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ORIGINAL ARTICLE

Detection of *Helicobacter pylori* by Phenotypic and Genotypic Methods

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Abstract
Aims This research evaluated the utilization of a urease in-house test, culture and molecular method (*ure*A PCR) as a diagnostic tool for *Helicobacter pylori* infection. Furthermore, we assessed the presence of the *cag*A gene in the specimens and in isolated strains that were positive for *ure*A by PCR positive.
Results Sensitivity and specificity, respectively, were 100 and 95.8% for the urease in-house test 93.3 and 95.8 for the *ure*A PCR assay of the specimen and 100 and 100% for the culture. The presence of the *cag*A gene was observed in eight (53%) *ure*A-positive samples.
Conclusions In this study, we found that the PCR technique has applicability in the study of *cag*A, and other genes related to the *H. pylori* pathogen. This method can be applied to samples directly from biopsy or isolated from the bacteria.

Keywords *H. pylori* · Diagnostic · *cag*A · *ure*A

Introduction

The identification of *Helicobacter pylori* in 1982 was the start of a revolution regarding the conceptualization and management of gastroduodenal diseases, since this microorganism is related to the pathogenesis of chronic gastritis, peptic ulcer, lymphoma of the gastric mucosa-associated lymphoid tissue (MALT), and gastric cancer [1, 2].

H. pylori is a Gram negative bacteria that colonizes the gastric mucosa and the microvilli of the epithelial cells and has been implicated in the destruction of gastric cells, resulting in vacuolization and production of cytotoxins and toxic enzymes, especially lipase, protease, and urease. These are probably deregulated factors of the epithelial defense mechanism [3].

This bacterium infects around half the world's population, mainly in developing countries [4]. However, the majority of infected individuals have no clinical signs of disease. This contradiction suggests the involvement of factors such as genetic predisposition and socioeconomic conditions of the host, as well as the genomic variability of *H. pylori* [5, 6].

The association between *H. pylori* and gastroduodenal diseases demonstrates the need to find diagnostic methods that detect the presence of bacteria in dyspeptic patients [2, 7, 8]. Several techniques with different sensitivities and specificities are available for identification of *H. pylori* infection. These methods can be classified as invasive, including use of endoscopy for the collection of gastric biopsies, and non-invasive, such as identification of antigens, antibodies, specific genomic *loci* and enzyme activities [5, 9]. To date, there is no diagnostic method that can meet, on its own, the criteria for acceptable sensitivity and specificity in the detection of *H. pylori* infection. Instead, diagnostic methods are recommended in combination of two or more in order to meet diagnostic criteria [7, 9, 10].

Histological analysis is one of the most widely used diagnostic methods, allowing for detection of the presence of the bacteria as well as assessment of the type and intensity of inflammation in the gastric mucosa [7, 9, 11].

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The primary limitation of histology is the subjectivity of assessment and the associated interobserver variation [11]. Other limitations are related to variations of the bacterial density and the location in the stomach [9, 12, 13].

Urease activity detection can be simply executed, has a low cost, and provides quick results [7]. However, this test does not supply information on the intensity of inflammation and is affected by the use of antimicrobials and proton pump inhibitors (PPI), which inhibit urease activity [9, 12]. Furthermore, the presence of other microorganisms that produces urease can lead to false-positive results [9, 12].

Culture of *H. pylori* allows the detection of factors and mechanisms related to pathogenicity, molecular epidemiology, and drug susceptibility testing (DST) [9, 11, 14]. However, growth in culture can be affected by factors such as concentration of bacteria in the specimen obtained from biopsy, transport conditions, use of antimicrobials, and PPI [9, 11, 15–17].

Molecular methods allow rapid detection of *H. pylori*, as well as the determination of their genotype [13, 18]. 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 *ure*A and 16S rRNA, may fail to detect the bacteria [2, 9].

These methods present sensitivity and specificity variables and are unable to identify the potential of pathogenicity of the microorganism. In recent years, various pathogenic markers related to clinical manifestations have been described [19].

One of the first markers of pathogenicity of *H. pylori* to be studied was the surface antigen *cag*A, encoded by the *cag*A gene (cytotoxin-associated gene). *Cag*A has been used as an indicator of the pathogenicity A island (PAI) [8, 20]. Many of the activities performed by the proteins produced by *cag*-PAI are still unknown. However, it has been shown that their presence is associated with increased secretion of interleukin 8 (IL-8) by epithelial cells, which is important for chemotaxis and activation of neutrophils in the inflammatory process [5, 14]. Although *cag*A is not a tool for *H. pylori* infection determination, it is important to determine prognostic and relationship infection and disease.

H. pylori detection still represents an arduous task, due to difficulties in accessing its ecological niche and the fastidious nature of the bacteria [9]. This study aimed to evaluate any diagnostic methods, including an in-house urease assay, bacterial culture, and PCR. Results were compared to the gold standard (histological analysis and rapid urease test) for *H. pylori* detection. Several studies recommend using two or more diagnostic methods to determine *H. pylori* infection. The combination of histological examination and rapid urease test employed in this study as a gold standard is the most frequently used [8, 9, 13]. Finally, we also determined the presence of *cag*A in *ure*A positive samples.

Methods

Patients and Samples

A transversal study was carried out between January and November 2007, with 39 patients with dyspeptic symptoms and submitted for endoscopy in the Integrated Center for Gastroenterology at the Hospital of the Federal University of Rio Grande, Rio Grande do Sul, Brazil. Patients that had recently (within 15 days) used antibiotics or non-steroidal anti-inflammatory drugs (NSAIDs) or had been treated for *H. pylori* or gastrointestinal bleeding in the last 7 days were excluded from the study.

This study was approved by the Research Ethics Committee of the Area Health (FURG—process number 23116.3631/7.29). Informed consent was obtained from all patients.

During digestive endoscopy upper (DEU), two fragments of the antrum, two of the incisures angularis, and two of the corpus were collected by biopsy and used for pathological examination. Six biopsies of the gastric antrum were performed to assess the presence of *H. pylori* using the rapid urease test (one specimen), the urease test in-house (one specimen), culture (two specimens), and PCR (two specimens).

After each endoscopy, the equipment was subjected to cleaning and disinfection procedures established by the Brazilian Society of Digestive Disease (SOBED) [21].

Histological Examination

The biopsies for histological examination were fixed in formalin and stained with H&E and Giemsa. The characterization of gastritis was established in accordance with the System Sydney [22].

Rapid Urease Test

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

In-House Urease Test

A second biopsy, also of the angular incisure, was incubated in 1 ml of urea broth (Isofar, BR), prepared according manufacturer's instructions and stored at 4°C until the time of use. The test was considered positive when, after 24 h of incubation at 25°C, there was a change in color of the broth, from yellow/orange to pink/purple.

Culture

Specimens from the gastric antrum to be used for culture were kept in 1 ml thioglycolate broth (Laborclin, BR) and refrigerated (4°C) for a maximum of 5 h [9, 23]. After, this broth had been shaken vigorously for 2 min, 50 µl was deposited on a slide, and slides were fixed and stained by the method of Gram [3]. Two hundred microliters were added to selective Columbia agar (Oxoid, UK), supplemented with 7% sheep's blood and mixed for selective isolation of *Helicobacter* sp. (CEFAR, BR). Cultures contained 6 mg/l of vancomycin, 20 mg/l nalidixic acid and 2 mg/l of amphotericin B. This medium has been designated in this study as modified Columbia agar (MCA).

The medium was incubated at 37°C in an anaerobic jar (JA 0400; Permution, BR) under microaerophilic conditions (5–15% O_2 and 10% CO_2), using an atmospheric generator (Microaerobac; Probac, BR) for a period of 4–8 days. Bacterial growth was monitored every 48 h [9, 24].

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 were performed: catalase, oxidase, and urease activity [8, 9, 14]. The identification of *H. pylori* was also carried out by PCR amplification of a *ure*A fragment. Pure cultures were preserved in brain heart infusion (BHI; Acumedia, USA) broth with 20% glycerol at −70°C [12, 24].

Extraction of Genomic DNA

DNA was extracted from biopsies of the gastric antrum using DNAzol® Reagent (Invitrogen™ Life Technologies, CA) and 10 mg/l of Proteinase K (Invitrogen™). The specimens were separated from the broth and resuspended in 100 µl of Proteinase K and 500 µl of DNAzol. The mixture was incubated at 55°C for 3 h and, after this period, another 500 µl of DNAzol® 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.

DNA extraction from the culture was performed after 48 h of bacterial growth in MCA, 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 discarded. The genomic DNA from the clinical isolates was then extracted according to the protocol for extraction by DNAzol.

PCR

Both samples taken from biopsy specimens and cultures were used as templates in the PCR reaction using the primers UREA1 (5′-CTCCTTAATTGTTTTTAC) and UREA2 (5′-GCCAATGGTAAATTAGTT). These primers amplify a fragment of 394 base-pairs (bp) of *ure*A [18]. The reaction was performed in a final volume of 50 µl, containing 20 ng, 20 nM of 10× buffer (500 mM KCl, 200 mM Tris–HCl—pH 8.3), 1.5 mM $MgCl_2$, 25 pmol of each primer, 200 mM of dNTPs (Invitrogen™), 2.5 units of Taq DNA polymerase enzyme (Invitrogen™) and H_2O Milli-Q. The PCR reactions were pre-incubated in a thermocycler (MJ Research, MA, USA) for 5 min at 94°C, followed by 35 cycles of 1 min at 94°C, 1 min at 45 and 72°C for 1 min, followed by a final extension of 7 min at 72°C [18, 20].

*Cag*A Detection

The presence of the *cag*A in samples identified as *H. pylori*-positive was investigated by an amplification of the 3′ conserved region this gene. The primers used were previously described [25], *cag*A/conF (5′-GTGCCTGCTAGTTTGTCAGCG) and *cag*A/conR (5′TTGGAAACCACCTTTTGTATTAGC) and amplify a fragment of 402 bp.

The PCR reaction was performed in a final volume of 50 µl, containing 20 ng of DNA, 20 nM of 10× buffer (500 mM KCl, 200 mM Tris–HCl—pH 8.3), 2.5 mM $MgCl_2$, 25 pmol of each primer, 200 mM of dNTPs (Invitrogen™), 2.5 units of Taq DNA polymerase enzyme (Invitrogen™) and H_2O Milli-Q. The conditions were: pre-incubation of 3 min at 94°C, followed by 35 cycles of 30 s at 94°C, 30 s at 50°C, and 30 s at 72°C, followed by a final extension of 7 min at 72°C [20, 25].

Statistical Analysis

For statistical analyses, 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 number 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 (IC95%) were calculated for each of the testing methods, using as gold standard a positive result in both histological examination and rapid urease test, as previously stated. Analysis were performed in Stata 9.0 [26].

Results

Determination of Infection with *H. pylori* and Relationship to Endoscopic Diagnosis

Thirty patients were female and nine male, aged between 16 and 74 years (average 47.9, median 51, SD 14.7 years).

According to the gold standard used in this study (correlation between the results of histological analysis and rapid urease test), 15 (38%) patients were infected with *H. pylori*, while 24 (62%) were classified as not infected. Among patients that were *H. pylori*-positive, 67% (10/15) had erosive gastritis or duodenal ulcers and 33% (5/15) had gastritis enanthematic. The association between endoscopic results and the diagnostic criteria chosen was not statistically significant ($P = 0.440$).

Comparative Study Among Different Diagnostic Methods

Considering the combination of histological analysis and rapid urease test as a gold standard, the culture and in-house urease test were the most sensitive (100%), followed by PCR (93.3%). One test was positive for the in-house urease test and amplification of *ure*A but the histological examination was negative. Culture was the most specific method (100%), followed by PCR and in-house urease test (95.8%). The PPV, NPV and accuracy of each method evaluated can be seen in Tables 1, 2 and 3.

Table 1 Validation of in house urease

Urease	Gold standard	
	Pos	Neg
Pos	15	1
Neg	0	23
	%	95% CI
Se	100	78.2–100
Sp	95.8	78.9–99.9
PPV	93.8	69.8–99.4
NPV	100	85.2–100

Table 2 Validation of *ure*A PCR

*ure*A PCR	Gold standard	
	Pos	Neg
Pos	14	1
Neg	1	23
	%	95% CI
Se	93.3	68.0–99.8
Sp	95.8	78.9–99.9
PPV	93.3	68.0–99.8
NPV	95.8	78.9–99.9

Table 3 Validation of cultura

Cultura	Gold standard	
	Pos	Neg
Pos	9	0
Neg	0	13
	%	95% CI
Se	100	66.4–100
Sp	100	75.3–100
PPV	100	66.4–100
NPV	100	75.3–100

*Cag*A Detection

Of the 15 biopsy specimens in which *urea* was detected, 8 (53%) were *cag*A positive. Of these, 6 (75%) were patients with an endoscopic diagnosis of erosive gastritis and 2 (25%) were individuals with enanthematic gastritis.

Discussion

H. pylori infection is related to the pathogenesis of chronic gastritis, peptic ulcer, cancer, and gastric MALT (mucosal-associated lymphoid tissue) [2, 5, 8]. Factors related to the host and the environment are considered important to disease determination; however, specific genotypes of *H. pylori* have also been identified as associated with increased pathogenicity [4, 6].

Currently, the correlation of results between two or more diagnostic methods has been used as diagnosis criterion of infection. Histological examination and the urease test are commonly used as a gold standard for diagnosis. However, this approach can lead to errors in the classification of patients since the combination of a few methods as a diagnostic criterion tends to increase false-positive results [7].

The urease test has the main advantage of practical implementation and low cost, and it can be used in all patients undergoing endoscopy [14]. The detection of *ure*A, in addition to its high sensitivity, can be used as a non-invasive method in samples of stool and saliva. It can also be used to monitor gastroduodenal diseases since treatment reduces the number of bacteria in the gastric mucosa, often making them undetectable by other methods [2, 8].

With few exceptions, the culture method is most prominently used as a medical microbiology diagnostic. Although *H. pylori* is a fastidious organism and its cultivation is laborious, the microorganism isolation is important because it is the most specific diagnostic method and allows one to perform the DST. This is increasingly important, since the bacteria are increasingly resistant to standard treatments, leading to an increase in rates of treatment failure [8, 11, 27].

However, the culture sensitivity is variable, possibly because the viability of the microorganism is reduced considerably upon exposure to adverse conditions, such as exposure to oxygen. Concentration of bacteria in the biopsy specimen, presence of coccoid forms, viability, and cultivation ability and failure of the patient to report use of drugs such as PPIs and antibiotics can also affect the sensitivity of this test [4, 9, 15, 17].

Genotypic methods, such as PCR [10], along with the ability to perform molecular epidemiological studies and to identify not only the presence of the microorganism but also markers of pathogenicity and drug resistance, will extend its utilization [7–9, 13].

The enzyme urease, produced by *H. pylori*, seems to be necessary for the survival of the organism in the acidic environment of the stomach, suggesting a strong selective pressure to maintain the *ure*A *locus* [4]. However, Lu et al. [28], compared five methods of PCR-based detection of *H. pylori* in gastric biopsies and observed that the amplification of the *ure*A gene, although highly specific, was less sensitive than three other gene targets. Smith et al. [29], in contrast, reported low specificity and high sensitivity for *ure*A detection. In this study, *ure*A amplification showed 93% sensitivity, and failed to detect the bacteria in only 1 of the 15 *H. pylori*-positive biopsies. This difference may be explained by a possible absence of the pathogen in the sample, due to an irregular distribution of the bacteria among the gastric mucosa. Alternatively, the presence of inhibitors of PCR or gene plasticity may account for the failure to detect the b [7, 13, 28].

The frequency of *cag*A was 53% among the 15 *ure*A positive samples. Although the *cag*A genotype is associated with more severe gastric lesions, the *cag*A gene was not identified in samples from two patients in this study with peptic ulcers. One patient with gastric ulcers also did not show the *ure*A. In another patient with a duodenal ulcer, the *ure*A was detected but with genotype *cag*A-negative. The presence of peptic ulcer may occasionally be explained by other factors besides bacteria, including the use of NSAIDs, variation of the *cag*-PAI *locus*, or other forms of pathogenicity [6, 20, 21, 30].

Patients without ulcers but with the *cag*A genotype could be at higher risk to develop ulcers, or the plasticity of the region 3′ of *cag*A could determine differences in pathogenicity [6, 21, 30]. It is also speculated that these patients may not have developed the disease because they were younger than the patients with ulcers were, suggesting that age is an important prognostic factor [6, 30].

Considering the demonstrated plasticity of the genome of *H. pylori*, genomic polymorphism may interfere with both the activity of pathogenic factors and the diagnosis itself [6]. Therefore, the characterization of the strains prevalent in a given population is essential in order to establish the sequences of more appropriate primers, minimizing the possibility of false negative results by the technique of PCR [6, 20, 21].

The diagnosis and prognosis of dyspeptic patients are challenges in the field of gastroenterology and medical microbiology. The use together of various methods based on different principles can work synergistically to raise the sensitivity and specificity of diagnostic. However, identification of the presence of the organism does not necessarily confirm its involvement in the pathogenesis. The molecular characterization of strains of *H. pylori*, through the identification of genes involved in pathogenicity, such as *cag*A, *ice*A, and *vac*A, could allow a better assessment of the potential pathogenesis of the strains identified.

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