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Original Investigation

Comparison of Culture, Real- time-PCR, ELISA, and Histopathological Examination Methods for Identification of *Helicobacter pylori*

Salih Maçin[1], Alpaslan Alp[2], Burçin Şener[2], Cenk Sökmensüer[3], Diclehan Orhan[3], Hasan Özen[4], Taylan Kav[5], Yakut Akyön[2]

Abstract

Introduction: There are several methods used for the diagnosis of *Helicobacter pylori* infections, and there is an increasing demand for the use of non-invasive, more rapid tests. The aim of the present study was to compare different diagnostic methods.

Methods: A total of 87 patients who had undergone esophagogastroduodenoscopy were included in the study. Biopsy samples obtained from these patients were used for culture, real-time polymerase chain reaction (RT-PCR), and histopathological examination. Stool samples were also collected from these patients and were tested using the *Helicobacter pylori* stool antigen (HpSA) kit. Histopathological examination was accepted as the gold standard test.

Results: *H. pylori* was identified by histological examination in 77/87 (87.5%) patients, whereas it was negative in 10/87 (12.5%) patients. Furthermore, positive results were obtained in 55 (63.2%), 71 (81.6%), and 77 (87.5%) patients using the culture method, HpSA analysis, and RT-PCR method, respectively. The sensitivity and specificity of culture, HpSA, and PCR tests were determined as 71.4% and 100%, 87% and 60%, and 97.4% and 80%, respectively. Antibiotic susceptibility tests were performed on 48 out of the 55 culture positive samples. Resistance to clarithromycin was found in 28 (58.3%), metronidazole in 14 (29.2%), and levofloxacin in 4 (8.3%) of the isolates. Resistance to amoxicillin and tetracycline was not observed.

Conclusion: There are currently several invasive and non-invasive diagnostic tests for the detection of *H. pylori* infections. Each test has some advantages and disadvantages. The diagnostic method of choice should be easy and applicable to all age groups.

Keywords: Antimicrobial resistance, diagnosis, *Helicobacter pylori*, methods, upper gastrointestinal bleeding

Introduction

Helicobacter pylori is a common pathogen that colonizes the gastric epithelium with a high infection prevalence (1). *H. pylori* infection risk increases due to insufficiency of socio-economic conditions and inability to create healthy living conditions. Many gastrointestinal illnesses are associated with *H. pylori* such as gastritis, gastric and duodenal ulcer, and malignancies (2).

Gastroduodenal ulcers can cause gastrointestinal bleeding. *H. pylori* is detected in more than 70% of cases of gastric ulcer and 90% of cases of duodenal ulcer (3). Its eradication leads to a significant reduction in the incidence of recurrent upper gastrointestinal bleeding (4). Many conventional diagnostic methods fail to identify *H. pylori* especially in patients with upper gastrointestinal bleeding. However, some studies have shown that polymerase chain reaction (PCR)-based methods are more reliable than the other techniques for the diagnosis of cases with upper gastrointestinal bleeding (5).

The accurate detection of *H. pylori* is essential for the management and eradication of bacteria in related cases. The diagnosis is based on both invasive and non-invasive methods. Invasive diagnostic tests include endoscopy followed by histopathological examination of biopsy specimens, fast urease test, and direct identification of the microorganism using culture. Non-invasive methods comprise urea breath test, antibody detection using serology, and stool antigen test (6).

The aim of the current study was to compare invasive and non-invasive tests for the detection of *H. pylori* in patients with gastroduodenal disease. Antibiotic susceptibility test results were also evaluated for *H. pylori* strains.

Methods

Patients

The present study was planned retrospectively. Data included 87 patients who had been evaluated at Departments of Pediatric Gastroenterology and Internal Medicine, Hacettepe University School of Medicine. Biopsy specimens were obtained during esophagogastroduodenoscopy. The study was conducted in accordance with the Declaration of Helsinki.

ORCID IDs of the authors: S.M. 0000-0002-9202-4047; A.A. 0000-0001-7856-7590; B.Ş. 0000-0002-0724-3166; C.S. 0000-0001-7637-8745; D.O. 0000-0003-3637-5392; H.Ö. 0000-0002-9063-3893; T.K. 0000-0003-2879-7739; Y.A. 0000-0002-0919-5508.

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[1]Department of Medical Microbiology, Selçuk University School of Medicine, Konya, Türkiye
[2]Department of Medical Microbiology, Hacettepe University School of Medicine, Ankara, Türkiye
[3]Department of Medical Pathology, Hacettepe School of Medicine, Ankara, Türkiye
[4]Departments of Pediatrics, Gastroenterology Unit, Hacettepe University School of Medicine, Ankara, Türkiye
[5]Departments of Internal Medicine, Gastroenterology Unit, Hacettepe University School of Medicine, Ankara, Türkiye

Address for Correspondence:
Salih Maçin
E-mail: salihmacin@hotmail.com

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Culture

Samples were inoculated onto brain heart infusion (BHI; Oxoid, England) agar containing 7% horse blood and antibiotics (10 mg/L vancomycin, 5 mg/L trimethoprim, 5 mg/L cefsulodin, and 5 mg/L amphotericin B). All samples were incubated at 35°C-37°C for 5-7 days under microaerobic conditions. Bacterial isolates were identified by Gram staining, colony morphology, and urease, catalase, and oxidase reactions (7).

Antibiotic Susceptibility Tests

Antibiotic susceptibility testing was performed using the gradient strip method. Amoxicillin, clarithromycin, tetracycline, levofloxacin, and metronidazole were tested. The identified strains were sub-cultured onto antibiotic-free 5% horse blood containing BHI agars and incubated at 35 °C for 3 days. Samples were inoculated onto Mueller-Hinton agar (Oxoid, England) containing 5% horse blood to perform antibiotic susceptibility testing. The European Committee on Antimicrobial Susceptibility Testing guidelines were used in order to evaluate the results (8). The *H. pylori* NCTC 11637 standard strain was used as the control strain.

Helicobacter pylori Stool Antigen (HpSA) Test

Stool samples obtained from the patients were stored at –20°C until use. Samples were tested for *H. pylori* antigen using the commercially available HpSA kit (GA Generic Assays GmbH, Germany). This is an indirect two-site immunoassay based on polyclonal antibodies, leading to qualitative determination of the antigen in stool samples.

Real-time (RT) PCR Method

DNA isolation was performed with MagNA Pure LC Total Nucleic Acid Isolation Kit (Roche Applied Science, Germany) in automated MagNa Pure nucleic acid isolation instrument. RT-PCR was performed in capillary tubes in the LightCycler 2.0 (Roche Diagnostics, Germany). Cycling conditions were denaturation at 95 °C for 10 min, followed by 40 cycles of amplification at 95°C for 10 s, 55°C for 10 s (with single acquisition of fluorescence), and 72°C for 15 s. Melting conditions were at 95°C for 10 s, 50 °C for 5 s, and 80°C for 0 s. Finally, a cooling step was applied at 40°C for 30 s (9).

Histopathological Examination

Two gastric biopsy specimens, one from the antrum and one from the corpus, were fixed in 10% formalin. Prepared sections (4 µm thickness) were placed on poly-l-lysine-coated adhesive microscope slides for immunohistochemical staining. All sections were first dewaxed (heating at 60°C in an autoclave) and then embedded in xylol for 10 min. Automatic immunohistochemical staining was performed using a Leica DS9800 system (New Castle, United Kingdom). Antigen retrieval was performed with citrate buffer (pH 6.0, 20 min, 95°C) for 10 min. Sections were incubated with *H. pylori* rabbit polyclonal antihuman antibody (215A-70; Cell Marque, CA, USA) at a dilution of 1:100 for 1 h. A polymer detection kit (DS9800; New Castle, United Kingdom) was used to detect immunostaining. Sections were treated with diaminobenzidine as chromogen. *H. pylori*-infected tissues were used as positive controls. The primary antibody solutions were substituted with phosphate buffer solution in the negative staining controls.

Statistical Analysis

All the analysis was performed by SPSS (Statistical Packege for Social Sciences) for Windows version 15.0 (SPSS Inc.; Chicago, IL, USA). A $p \leq 0.05$ was considered statistically significant. Pearson correlation analysis was also conducted. P value was calculated using the McNemar test.

Results

The mean age of the patients was 17.8±10.6 years (min-max: 5-64 years), and 45 (51.7%) were men. Histopathological examination identified 77/87 (87.5%) patients as positive, whereas 10/87 (12.5%) patients were negative. Positive results were obtained in 55 (63.2%), 71 (81.6%), and 77 (87.5%) patients by the culture method, HpSA analysis, and PCR method, respectively (Table 1).

Histopathological examination results were accepted as the gold standard, and specificity, sensitivity, positive predictive value (PPV), and negative predictive value (NPV) were calculated for each method (Table 2). The specificity of culture was 100%, and sensitivity was 71.4%. The sensitivity and specificity of HpSA and RT-PCR tests were found as 87% and 60% and 97.4% and 80%, respectively (Table 3).

Histopathological examination results revealed that false-positive results were detected by HpSA in 5.6% (4/71) and by RT-PCR in 2.6% (2/77) of the patients. A higher rate of false-negative results was obtained with the culture method (22/32; 68.7%). Culture method, RT-PCR, and HpSA tests were found to correlate with the Pearson correlation analysis (Table 4).

In 20 patients who had upper gastrointestinal bleeding, *H. pylori* was detected in all patients by RT-PCR, detected in 14 patients by culture, and detected in 16 patients by HpSA (Table 5).

Antibiotic susceptibility tests were performed on 48 out of the 55 culture positive samples. Among 48 isolates, none of them were

Table 1. The evaluation of the diagnostic tests used for the detection of *H. pylori* in the study samples

Method	Positive samples (%)	Negative samples (%)	Total
Histopathology	77 (87.5)	10 (12.5)	87
Culture	55 (63.2)	32 (36.8)	87
HpSA	71 (81.6)	16 (18.4)	87
RT-PCR	77 (87.5)	10 (12.5)	87

HpSA: *Helicobacter pylori* stool antigen; RT-PCR: real-time polymerase chain reaction

Table 2. Comparison of the histopathology results with culture, HpSA, and RT-PCR results for the detection of *H. pylori* in the clinical specimens

	Culture (+)	Culture (–)	HpSA (+)	HpSA (–)	RT-PCR (+)	RT-PCR (–)
Histopathology positive (n: 77)	55	22	67	10	75	2
Histopathology negative (n: 10)	0	10	4	6	2	8
Total	55	32	71	16	77	10

HpSA: *Helicobacter pylori* stool antigen; RT-PCR: real-time polymerase chain reaction

Table 3. Comparison of the results of the *H. pylori* diagnostic methods when histopathology was considered as the gold standard

Method	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Culture	71.4	100	100	31.2
HpSA	87	60	94.4	37.5
RT-PCR	97.4	80	97.4	10.4

PPV: positive predictive value; NPV: negative predictive value; HpSA: *Helicobacter pylori* stool antigen; RT-PCR: real-time polymerase chain reaction

Table 4. Correlation of *H. pylori* test results to histopathology results

Method	False-positive	False-negative	r[a]	p
Culture	0	22	0.472	<0.0001
HpSA	4	10	0.387	0.180
RT-PCR	2	2	0.774	<1

[a]Correlation is significant at the 0.01 level.
HpSA: *Helicobacter pylori* stool antigen; RT-PCR: real-time polymerase chain reaction

Table 5. Detection of *H. pylori* infection in patients with upper gastrointestinal bleeding

Method	Positive samples (%)	Negative samples (%)	Total
Histopathology	20 (100)	0	20
Culture	14 (70)	6 (30)	20
HpSA	16 (80)	4 (20)	20
RT-PCR	20 (100)	0	20

HpSA: *Helicobacter pylori* stool antigen; RT-PCR: real-time polymerase chain reaction

Table 6. Antimicrobial resistance of the tested *H. pylori* strains (n=48)

Antibiotics	Resistance, n (%)
Amoxicillin	0
Clarithromycin	28/48 (58.3%)
Levofloxacin	4/48 (8.3%)
Metronidazole	14/48 (29.2%)
Tetracycline	0

resistant to amoxicillin and tetracycline. Resistance to clarithromycin was found in 28 (58.3%), metronidazole in 14 (29.2%), and levofloxacin in 4 (8.3%) of the isolates (Table 6).

Discussion

Currently, several different tests for the diagnosis of *H. pylori* infections exist. Each test has both advantages and disadvantages. Several studies have examined the diagnostic performance of invasive and non-invasive methods (10, 11). However, these studies demonstrated a lack of agreement. Discrepancies in the diagnostic performance of different tests in different studies might be attributed to the selection of different methods as the gold standard.

Among various diagnostic methods, histopathological examination of endoscopic biopsy specimens provides more information about the degree of inflammation and associated pathology (12). It is also the most reliable test in the presence of upper gastrointestinal bleeding. Proton pump inhibitors should be stopped prior to gastroduodenoscopy, since they may decrease the sensitivity of the histopathological examination.

Routine cultivation is difficult to perform in microbiology laboratories, since it is time consuming and hard to maintain microaerophilic conditions. However, bacterial growth in cultures provides definitive diagnosis and also enables antibiotic susceptibility testing to guide specific treatment. Gisbert and Abraria (13) reported three studies with culture sensitivity of 45% and specificity of 98%. Aktepe et al. (14) reported that the sensitivity of the culture method is 61%, and specificity is 91%. In the present study, sensitivity was 71.4%, and specificity was 100% for culture. The low sensitivity and high specificity of the culture-based methods might be correlated with inappropriate biopsy site and inadequate specimens. However, culture-based methods enable specific antibiotic susceptibility testing of the strains, thus providing important data especially in populations with a high rate of drug resistance among *H. pylori* strains.

Helicobacter pylori stool antigen test, which has been introduced as a non-invasive diagnostic alternative, has the advantages of being relatively inexpensive, easy to perform, and can be used in pregnant women, children, and the elderly. It can easily be performed in routine laboratories as it does not require complicated laboratory facilities. In a Japanese study, the sensitivity and specificity of the HpSA test were 93.9% and 95.7%, respectively, when compared with histopathological examination (15). A study from Turkey reported the HpSA test sensitivity as 72% and specificity as 67% (14). In our study, sensitivity and specificity were 87% and 60%, respectively. There were four false-positive samples using the HpSA test. Two of these false-positive samples were negative using culture and RT-PCR. The two other samples were negative using histopathology and culture, but positive with RT-PCR method. Thus, this false-positive may be attributed to the lack of detection by histopathology that was considered as the gold standard.

The presence of *H. pylori* and specific antibiotic-resistant genes can be investigated by RT-PCR from gastric biopsy specimens. RT-PCR has a high sensitivity and specificity and can be used as a follow-up assessment after therapy (16). The contamination may occur during the DNA extraction step or the presence of inanimate microorganisms residual chromosomal DNA, and this may lead to false-positive results (17). In our study, biopsy PCR studies had a sensitivity of 97.4%, specificity of 80%, NPV of 10.4%, and PPV of 97.4%. There were only two false-positive samples by RT-PCR. These two samples were also positive by HpSA test. Thus, obtaining positive results with two different methods provided strong information that this patient was infected with *H. pylori*, and the "false" false-positive evaluation in that specific case was attributed to choosing histopathology as the gold standard.

Many conventional *H. pylori* diagnostic methods show a significant decrease in their sensitivity in patients with upper gastrointestinal bleeding. However, in cases of upper gastrointestinal bleeding, PCR techniques and histopathological examination are more reliable than rapid urease test, HpSA test, or culture (5, 18). A Korean

study that evaluated the patients with gastrointestinal bleeding (n=157) found that sensitivity and specificity were 92.5% and 96% for histopathological examination, 40% and 100% for culture, and 97% and 56% for serology. The HpSA method showed relatively high sensitivity, but cannot be recommended as the primary diagnostic method in the bleeding situation, because of its low specificity (19). In our study, *H. pylori* was correctly detected by RT-PCR and histopathological examination in 20 patients who had upper gastrointestinal bleeding, culture in 14, and HpSA in 16. The findings of the current study suggested that histopathological examination and RT-PCR assay were the most appropriate methods for the detection of *H. pylori* in patients with upper gastrointestinal bleeding.

Antibiotic susceptibility testing provides valuable information for choosing the right treatment. Resistance to metronidazole is reported to be between 15.9% and 77.9% (20, 21). In accordance with the literature, metronidazole resistance was 29.2% in the current study. Resistance to clarithromycin was reported to be 34% in Austria, 54.6% in Spain, and 30.1% in Turkey (22-24). Clarithromycin resistance was also found to be high (58.3%) in the present study. Resistance to amoxicillin and tetracycline is very rare. Agudo et al. (23) revealed no resistance to amoxicillin, rifampicin, and tetracycline. Vécsei et al. (20) reported tetracycline resistance as 0.9%; however, they did not report any amoxicillin resistance. In our study, no resistance to amoxicillin and tetracycline was found. The frequency of levofloxacin resistance is reported to be 5.9%-18.2% in Turkey, 22.1% in Italy, and 34.5% in China (25-28). In our study, levofloxacin resistance was also found to be compatible with previous studies from Turkey (8.3%).

Conclusion

There are a variety of tests available for the diagnosis of *H. pylori*. In cases where endoscopy could not be performed, non-invasive, simple, rapid, and practical HpSA test with acceptable results can be used to provide diagnosis and to monitor treatment. RT-PCR method has a high sensitivity and specificity in comparison with histopathological examination accepted as the gold standard. Both RT-PCR and histopathological examination are also reliable diagnostic methods in patients with upper gastrointestinal bleeding. When available, culture should be performed for antibiotic susceptibility tests, especially in the case of treatment failure. Thus, each laboratory should better establish its own diagnostic algorithm for the accurate and appropriate diagnosis of *H. pylori* infection in their patient population, according to their laboratory facilities and clinical setting.

Ethics Committee Approval: Since our study was retrospective and we compared the tests previously used for Helicobacter pylori in our hospital, no ethics committee approval was applied.

Informed Consent: Informed consent is not obtained due to the retrospective nature of this study.

Peer-review: Externally peer-reviewed.

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