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

**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 laboratory 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 35°C–37°C 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).
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 $10^6$ *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 of 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 mini kit® (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, totaling 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).

![Figure 1](image1.png)

**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

![Figure 2](image2.png)

**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

| 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 |

| 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 |
| Positive | 15 | 70 | 66 |
| Negative | 70 | 15 | 19 |
| Total | 85 | 85 | 85 |

RUT: rapid urease test; PCR: polymerase chain reaction; rRNA: ribosomal ribonucleic acid.
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.\(^{(17)}\)

False-positive results observed in the RUT may be due to
the presence of other microorganisms that have urease activity
as *Proteus*, *Versinia*, *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\(^{\circ}\).\(^{(19)}\)

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 *Duca*, 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.

**ACKNOWLEDGMENTS**

The authors would like to thank the gastroenterologist Dr.
Fernando Faria for the collection of samples and providing the
data, the researcher Dr. Lucas Trevizani Rasmussen for sending
the positive control for *H. pylori*, professors Dr. Iderval da Silva
Junior Sobrinho and Dr. Elaine Cristina Castelhano of Regional
Jataí Genetic department, who contributed to the study.

**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 esfígamo 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.

REFERENCES


Mônica Santiago Barbosa
Rua 235, s/n; Setor Universitário; CEP: 74605050; Goiânia-GO, Brasil; e-mail: santiagosant@gmail.com.

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