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Molecular technique for detection and identification of *Helicobacter pylori* in clinical specimens: a comparison with the classical diagnostic method

Técnica molecular para detecção e identificação de Helicobacter pylori *em espécimes clínicas: comparação com o método clássico de diagnóstico*

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ABSTRACT

Introduction: *Helicobacter pylori* is a bacterium found in human epithelial cells of the gastrointestinal tract. Its infection is related to different diseases, such as chronic gastritis, peptic ulcers, gastric lymphoma and adenocarcinoma. The infection by *H. pylori* is present in more than a half of the world population. **Objectives:** To detect *H. pylori* and to compare the diagnostic methods of the rapid urease test (RUT) and polymerase chain reaction (PCR). **Materials and methods:** The study was conducted between April and July, 2015. For such, three biopsies were collected from each patient. Two were used for PCR and one for RUT. **Results:** A total of 85 samples were collected from patients undergoing endoscopy, with 56 (65.88%) females and 29 (34.11%) males. From the total samples subjected to RUT, 15 (17.64%) were positive and 70 (82.35%), negative. In PCR for detection of gene 16S ribosomal ribonucleic acid (rRNA) of *H. pylori*, 66 (77.64%) presented positive results and 19 (22.35%), negative results. For the analysis of the presence of *UreA* gene in all samples, positive results were found in 70 (82.35%), and negative in 15 (17.64%). According to the results, RUT and the molecular test presented statistical difference. **Conclusion:** PCR is a useful method in the laboratorial routine to detect the presence of *H. pylori* in the stomach tissue, due to high sensitivity and specificity, but it requires a more careful analysis and standardization.

Key words: bacteria; polymerase chain reaction; urease.

INTRODUCTION

Helicobacter pylori is a gram-negative microaerophilic bacterium, with spiral morphology. It is found deep in epithelial cells that secrete mucus in the human stomach, where neutral pH is prevalent. This bacterium is reported as a major cause of various gastrointestinal diseases, chronic gastritis, peptic ulcer, gastric cancer and gastric mucosa-associated lymphoid tissue (MALT) lymphoma, which is classified by the World Health Organization (WHO) as type I carcinogen[1-3].

Its distribution is universal and affects more than 90% of the world population, but it is more common in developing countries, probably due to the possible fecal-oral route of transmission and the precarious sanitation conditions in these countries[4]. Although *H. pylori* infection is associated with gastroduodenal lesions, not all infected individuals present clinically significant alterations[5]. Several studies are performed in Brazil and all over the world for a better comprehension of the disease pathogenesis, to analyze epidemiological and diagnostic aspects in order to reduce the number of *H. pylori* infections, and to improve patients' prognosis.

The bacteria may be cultured in a non-selective blood medium, producing small, gray and translucent colonies. The ideal temperature for their isolation is 35ºC-37ºC during three to five days, but this method has low sensitivity mainly due to the use of antibiotics and proton-pump inhibitor drugs[6]. The smears may reveal short bacilli, but for a more precise identification catalase, oxidase and urease tests must prove positive[1, 7].

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The ideal diagnostic method for detection of *H. pylori* does not exist at this moment, although there are various methodologies presenting advantages and limitations. Thus, clinical indication, costs and the available resources should be considered when choosing type and number of specimens, and also the method to be used. Undoubtedly, patients with gastric disorders require a reliable diagnosis and a rigorous treatment to prevent an increase in bacterial resistance[8].

The rapid urease test (RUT) is an indirect test for the presence of *H. pylori* based on the presence of urease in or on the gastric mucosa. In the presence of *H. pylori* urease, urea is hydrolyzed to produce ammonia and bicarbonate, leading to a pH increase in the gastric mucosa, which is indicated by a change in the color of phenol red from yellow to pink or red. A positive RUT requires approximately 10^5 *H. pylori* in the biopsy sample to change color. The time the test turns positive depends on the concentration of bacteria and the temperature. Most will turn positive within 120 to 180 minutes, but it is best to hold those that appear negative for 24 hours. After 24 hours, the test may turn positive from the presence non-*H. pylori* urease-containing organisms. Positive results after 24 hours are most often false positive and should not be used for treatment decisions[9, 10].

Molecular methods are used with expansion in the diagnosis of infections caused by *H. pylori* along with virulence and drug resistance analysis, due to the high sensitivity and specificity[11]. The present study aimed to evaluate and to improve the use of polymerase chain reaction (PCR) within *H. pylori* detection and to compare it with the RUT, in addition to analyze the infection prevalence in this region, since no study has been carried out in this context in southwest Goiás.

MATERIALS AND METHODS

Ethics

The project was accepted by the Ethics Committee on 12/15/2004, Res CNS 196/96, with registration CEP: 038/04. Specimens were included from patients who underwent endoscopic examination and consented to participate in the study by signing the term of consent. Patients with gastric bleeding were excluded, as well as those who refused to sign the term of consent.

Specimens

Gastric tissue samples were collected from 85 patients who were subjected to endoscopic examination. The examination was conducted in an endoscopy clinic in Rio Verde (GO), Brazil, between April and May, 2015. One biopsy was collected for RUT, which was performed by the doctor at the examination site; two other biopsies, one of the gastric antrum and another of the gastric body, were obtained for the molecular diagnosis. These two fragments were jointly stored in 1.5 ml dry, sterile and identified plastic tubes. Specimens were stored in liquid nitrogen and transported to the Molecular Diagnostic Laboratory of Universidade Federal de Goiás (UFG), Regional Jataí, where they remained frozen until molecular extraction.

Deoxyribonucleic acid (DNA) extraction

DNA extraction was performed at the Molecular Diagnostic Laboratory of UFG, Regional Jataí, according to KitQIamp DNA minikit® (Qiagen, Valencia, CA, United States) protocol. A 10-µl aliquot from each sample was used for DNA quantification and purity analysis with NanoDrop® (ND-1000 UV-Vis) at the Genetics Laboratory of UFG, Regional Jataí.

PCR conditions

Genomic DNA was amplified by the PCR method with conditions and oligonucleotides sequence already described by Luscenti and Gatti (2008)[12], which were synthetized by Exxtend® Company (Campinas, SP, Brazil). The amplification was held in thermal cycler Amplitherm® TX96 (Thermal Cyclers), and each reaction consisted in: 0.5 µl Taq DNA polymerase (2.5 units), 5 µl PCR buffer CoralLoad 10× (QIAamp, Qiagen) containing MgCl2 (1.5 mM), 2 µl (2.5 mM) of dNTP (desoxyribonucleotides 5'-triphosphate – dATP, dCTP, dGTP, dTTP), 4 µl of each oligonucleotide pair (10 pmol each), 33.5 µl of ultrapure milli-Q water and 5 µl (50 ng) of genomic DNA, totalizing 50 µl per reaction. For each assay a negative and a positive control were used, with an *H. pylori* DNA aliquot kindly given by researcher Dr. Lucas Trevizani Rasmussen, from Universidade do Sagrado Coração (Bauru, SP, Brazil).

Visualization of amplicons

All PCR products were analyzed through a 1.6% agarose gel stained with ethidium bromide (10 mg/ml) in a horizontal tank. Upon electrophoresis, each gel was visualized under ultraviolet light and the image was captured with a camera.

The samples were considered positive by analysis of the molecular weight marker according to the positive control for each oligonucleotide. Fragment sizes of 150, 296 and 411 base

pairs were considered positive results of PCR primers HpX/HpX1 [16S ribosomal ribonucleic acid (rRNA) gene], H3/H4 (antigenic protein of 26 kDa specie specific) and H5/H6 (*ureA* gene) respectively.

Result analysis

The results from amplifications were analyzed using the Chi-square statistic test with $p < 0.05$, through GraphPad Prism 5.00 (Trial, 2007), to identify the possible variables found in the study and to compare results obtained by the PCR method and by RUT.

RESULTS

Gastric biopsies from 85 patients were analyzed, 56 (65.88%) women and 29 (34.11%) men. Participants' ages varied from 15 to 89 years, with an average of 40.77 years, and 27 of the total (31.76%) being 50 years or older. The endoscopic diagnoses provided in the medical reports were: 20 (23.52%) patients with normal examination, 29 (34.11%) patients with slight/moderate esophagitis, 30 (35.29%) patients with slight/moderate gastritis, three (3.52%) patients with ulcers and three (3.52%) with other diseases (**Table 1**).

From the total of 85 samples, RUT had 15 (17.64%) positive and 70 (83.35%) negative results. Among the samples analyzed by the PCR method for *H. pylori* gene 16S rRNA (HpX/HpX1), 66 (77.64%) were positive, amplifying 150-bp fragments, and 19 (22.35%) were negative (**Figure 1**).

In the amplification reactions for *UreA* gene (H5/H6), 70 (82.35%) samples were positive, with 411-bp fragments, and the other 15 (17.64%) were negative (**Figure 2**).

Table 2 shows the results from both diagnostic tests applied in the study.

UreA gene (H5/H6) is an important virulence factor and ensures that bacteria have the capacity to resist in the acid environment of the gastric region. Initially, the result of *UreA* gene (H5/H6) amplification was compared with results from RUT (**Figure 3**). The calculated results were statistically significant when $p < 0.0001$.

The 16S rRNA (HpX/HpX1) gene is a conserved region of prokaryotic DNA and allows specific identification. When comparing RUT results with amplification of 16S rRNA gene, the results were statistically significant as $p < 0.0001$ (**Figure 4**).

TABLE 1 – Distribution of gender, age and endoscopic diagnosis of *Helicobacter pylori*, Rio Verde (GO), 2015

Variables	*n*	%
Gender (*n* = 85)		
Women	56	65.88
Men	29	34.11
Age (*n* = 85)		
> 50 years	27	31.76
< 50 years	58	68.24
Endoscopic diagnosis (*n* = 85)		
Normal	20	23.52
Slight/moderate esophagitis	29	34.11
Slight/moderate gastritis	30	35.29
Ulcers	3	3.52
Others	3	3.52

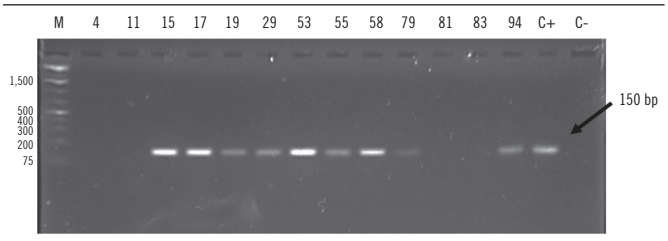

FIGURE 1 – *Photograph of 1.6% agarose gel stained with ethidium bromide as a result of gene amplification of the 16S rRNA (HpX/HpX1) with the expected size of 150 bp*

rRNA: ribosomal ribonucleic acid; M: deoxyribonucleic acid (DNA) marker; C+: positive control; C-: negative control.

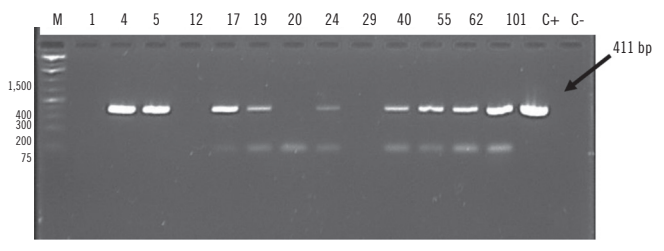

FIGURE 2 – *Photograph of 1.6% agarose gel stained with ethidium bromide as a result of amplification of the* UreA *gene (H5/H6) with the expected size of 411 bp*

M: deoxyribonucleic acid (DNA) marker; C+: positive control; C-: negative control.

TABLE 2 – Results from RUT and molecular diagnosis (PCR) for genes *UreA* (H5/H6) and 16S rRNA (HpX/HpX1), Rio Verde (GO), 2015

Results	RUT	PCR	
		H5/H6	HpX/HpX1
Positive	15	70	66
Negative	70	15	19
Total	85	85	85

RUT: rapid urease test; PCR: polymerase chain reaction; rRNA: ribosomal ribonucleic acid.

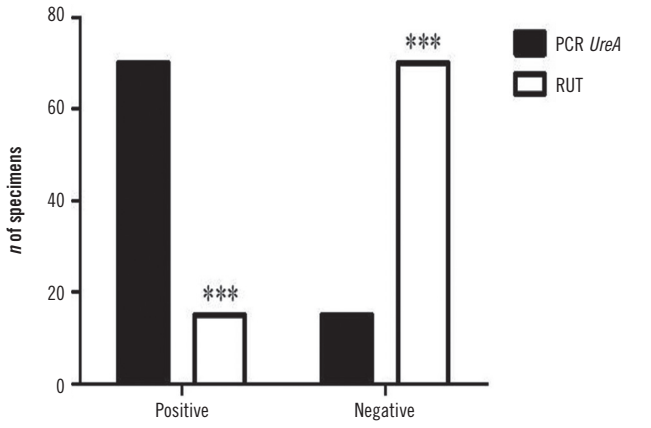

FIGURE 3 – *Comparison between the number of samples and the results obtained by the molecular diagnostic method using the* UreA *gene and the rapid urease test. There was a significant difference between the groups (*$p < 0.0001$*)*

PCR: polymerase chain reaction; RUT: rapid urease test.

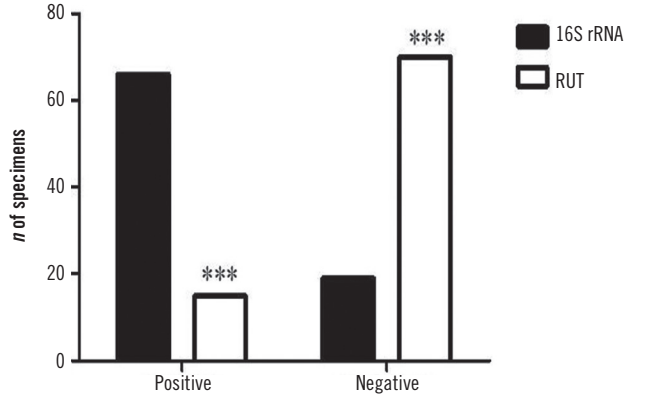

FIGURE 4 – *Comparison between the number of samples and the results obtained by the molecular diagnostic method using the 16S rRNA gene (HpX/HpX1) and the RUT. There was a significant difference between the groups (*$p < 0.0001$*)*

rRNA: ribosomal ribonucleic acid; RUT: rapid urease test.

A comparison between the results from molecular diagnosis using both genes did not show a statistically significant difference ($p < 0.5656$) ("Data not shown").

DISCUSSION

In order to diagnose *H. pylori*, several methods are ready for use, and the best choice must consider aspects as sensitivity, specificity, clinical condition, availability and cost. That is the reason why several studies compare and correlate the detection of *H. pylori* through different methods, invasive or non-invasive[6].

In the current study, we drew a comparison of the molecular method, through the target of the 16S rRNA and *UreA* genes, with the RUT. Both are invasive tests and require biopsy. Their sensitivity depends on some factors: the number of biopsies performed, the density of bacteria present in each biopsy, the presence of *H. pylori* in endoscopic material and the presence of microorganisms besides *H. pylori*[5, 13].

The advantages of PCR are uncounted, such as a possibility of genotyping of samples to identify different strains of the species; the use of retrospective materials in research, which is of great relevance to avoid the need to repeat the invasive procedure to collect another sample; the detection in samples that contain small DNA quantity, in addition to allowing amplification of resistance genes to antibiotics without the need to perform a conventional antibiotic susceptibility testing[14].

The disadvantages are the high cost of the technique and the possibility of sample contamination, but they can be avoided by the use of biosafety standards during all phases of the process. It should also be considered that after antibacterial therapy, the number of bacteria decreases and the microbiological and histological tests can produce false-negative results. However, the PCR is a sensitive method that allows amplification of small amounts of bacterial DNA in various types of biological samples[15].

The prevalence of *H. pylori* in this study based on a molecular method is in accordance with studies by Redéen *et al*. (2011)[16], which evaluated 304 individuals and found that one-third had infection. Studies by Rasmussen *et al*. (2010)[17] also corroborate this current result: it was possible to observe that most of the samples were diagnosed by PCR for detection of *H. pylori* in the gastric tissue.

In this study, 85 samples were subjected to the RUT, only 15 (17.64%) were positive and 70 (82.35%) were negative. The high probability of false-negative results in the RUT is due to the likely reduction in urease activity, which may be caused by the recent use of antibiotics, bismuth compounds or proton-pump inhibitors; noting that individuals who were using these drugs were not excluded from this study. In addition to these factors, the sensitivity of RUT can be affected by the amount and viability of the bacterium present in the biopsy; it is further proposed that specificity decreases with increasing time of incubation, concentrations of the test components and the total concentration of urease production[18]. These false-negative results of RUT can also be observed when *H. pylori* is present in coccoid form, and urease activity is decreased[13, 19].

False-negative results, which were predominant in this study, can be a major problem when *H. pylori* is not successfully detected in patients with diseases associated with the presence of the bacterium; endoscopic biopsies and other procedures are required,

what can be costly for doctors and patients. However, molecular tests using biopsies already used in the RUT method may reduce the need of endoscopy for obtaining biopsy. In particular, its use is more useful when the RUT is negative and there is still suspicion of *H. pylori* infection[11].

False-positive results observed in the RUT may be due to the presence of other microorganisms that have urease activity as *Proteus*, *Yersinia*, *Klebsiella* and *Pseudomonas*. Conversely, Garza-González *et al*. (2014)[19] argue that the oropharynx microbiota, which also produce urease, can be swallowed with saliva, but the enzyme is denatured due to the high acidity of the stomach, not affecting the outcome of RUT when gastric biopsies are used. Rapid tests of commercial urease show specificity between 95%-100%, but their sensitivity is somewhat lower, 85%-95%[19].

Analysis of the results obtained in this study showed a statistically significant difference between the molecular detection and RUT, and the presence of *H. pylori* was lower in RUT than in PCR. Other studies also show a lower detection of *H. pylori* by the RUT method compared to other methods of classic diagnosis, although some showed no statistically significant difference[6, 8, 11, 19-24].

In a study comparing various *H. pylori* detection methods used in 78 patients, 46 (59%) were positive by PCR. Analyzing these samples added to the technique of Southern blotting, this rate rose to 66 (84.6%); histological examination detected the presence of bacteria in 21 (27%) patients; and RUT, in 30 (38.5%). Their results showed that the prevalence of the organism in adults exceeded 80%, which is in agreement with other authors and the results of this study, in which most patients infected by the bacteria were adults[17].

The presence of *H. pylori* was assessed in samples of dental plaque, saliva and stomach through the RUT, histology [hematoxylin and eosin (HE)] and PCR followed by Southern blot hybridization. Results were positive in 50/62 (80.6%) by PCR, 19/62 (30.6%) by the histological method and 27/62 (43.5%) by RUT. This result was expected, since the PCR with Southern blotting is more sensitive when compared with histopathology and RUT[25].

PCR can be used to identify strains of bacteria and correlate them to different gastric diseases, in addition to being applied in epidemiological studies. A considerable disadvantage of PCR is that it can detect and amplify DNA from dead bacteria present in the gastric mucosa of patients after treatment with antibiotics, and consequently, generate false-positive results. Due to the increasing prevalence of antibiotic resistance in populations with high prevalence of *H. pylori*, molecular tests may be relevant alternatives for diagnosis[19].

This study enabled analysis of the prevalence of *H. pylori* infection in this region of Goiás, and proved that the RUT adopted in the diagnosis of *H. pylori* can generate many false-negative results, harming patients in need of antibiotic treatment. For this reason, care should be taken when making the diagnosis. It is necessary to use more than a confirmatory test such as PCR, in the case of negative results by RUT, especially in patients with clinical suspicion.

Later, there will be the evaluation of other important genes in the bacterial virulence, such as *VacA*, *CagA* and *DupA*, so it will be possible to combine the bacterial strain with clinical manifestations and age of those patients, an epidemiological study of a descriptive character. Such data will help to create a clearer picture of host/parasite relationship, contributing more directly to clinical medicine.

CONCLUSION

The success rate in the detection of *H. pylori* by the molecular method of 82.35% was significantly higher compared to the RUT. PCR is not used in routine diagnosis of *H. pylori*, a fact that has been changing over the years, because of achieved results with standards of 100% sensitivity and specificity.

The results indicate a high rate of *H. pylori* infection in the region studied and could lead to further analysis as to virulence factors, as well as epidemiological intervention to control the means of transmission and clinical prevention measures.

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CONFLICT OF INTEREST

None of the authors has any conflicts of interest regarding this paper to declare.

RESUMO

Introdução: Helicobacter pylori *é uma bactéria encontrada nas células epiteliais do trato gastrointestinal humano. Sua infecção relaciona-se com diferentes patologias, como gastrite crônica, úlcera péptica, linfoma gástrico e adenocarcinoma. A infecção por* Helicobacter pylori *está presente em mais da metade da população mundial.* ***Objetivos:*** *Detectar a presença de* H. pylori *e comparar os métodos diagnósticos do teste rápido de urease (TRU) e reação em cadeia da polimerase (PCR).* ***Materiais e métodos:*** *No estudo, realizado entre abril e julho de 2015, três biópsias foram coletadas de cada paciente. Duas foram usadas para realizar PCR e uma, para TRU.* ***Resultados:*** *Oitenta e cinco amostras foram coletadas dos pacientes por meio de endoscopia, sendo 56 (65,88%) mulheres e 29 (34,11%) homens. Do total dos indivíduos sujeitos ao TRU, 15 (17,64%) foram positivos e 70 (82,35%), negativos. Na PCR, na detecção do gene 16S ácido ribonucleico ribossômico (rRNA) de* H. pylori*, 66 (77,64%) apresentaram resultados positivos e 19 (22,35%), negativos. Para a análise da presença do gene* UreA *em todas as amostras, resultados positivos foram encontrados em 70 (82,35%) e negativos em 15 (17,64%). De acordo com os resultados, o TRU e o teste molecular apresentaram diferenças estatísticas.* ***Conclusão:*** *A PCR é um método útil na rotina laboratorial para detectar* H. pylori *em tecido de estômago devido à sua alta sensibilidade e especificidade, mas é necessária maior atenção na análise e na padronização.*

Unitermos: *bactérias; reação em cadeia da polimerase; urease.*

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