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Contribution of genetic amplification by PCR for the diagnosis of *Helicobacter pylori* infection in patients receiving proton pump inhibitors

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Abstract

Background: *Helicobacter pylori* detection by standard methods may be altered by proton pump inhibitor (PPI) use. However, some patients cannot or should not interrupt PPI use before undergoing testing for *H. pylori*. Polymerase chain reaction (PCR) could allow more reliable *H. pylori* detection even in patients taking PPIs.

Objective: The aim of our study is to compare the *H. pylori* infection diagnostic value of histological examination without and with immunohistochemical staining, bacterial culture and PCR, in PPI-treated vs untreated patients.

Methods: Patients undergoing a gastric endoscopy for upper digestive symptoms were included. Gastric biopsy samples were obtained. The impact of taking PPI on the diagnostic performance of the different methods was studied. PCR results were confirmed by sequencing the *glmM* gene.

Results: A total of 497 patients were included, of whom 192 were *H. pylori* positive. Fifty-two patients received PPIs during the 14 days preceding the endoscopy while 140 did not. All methods had lower sensitivity than PCR, in all cases (PPI treatment or not). PPI use did not change significantly the methods' sensitivities.

Conclusion: The PCR method showed the best performance for the detection of *H. pylori* in gastric samples, whether or not patients received previous PPI treatment. This diagnosis test could become a new gold-standard test, especially in patients undergoing PPI treatment.

Keywords

Helicobacter pylori, proton pump inhibitors, histology, real-time PCR, glmM

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Key summary

Established knowledge

- Proton pump inhibitor (PPI) use decreases the sensitivity of direct and indirect tests for the diagnosis of *Helicobacter pylori* infection.
- Suspending PPI treatment can lead to recurrence of symptoms.
- In case of PPI use, polymerase chain reaction exhibits a higher sensitivity for the diagnosis of *H. pylori* infection than standard histology and the rapid urease test.
- PCR diagnosis performance for the diagnosis of *H. pylori* infection has not been assessed since the introduction of immunohistochemical staining (IHC).

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Significant findings

- The PCR is more sensitive for the diagnosis of *H. pylori* infection than histology alone, IHC or culture, regardless of whether the test is performed under PPI.
- PCR detection sensitivity does not decrease in case of PPI use, allowing their continuation.
- The PCR diagnostic test should be considered as the new gold-standard to detect *H. pylori* infection in gastric biopsies.

Introduction

Helicobacter pylori infection can be diagnosed by a variety of tests, either invasive (requiring an endoscopy) or noninvasive. Invasive tests include histological examination without or with immunohistochemical stain (IHC), rapid urease test (RUT), culture, and polymerase chain reaction (PCR); noninvasive tests include respiratory urea breath test, stool antigen detection and serology.

In current practice, these tests should be carried out only four weeks after the completion of an antibiotic treatment. In addition, proton pump inhibitors (PPIs) should be discontinued at least two weeks before testing for *H. pylori* infection. Indeed, recent use of PPI can induce false-negative results.[1] Because of their in vitro anti-*H. pylori* activity,[2] PPIs decrease the load of *H. pylori*[3–5] and inhibit *H. pylori* urease activity.[6] A previous study has shown that PPIs decrease histological examination, RUT,[7] urea breath test, culture and stool antigen test[8] sensitivity.

Histological examination without specific stain leads to more than 10% diagnosis of atypical biopsies (without typical chronic active gastritis with numerous *H. pylori*), responsible for false-negative diagnosis of *H. pylori* infection. These atypical biopsies seem to be mainly due to PPI consumption or previous eradication treatment.[9] This stresses that a special stain for *H. pylori* must be used and, in particular, an IHC, which has a lower interobserver variation.[1]

PCR is a highly sensitive method that can detect very small amounts of DNA. PCR may be useful, therefore, in detecting the presence of *H. pylori* even when the organism is present at a low bacterial load. In particular, quantitative real-time PCR targeting 23S ribosomal DNA (rDNA) allows both *H. pylori* detection and its clarithromycin resistance.[10,11] PCR was described as more sensitive than RUT and histology in patients taking PPIs,[12] suggesting that this method could be interesting in patients who cannot or should not stop PPIs before an endoscopy.

The effects of PPI on *H. pylori* detection have not been assessed since the introduction of IHC and PCR.

The aim of our study is to compare the *H. pylori* infection diagnostic value of histological examination without and with IHC, bacterial culture and PCR, in PPI-treated vs PPI-untreated patients.

Material and methods

Patients

Adult patients attending the gastroenterology unit in Ambroise Paré Hospital for an upper endoscopy were included. All suffered from dyspeptic symptoms, gastric disorders or gastric cancer suspicion.

Patients with a history of any gastric surgeries such as bariatric surgery and patients with hemostatic disorders were excluded, as were patients with previously diagnosed gastric tumors or lymphomas. The intake of PPIs during the previous 14 days was recorded either on the day of endoscopy or later by phone.

At least four biopsies were performed during each endoscopy, two antral biopsies and two corporeal biopsies that were used for histology. Two supplemental biopsies for culture and PCR were sampled in antrum and fundus, dipped in a dry tube and frozen immediately in liquid nitrogen until processing. The endoscopic apparatus—an Olympus GIF-IT—including all channels were cleaned and disinfected using the standard disinfection protocol. For each patient one single-use biopsy forceps was used to contain cross-contamination and the biopsy was extracted from the forceps without formalin contact.

Ethics

An information notice was signed by all participants. All the procedures were performed as routine, with absence of impact on patient care. The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki.

This study was retrospective. All the diagnosis methods were routinely performed. All patients were investigated in a hospital setting, according to good clinical practices, with informed consent of the endoscopic procedure followed, when applicable, by the appropriate treatment. In this routine process, the consent for the endoscopic procedure is always written and kept in the patient's medical record. No extra biopsy sample or additional endoscopy

was required to evaluate the *H. pylori* status of the patients.

Histology and IHC

Giemsa staining was carried out to ascertain the presence of *H. pylori*. The presence of mucosal infiltration by lymphocytes and polymorphonuclear cells was determined after hematoxylin and eosin (H&E) staining. The absence of gastritis was defined as the absence of lymphocytes or polymorphonuclear infiltration, corresponding to 0 subscores using the Sydney System Classification.[13] All biopsies underwent IHC, which was performed on 4-µm thick sections of formalin-fixed paraffin-embedded tissue samples with Bond autostainer (Leica, Newcastle upon Tyne, UK) after antigen retrieval in pH 6 citrate buffer (Leica) and endogenous peroxidase inhibition with H_20_2 (3%). The primary antibody against *H. pylori* was polyclonal rabbit IS523 (Dako, Glostrup, Denmark). Revelation and amplification of the signal was performed with Bond Polymer Refine Detection (Leica, Biosystems Newcastle Ltd) and counterstaining with hematoxylin.

Culture

Gastric biopsies were homogenized using a glass Griffith's tube, and *H. pylori* was cultured according to a previously described method.[14] Colonies obtained from the primary culture were harvested together to be tested for antimicrobial susceptibility. Clarithromycin resistance was detected by antimicrobial susceptibility testing according to the E-test method (AB Biodisk®, Solna, Sweden) using Mueller-Hinton agar (Oxoïd®, Dardilly, France) supplemented with 10% horse blood and incubated in microaerophilic conditions for three days. Strains were considered resistant when the minimum inhibitory concentration (MICs) levels were >0.5 mg/l.[15] Strains were stored at –80° C in 3 ml meat-liver media (Mérieux®, Marcy l'Etoile, France) added to 0.3 ml of glycerol. Amoxicillin, metronidazole, tetracycline, rifampicin and ciprofloxacin susceptibilities were also determined by the E-test method (AB Biodisk®, Solna, Sweden). The strains were considered amoxicillin, metronidazole and ciprofloxacin resistant with MICs equal to or greater than 1 mg/l, 8 mg/l, and 1 mg/l.[15]

PCR

A quantitative PCR (qPCR) assay was used directly on DNA obtained from gastric biopsies to detect both the presence of *H. pylori* and the point mutations conferring resistance. DNA was isolated by using a QIAamp DNA mini kit (Qiagen SA®, Courtaboeuf, France). A 267-bp fragment of the 23 S ribosomal RNA (rRNA) gene of *H. pylori* was amplified by using primers HPYS and HPYA as previously described.[16] By using the LightCycler thermocycler (Roche Diagnostics®, Neuilly sur Seine, France) the PCR and hybridization reactions were carried out in glass capillaries in a volume of 20 µl containing 3 µl of template DNA, 1.6 µl of $MgCl_2$ (25 mM), 0.4 µl of forward and reverse primers (20 µM each), 0.2 µl of sensor and anchorprobes (20 µM each), and 2 µl of FastStart DNA Master Hybridization Probes (Roche Diagnostics®, Neuilly sur Seine, France). PCR amplification comprised an initial denaturation cycle at 95°C for 10 minutes, followed by 50 amplification cycles (with a temperature transition rate of 20°C/s) consisting of 95°C for 0 seconds, annealing at 60°C for 10 seconds, and extension at 72°C for 17 seconds. After amplification a melting step was performed, consisting of 95°C for 0 seconds, cooling to 45°C for 30 seconds (with a temperature transition rate of 20°C/s), and finally a slow rise in the temperature to 85°C at a rate of 0.1°C/s with continuous acquisition of fluorescence decline. The sensitivity and specificity of the real-time PCR were 98.4% and 94.1%, respectively. Each run included positive and negative controls, the former prepared from 10^{-2}, 10^{-4}, and 10^{-6} dilutions of 45-µg/ml DNA from *H. pylori* strain H37Rv, and the latter consisted of sterile water. Quality control was acceptable when the negative control had an undetectable cycle threshold (and the 10^{-2}, 10^{-4}, and 10^{-6} dilutions of *H. pylori* DNA McFarland 1, had cycle threshold values between 17 and 19, and 27, and 33, respectively. The specificity of the PCR method was evaluated in 20 biopsies samples without *H. pylori* infection but positive for different microorganisms (*Enterococcus faecalis, Pseudomonas aeruginosa, Coagulase-negative Staphylococcus, Candida albicans, Escherichia coli, Klebsiella pneumoniae* and *Campylobacter sp*) for which the PCR remained negative.

When the PCR method was positive and the culture negative, results were confirmed by PCR amplification of the *glmM* gene, a housekeeping gene coding for a phosphoglucosamine mutase, essential for the development of the cell wall in bacteria as well as for the growth of the microorganism.[17] Briefly, a 294-bp fragment internal to the *glmM* gene was PCR-amplified with 5'- GGATAAGCTTTTAGGGGTGTTAGGG G-3' and 5'- GCTTACTTTCTAACACTAACGCGC-3'.[18,19] The 50 µl PCR mixture contained 50 pmol of each primer, 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% w/v gelatin, 0.2 mM of each deoxynucleotide, 5 µl of DNA, and 2.5 U Taq DNA polymerase. PCR consisted of 30 cycles as follows: 94°C for one minute, 56°C for one minute, and 72°C

for two minutes.[18] The completed reactions were analyzed by gel electrophoresis.

Positive and negative cases

To consider a patient as *H. pylori* positive, at least one of the four methods (histology, IHC, culture or PCR) had to be positive. Conversely, a patient was considered as negative if none of the four methods was positive.

Statistical analysis

For the nominal qualitative variables, a chi-square test or an exact Fisher test was performed. For the quantitative variables, a Wilcoxon nonparametric test or a Student *t* test was performed depending on the normal distribution of the values. α risk was set at 5%.

Statistical analysis was performed with Excel® and JMP 10 software (SAS® Institute).

Results

Population characteristics

From January 2011 to September 2015, 497 patients were included. Following the above definition, 192 were *H. pylori* positive whereas 305 patients were *H. pylori* negative. Demographic characteristics of the study population are presented in Table 1. Of the 192 positive patients, the male-to-female ratio was 1/1.1. The mean age was 52 ± 17.8 years for *H. pylori*-positive patients, and 57 ± 18.2 years for *H. pylori*-negative patients ($p = 0.13$). The mean number of biopsies performed for one patient was 4.4 ± 1.8. At least one fundic biopsy was taken in 180 (94%) patients.

Out of the 192 *H. pylori*-positive patients, 52 (27%) received PPIs during the 14 days preceding the endoscopy while 140 (73%) did not. The endoscopic findings were gastric ulcerations in 32/192 (17%) patients, duodenal ulcerations in 25/192 (14%), esophagitis in 9/192 (4%), and gastric carcinoma in 2/192 (1%).

No statistically significant difference was found between PPI-treated patients and untreated patients in terms of age, endoscopically diagnosed pathology and number of biopsies taken (4.5 ± 1.0 vs 4.5 ± 2.0, $p = 0.94$).

Table 1. Demographic and clinical characteristics of patients.

	PPIs −	PPIs +
n	140 (72%)	52 (28%)
Age	56 ± 18	54 ± 16
Sex ratio (M/F)	1.3	0.7
Endoscopy findings		
Normal	54 (28%)	16 (8%)
Gastric ulcer	18 (9%)	14 (7%)
Duodenal ulcer	15 (8%)	9 (5%)
Esophagitis	7 (4%)	2 (1%)
Gastric cancer	0 (0%)	2 (1%)

Values are means (%) ± standard deviation.
F: female; M: male; PPI: proton pump inhibitors.

Comparison of diagnosis performances of different tests

First, sensitivities of the different methods for the diagnosis of the *H. pylori* infection were compared, taking PCR as the gold standard. Histology, IHC and culture had a lower sensitivity (95% confidence interval) compared to PCR, regardless of PPI use, 42.3% (26.7–56.8), 59.6% (45.1–73.0) and 51.9% (37.6–66.0) vs 100%, respectively.

Taking PPIs during the previous 14 days tended to decrease the sensitivity of histology and culture as compared to controls (Table 2, not significant (NS)).

For the patients not treated by PPIs, culture and IHC had the same sensitivity (Table 2), not statistically different from histology alone ($p = 0.4$).

Histology alone exhibited 50% false-negative results in the absence of PPIs, and 70% in case of PPI use ($p = 0.5$). Overall, the sensitivity of PCR was higher than that of all the other tests in both groups. This difference between the groups was statistically significant ($p < 0.001$ in both groups). The specificity of histology, IHC and culture was 100%.

The results of the four methods were then compared according to PPI use (Table 2). For histology, IHC and culture, the rate of positive results was not statistically different in case of PPI use, unlike PCR (37% vs 23%, $p = 0.03$).

PCR-only was positive for 14 patients, confirmed by the amplification of the *glmM* gene. Among these 14 patients, three showed 10^1 copies of qPCR bacterial load, six showed 10^2 copies, and five showed 10^3 copies. The bacterial load was higher in PCR-positive patients in whom at least one other method including culture was positive: three patients were positive with 10^2 copies, nine with 10^3 copies, and 11 with 10^4 copies. In 11 PCR-positive patients in whom at least one other method except culture was positive, the amplification of the *glmM* gene was also performed and confirmed the results.

For these 14 patients for whom PCR-only was positive, an upper endoscopy was performed for eight gastro-duodenal ulcer controls, for one *H. pylori* eradication control, and in two cases to explore upper digestive symptoms. Out of these, four patients suffered from gastro-duodenal ulcers and two from gastric cancer.

Table 2. *Helicobacter pylori* diagnosis performance comparison between histology, immunohistochemical stain and culture with PCR as a gold-standard test.

	PPI− ($n=140$)	PPI+ ($n=52$)	Total positive	p
PCR				
Positive	32 (22.8%)	20 (38.4%)	52	0.03
Negative	108	32		
Histology				
Positive	16 (11.4%)	6 (11.5%)	22	0.99
Negative	124	46		
Sensitivity % (95% CI)	50 (32.7–67.3)	30 (9.9–50.1)	42.3% (26.7–56.8)	
IHC				
Positive	19 (13.7%)	12 (23%)	31	0.11
Negative	121	40		
Sensitivity % (95% CI)	56 (42.4–76.4)	60 (38.5–81.5)	59.6% (45.1–73.0)	
Culture				
Positive	19 (13.7%)	8 (15.3%)	27	0.75
Negative	121	44		
Sensitivity % (95% CI)	56 (42.4–76.4)	40 (18.5–61.5)	51.9% (37.6–66.0)	

p: comparison between PPI negative (−) and PPI positive (+).
CI: confidence interval; IHC: immunohistochemical stain; PCR: polymerase chain reaction; PPI: proton pump inhibitors.

In five patients no gastritis was found even after careful reexamination of biopsies. Four (29%) of these patients were taking PPIs vs 48 (27%) of the 178 other patients ($p=0.99$).

Among patients not treated by PPIs, 8/23 (35%) showed $<10^3$ copies of qPCR bacterial load and 15/23 (65%) showed $\geq 10^3$ copies of qPCR bacterial load. In patients undergoing PPI treatment, 4/14 (29%) showed $<10^3$ copies of qPCR bacterial load and 10/14 (71%) showed $\geq 10^3$ copies of qPCR bacterial load (NS).

Discussion

The aim of this study was to evaluate the performances of the different invasive tests for the diagnosis of *H. pylori* infection, requiring upper endoscopy to take gastric biopsies such as histology, IHC, culture and PCR.

At the Ambroise Paré Hospital Endoscopy Unit, a total of 1400 upper gastrointestinal endoscopy exams are performed annually. *H. pylori* detection using histology, IHC or culture in this cohort was similar to that, very variable depending on age, obtained from the global French population.[20] The high detection rate of the real-time PCR in this study can be at least partially attributed to the study population. Indeed, only symptomatic patients, who are more likely to be infected,[21] were tested.

In the non-PPI-treated group, culture had a sensitivity similar to IHC and a better sensitivity than histology alone. The slightly lower yield of histology in this group can be explained by the biopsy specimen containing a lower bacterial density of viable cells compared to biopsies performed in PPI-treated patients, which has already been reported,[22] thereby leading to the failure to identify *H. pylori* and finally resulting in false negatives. This is in contradiction with in vitro studies, but PPIs at recommended doses will not consistently achieve the sustained pH increase required to mimic the bactericidal effect that was observed in vitro. Moreover, change of spiral morphology to coccoid has been reported with PPI use, and could decrease histology sensitivity.[2]

When treated with a PPI, the distribution of *H. pylori* within the stomach changes so the density in the antrum is reduced and that in the corpus is relatively increased.[5] Moreover, as bacterial load is correlated with gastritis grade,[23] the low bacterial load in PCR-only-positive patients could explain the absence of histological gastritis in some of these patients, as has been shown in a pediatric cohort.[10] The fact that some PCR-positive patients, confirmed by two amplification methods (of 23S rRNA and *glmM* genes), do not exhibit histological signs of gastritis raises the question that *H. pylori* infection is not always associated with gastritis, contrary to widespread dogma.

The fact that PCR sensitivity is much higher than that of all other methods is striking. The specificity of 23S qPCR was tested with a variety of microorganisms (see the Methods section). All PCRs associated with negative culture were confirmed by *glmM* amplification ($n=25$). Lage et al.[24] showed a 100% specificity of

glmM (*ureC*) PCR against 25 isolated bacterial strains, and 97% specificity on biopsy specimen with culture as the gold-standard. Espinoza et al.[25] stressed that the use of the *glmM* primers described by Tomasini can lead to rare aspecific amplification, and proposed an alternative reverse primer (GLM MR1) that increases the specificity of amplification, combined with Tomasini forward primer. However, the amplification of two target genes (coupled PCR) as was the case in our study decreases in itself false-positive results.[26] Consequently, our results suggest a real superiority of coupled PCR compared to other methods, including culture. The tendency of PPIs to reduce histology and culture sensitivity, but not PCR sensitivity, is consistent with previous studies.

In an earlier study *H. pylori* was detected by histological examination in 27 of 40 controls (68%) and in only 13 of 25 patients taking PPIs (52%).[24] A study conducted by Siavoshi et al. showed that *H. pylori* was detected in 40% of patients by culture and 48.3% by RUT. A strong correlation was found between PPI consumption and negative results of culture and RUT ($p < 0.05$).[27]

Yakoob et al. conducted a study that found no difference in the PCR detection rates between one group under PPI treatment and the control group (74% vs 75% in the antrum). It also concluded that the diagnostic performance of both RUT and histology without IHC was reduced and that PCR was more sensitive than both other methods.[12] The tendency of sensitivity reduction for histology, IHC and culture under PPI treatment in our study should be confirmed in a larger population.

One other study conducted by Chen et al. concluded there was no difference between two groups tested by multiplex PCR. By this method, *H. pylori* was detected in 143 (52.2%) of 274 cases in the control group and 130 (46.1%) of 282 cases in patients undergoing PPI treatment. Moreover, 241 (46.1%) of 523 cases detected by multiplex PCR were RUT negative, and PPI use decreased RUT sensitivity.[28]

Finally, previous studies have shown that IHC is at least as accurate as histology alone (Genta and H&E stains),[1,29] and has a lower interobserver variation,[30] which is consistent with our results.

Conclusion

Because of the multiple pathogenic effects of *H. pylori*, an accurate detection method is needed. Currently, there is no invasive gold-standard method. We tested techniques that are used in daily practice with proven clinical significance.

The implication of this study is that with prior use of PPIs, it is still possible to get a very high sensitivity by performing PCR in comparison with other invasive techniques such as IHC, culture and histology. PCR today is cheaper than culture, which is fastidious and dependent on transportation conditions. Real-time PCR could thus be used as a new gold-standard test to detect *H. pylori* in patients by designing extremely specific primers to *H. pylori* and targeting at least more than one conserved gene.[31] PCR would increase *H. pylori* detection rates, thereby increasing opportunities for medical interventions and allowing patients to be treated.

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All raw data are available on request.

Ethics approval

The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki.

Declaration of conflicting interests

None declared.

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Informed consent

All patients were investigated in a hospital setting, according to good clinical practices, with informed consent of the endoscopic procedure followed, when applicable, by the appropriate treatment. In this routine process, the consent for the endoscopic procedure is always written and kept in the patient's medical record.

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