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EVALUATION OF DIAGNOSTIC METHODS FOR THE DETECTION OF *HELICOBACTER PYLORI* IN GASTRIC BIOPSY SPECIMENS OF DYSPEPTIC PATIENTS

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ABSTRACT

Helicobacter pylori infects nearly 50% of the world's population. This microorganism is accepted as the most important agent of gastritis and as a risk factor for peptic ulcer disease and gastric adenocarcinoma. Currently many diagnostic methods exist for detecting *H. pylori*, however they all have limitations, thus it is recommend a combination of at least two methods. The aim of this study was to evaluate diagnostic methods, such as *in-house* urease test, culture and Polymerase Chain Reaction (PCR), for the detection of the *H. pylori* in gastric biopsy specimens of 144 dyspeptic patients, using as gold standard the association between histology and rapid urease test. According to the gold standard used in this study, 48 (33.3%) patients were infected with *H. pylori*, while 96 (66.7%) were classified as not infected. The *in-house* urease test and the PCR were the most sensitive methods (100%), followed by culture (85.4%). However, the *in-house* urease test and the culture were the most specific (100%), followed by PCR (75%). In conclusion, this study showed that, in comparison with the combination of histology and rapid urease test, the *in-house* urease test and the PCR presented 100% of sensitivity in the diagnosis of gastric infection by *H. pylori,* while the *in-house* urease test and the culture reached 100% of specificity. These finding suggest that the combination of two or more methods may improve the accuracy of the *H. pylori* detection.

Key words: *Helicobacter pylori,* histology, culture, urease test, Polymerase Chain Reaction.

INTRODUCTION

Helicobacter pylori is a Gram-negative, spiral and microaerophilic bacterium that is present in the human stomach of approximately 50% of the world´s population (1, 8). This microorganism plays a significant role in the pathogenesis of chronic gastritis, peptic ulcer disease and gastric adenocarcinoma (1).

This association between *H. pylori* and gastroduodenal diseases demonstrates the need to diagnose the presence of bacteria in dyspeptic patients. Traditionally, diagnostic methods for detecting *H. pylori* may be classified as invasive, which require endoscopy to obtain biopsies of gastric tissues, and non-invasive. The invasive methods include histology, culture, urease test and molecular methods, while the non-invasive methods include urea breath testing, serology, stool

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antigen testing and molecular methods (12).

Although there are several available diagnostic methods for detecting infection with *H. pylori,* there is no single technique that can meet, on its own, the criteria for acceptable sensitivity and specificity in identification of the bacterium. Therefore, diagnostic methods are recommended in a combination of two or more to meet diagnostic criteria (14, 19, 25).

Histology is considered the standard method for the diagnosis of *H. pylori* infection, providing additional and essential information on the status of the mucosa such as the presence of acute or chronic inflammation, lymphoid aggregates, intestinal metaplasia and glandular atrophy. The reliability of this method depends essentially on the number and localization of specimens collected (12).

Culture permits testing of the sensitivity of *H. pylori* to antimicrobial agents used for its eradication and detection of factors and mechanisms related to pathogenicity of the bacterium (14, 20).

The urease test is simple and provides quick results (11); however, this method is affected by the use of antimicrobial agents, proton pump inhibitors (PPIs) and bismuth-containing compounds, which inhibit urease activity (7). Furthermore, the presence of other microorganisms that produce urease can lead to false-positive results (19).

Finally, molecular methods are widely used for the diagnosis of *H. pylori* infection as well as for analyses of diversity, virulence and resistance patterns of these bacteria (4). However, the high degree of genomic plasticity between strains of *H. pylori* complicates the choice of target genes. Even nucleotide sequences that are highly conserved in different strains of the pathogen, such as *urease* A (*ure*A), *urease* C (*ure*C) and 16S rRNA, may fail to detect the bacteria (19, 23).

Each of the methods above has advantages and disadvantages, and none can be considered as a single gold standard. A combination of endoscopic biopsy-based methods usually gives the most reliable diagnosis (9). Thus, this study aimed to evaluate diagnostic methods, including an *in-house* urease test, culture and Polymerase Chain Reaction (PCR). The results were compared to the gold standard (histology and rapid urease test) for *H. pylori* detection. This combination employed as a gold standard is the most frequently used in studies (10, 16, 19).

MATERIALS AND METHODS

Patients and Samples

A transversal study was performed with 144 dyspeptic patients submitted to upper gastrointestinal endoscopy between October 2008 and March 2009 in the Integrated Center for Gastroenterology at the Hospital of the Federal University of Rio Grande, Rio Grande do Sul, Brazil. The study excluded those patients who presented recent use (within 15 days) of antibiotics or non-steroidal anti-inflammatory drugs (NSAIDs), those treated for *H. pylori* infection and those with gastrointestinal bleeding in the last seven days. This study was approved by the Research Ethics Committee (FURG — process number 23116.003335/2008-43). Informed consent was obtained from all patients.

Through endoscopy, 11 biopsy specimens were obtained from each patient. Of these, five were destined for histology (two from the gastric antrum, two from the gastric body and one from the angular incisure). The other six biopsy specimens were intended for the rapid urease test (one specimen from the angular incisure), the *in-house* urease test (one specimen from the angular incisure), culture (two specimens from the gastric antrum) and PCR (two specimens from the gastric antrum) (6, 13, 15, 26).

Histology

The biopsy specimens from each patient that were destined for histological examination, were fixed in formalin and stained with Hematoxylin-Eosin (H&E) and Giemsa. The classification of gastritis was established in accordance with

the Sydney System (21).

Culture

After collection, biopsy specimens intended for culture and obtained from the gastric antrum were kept in 1 mL Brain Heart Infusion (BHI; Acumedia®, USA) Broth with 20% glycerol and refrigerated (4 to 8 °C) for a maximum of 4 hours (19).

Subsequently, this broth was vortexed vigorously for 2 min. Two hundred microliters were added to selective medium Columbia Agar (Oxoid, UK) and supplemented with 7% sheep blood (Newprov, BR) and with a selective mixture for isolating *Helicobacter sp.* (Cefar, BR) containing: 6 µg/mL vancomycin, 20 µg/mL nalidixic acid and 2 µg/mL amphotericin B. The medium was incubated at **37°C** in an anaerobic jar (JA 0400; Permution, BR) under microaerophilic conditions (5 to 15% O_2 and 10% CO_2), using an atmospheric generator (Microaerobac; Probac, BR), for a period of 4 to 10 days. Bacterial growth was monitored every 48 hours (7, 19).

When growth in the culture medium was observed, microscopy was performed. When straight or curved Gram-negative bacilli were observed, the following tests for identification of *H. pylori* were performed: catalase, oxidase, and urease. Identification was also carried out by PCR amplification of a *ure*A fragment (7, 15).

Rapid Urease Test

From each patient, a biopsy specimen from the angular incisure was incubated immediately after collection in Pre-Made Broth (TUPF; Laborclin, BR) for the urease test. This test detects the presence of *H. pylori* urease. The test was considered positive when the color of the solution changed from yellow/orange to pink/purple within 2 hours of incubation at 25 °C.

In-House **Urease Test**

From each patient, a biopsy specimen from the angular incisure was incubated immediately after collection in 1 mL of urea broth (Isofar, BR). This broth was prepared according to the manufacturer’s instructions and stored at 4 °C until the time of use. The test was considered positive when, after 24 hours of incubation at 25 °C, there was a change in the color of the broth from yellow/orange to pink/purple.

Extraction of DNA

The DNA was extracted from biopsy specimens from the gastric antrum using DNAzol® Reagent (Invitrogen™, USA) and 10 µg/µL of Proteinase K (Promega, USA). The biopsies were separated from the broth and resuspended in 100 µL Proteinase K and 500 µL DNAzol® Reagent. The mixture was incubated at 55 °C for 3 hours, and after this period, another 500 µL of DNAzol® Reagent were added. After centrifugation at 14,000× *g* for 10 min, the supernatant was collected, and 500 µL cold absolute ethanol was added followed by centrifugation at 12,000× *g* for 10 min, after which the supernatant was discarded. The DNA pellet was washed two times with 800 µL 75% ethanol, air dried and resuspended in 50 µL of 8 mM NaOH. The DNA was stored at – 20 °C until used.

DNA extraction from the culture was performed after 48 hours of bacterial growth. The colonies were collected and resuspended in 500 µL of 1× TE buffer (10 mM Tris–HCl, 1 mM EDTA — pH 8.0). The suspension was centrifuged at 10,000× *g* for 5 min, and the supernatant was discarded. The total DNA from the clinical isolates was then extracted according to the protocol for extraction by DNAzol® Reagent and preserved in 50 µL of 8 mM NaOH at – 20 °C.

PCR

In this study, *ure*A was used for the detection of *H. pylori.* The total DNA of the clinical isolates and the biopsy specimens was amplified with the primers UREA1 (5′ - GCCAATGGTA AATTAGTT – 3′) and UREA2 (5′ - CTCCTTAATTGTTTTT AC – 3′) (Invitrogen®, USA). These primers amplify a fragment of 394 bp of *ure*A (5). PCR was performed as described by Rota *et al.* (22).

Statistical analysis

A previous description of the sample was performed, calculating means and standard deviations for continuous data and proportions for categorical data. For validation purposes, the numbers of positive and negative results for each test were calculated with their respective proportions. Sensitivity (Se), specificity (Sp), positive predictive value (PPV), negative predictive value (NPV), and 95% confidence intervals (95% CI) were calculated for each of the testing methods, using as the gold standard a positive result in both histology and rapid urease test, as previously stated. Analyses were performed in Stata 9.2.

RESULTS

Determination of infection with *H. pylori*

Of the 144 patients undergoing upper gastrointestinal endoscopy, 99 were female, and 45 were male. Their ages were between 14 and 80 years (mean age 46.5, SD 15.5 years).

Based on the gold standard used in this study (correlation between the results of histology and rapid urease test), 48 (33.3%) patients were infected with *H. pylori,* while 96 (66.7%) were classified as not infected. It should be emphasized, however, that the gold standard chosen may have introduced errors in patient classification as infected or not infected. Among patients that were *H. pylori*-positive, 47,9% (23/48) had endoscopic diagnosis of erosive gastritis, and 52,1% (25/48) had enanthematous gastritis.

Comparative study among different diagnostic methods

According to the results of the methods of histology, rapid urease test, *in-house* urease test, PCR and culture, the presence of *H. pylori*, respectively, were identified in 75.7% (109/144), 33.3% (48/144), 33.3% (48/144) 50% (72/144) and 28.5% (41/144) of patients.

Considering the combination of histology and rapid urease test as a gold standard, the *in-house* urease test and PCR were the most sensitive methods (100%), followed by culture (85.4%). The *in-house* urease test and culture were the most specific (100%), followed by PCR (75%).

The PPV, NPV and diagnostic accuracy of each method are noted in Table 1.

Table 1. Validation of *in-house* urease test, culture and *ure*A PCR in comparison with the combination of histology and rapid urease test

Combination of Histology and Rapid Urease Test	Urease		Culture		***ure*A PCR**	
	Pos.	Neg.	Pos.	Neg.	Pos.	Neg.
Pos.	48	0	41	0	48	24
Neg.	0	96	7	96	0	72
	%	95% CI	%	95% CI	%	95% CI
Se.	100.00	98.96 - 100.00	85.42	74.39 - 96.44	100.00	98.96 - 100.00
Sp.	100.00	99.48 - 100.00	100.00	99.48 - 100.00	75.00	65.82 - 84.18
PPV	100.00	98.96 - 100.00	100.00	98.78 - 100.00	66.67	55.08 - 78.25
NPV	100.00	99.48 - 100.00	93.20	87.86 - 98.55	100.00	99.31 - 100.00

Se.: sensitivity; Sp.: specificity; PPV: positive predictive value; NPV: negative predictive value; 95% CI: 95% confidence intervals.

DISCUSSION

Currently many diagnostic methods exist for detecting *H. pylori* infection. Each method has its own merits and drawbacks in terms of indication, sensitivity, specificity and cost (18). Thus, it is recommended that a combination of at least two methods based on different principles be used to detect colonization by *H. pylori* (15). In this study, the results of the *in-house* urease test, culture and PCR were compared to the gold standard (histology and rapid urease test) for bacterium detection.

The gold standard used in this study is frequently used by

authors because the rapid urease test allows rapid detection of *H. pylori*, and histology enables identification of bacteria and evaluation of the type and intensity of inflammation of the gastric mucosa (10, 16, 24). However, it is known that in the rapid urease test, false-negative results may occur because of irregular distribution of bacteria in the gastric mucosa or the use of antimicrobials or PPIs. On the other hand, the contamination of biopsy with saliva can cause false-positive results because bacteria from the oral flora can produce urease (7, 8, 19). It was previously shown histologically that the presence of structures similar to *H. pylori* could cause false-positive results (19). Thus, the gold standard chosen may have not been the most appropriate even though it is the most commonly used by authors.

In this work, the choice of collection site of the biopsies in the stomach for histology, rapid urease test, *in-house* urease test, PCR and culture was based on previous studies (6, 13, 15, 26). However, the irregular distribution of the *H. pylori* in the gastric mucosa could influence on the results obtained.

The *in-house* urease test reached 100% sensitivity and specificity in the diagnosis of gastric infection by *H. pylori.* This method has been widely used because it is inexpensive and easy to perform (19); however, does not provide information on the intensity of inflammation (7).

The urease enzyme, produced by *H. pylori*, seems to be necessary for the survival of this microorganism in the acidic gastric environment, suggesting a strong selective pressure to maintain the amino acid sequence of this enzyme, resulting in the observed conservations of the DNA sequence among strains (5). The *ure*A is a species-specific gene present in all samples of *H. pylori* (3).

In this study, PCR was performed by *ure*A detection. This method presented sensitivity of 100% and specificity of 75%. A result similar to this was reported by Smith *et al.* (24). In contrast, Lu *et al.* (17) compared PCR methods for the detection of *H. pylori* in gastric biopsy specimens and observed that the amplification of *ure*A, although highly specific, was less sensitive than for *ure*C, a 26-kDa species-specific antigen gene, and 16S rRNA gene detection.

The false-positive results found by PCR may have occurred because of sample contamination by PCR products or inadequate disinfection of the endoscopes (13). The advantage of the PCR is that it allows detection of specific genes relevant to pathogenesis and specific mutations associated with antimicrobial resistance in addition to detection of the microorganism (19).

The culture showed sensitivity and specificity of 85.4% and 100%, respectively. These values are similar to those found by other authors, who reported high specificity and significant fluctuation in sensitivity. The false-negative results of this method may have occurred due to the absence or low density of bacterium in the biopsy specimens, use of antimicrobials and PPIs, inappropriate conditions of transport or loss of viability of the microorganism due oxygen exposure (12, 13, 19).

However, the main advantage of culture is that, in addition to detecting *H. pylori*, it allows the testing of antibiotic sensitivity, which can be very useful in some patients who are not responding to treatment (2).

In conclusion, this study showed that, in comparison with the combination of histology and rapid urease test, the *in-house* urease test and the PCR presented 100% of sensitivity in the diagnosis of gastric infection by *H. pylori,* while the *in-house* urease test and the culture reached 100% of specificity. These finding suggest that the combination of two or more methods may improve the accuracy of the *H. pylori* detection.

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