submission.

We declare that we have no conflicts of interest.

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