JIDC | THE JOURNAL OF INFECTION IN DEVELOPING COUNTRIES

Original Article

Diagnostic performance of biopsy-based methods for determination of Helicobacter pylori infection in dyspeptic Mozambican patients

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Abstract

Background: *Helicobacter pylori (H. pylori)* is a Gram-negative bacterium capable of colonizing the human stomach, which can lead to various gastrointestinal conditions. Several invasive and non-invasive methods exist for diagnosing *H. pylori*; however, none can be considered the gold standard. This study aimed to evaluate the performance of three biopsy-based methods (rapid urease test - RUT, histopathology - HIST, and polymerase chain reaction - PCR) in diagnosing *H. pylori*, and to assess their combined effect in confirming the infection.

Methodology: Eighty dyspeptic patients were recruited for this study, and gastric biopsies were collected from each of them using upper digestive endoscopy. *H. pylori* was diagnosed using three biopsy-based methods: RUT, HIST, and PCR. RUT was performed using the commercially available PYLO DRYTM Kit, HIST was conducted with Hematoxylin & Eosin and Giemsa staining, and PCR was performed by amplifying the *16S rRNA* and *23S rRNA* genes. The patient had to test positive in at least two combined diagnostic methods to be confirmed as a case.

Results: The three biopsy-based methods (RUT, HIST, and PCR) showed positivity rates of 100% (80/80), 35% (28/80), and 65% (52/80), respectively. When all methods were combined to confirm *H. pylori* infection, 75% (60/80) of cases were confirmed, while the remaining 25% (20/80) were classified as undetermined, as they were positive only for RUT.

Conclusions: Despite slight differences, RUT and PCR performed well in diagnosing *H. pylori* compared to HIST. However, when all three methods were combined, they improved the accuracy of *H. pylori* diagnosis and infection confirmation.

Key words: *Helicobacter pylori*; dyspepsia; endoscopy; rapid urease test; histopathology; PCR; Mozambique.

J Infect Dev Ctries 2024; 18(11):1702-1708. doi:10.3855/jidc.19518

(Received 06 June 2023 – Accepted 25 December 2023)

Introduction

Helicobacter pylori (*H. pylori*) is a Gram-negative, microaerophilic, motile, spiral-shaped bacillus capable of colonizing the human stomach. It represents a significant public health concern, affecting approximately 4.4 billion individuals worldwide [1,2].

This pathogen is implicated in several gastrointestinal conditions, including dyspepsia [3,4]. Dyspepsia has been highlighted by many studies as a common and widespread condition of multifactorial origin; however, *H. pylori* is considered one of its main causes, as it is constantly found in the gastric mucosa of these individuals than in healthy individuals [1,5,6].

H. pylori infection is usually acquired in childhood and can persist throughout the host's life if not diagnosed and treated. The prevalence of this infection increases with age, low socioeconomic status, overcrowding, and poor environmental sanitation, and it can vary geographically [7-9]. Several epidemiological studies indicate that the infection rate is around 50% in developed countries, while in developing countries it can reach up to 90% [1,10,11].

For instance, higher rates of infection observed in many African countries have been linked to conditions of transmissibility [8,12]. However, this scenario is changing in some countries due to improved sanitation, rapid urbanization, and the implementation of eradication regimens [1,13,14].

In 2012, the World Health Organization classified *H. pylori* as a type I carcinogen due to its ability to cause gastric cancer in humans, and it is currently believed to contribute approximately 5.5% of the global cancer burden. Therefore, accurate diagnosis and eradication are crucial for altering this situation [8,15,16].

The available methods for diagnosing *H. pylori* infection are categorized into two groups: invasive and non-invasive. Invasive methods, often referred to as biopsy-based, include histopathology, rapid urease test (RUT), culture, and polymerase chain reaction (PCR). Non-invasive methods include the urea breath test (UBT), fecal antigen test, and serological test [16,17].

Among biopsy-based methods, microbiological culture is generally regarded as the reference. However, given the fastidious nature of *H. pylori*, it is advisable to combine it with alternative methods, such as histopathology and PCR, to achieve a reliable diagnosis [16,18,19]. Nevertheless, none of these methods should be considered the gold standard [20].

In developed countries, endoscopy is routinely performed, allowing the use of all three biopsy-based methods and comparing their results to achieve the most accurate diagnosis [19,20,21]. Ideally, this approach should be followed universally. However, in many developing countries, the cost of testing often dictates the choice of diagnostic method rather than its accuracy, which may contribute to the lack of a consensus approach for the diagnosis of *H. pylori* in these regions [19,22,23].

Due to limited resources, serological methods are commonly used for the routine diagnosis of *H. pylori* in Mozambique. When endoscopy is performed, histopathology is the preferred method rather than PCR. Incorporating PCR into routine diagnosis is challenging due to its cost and the shortage of trained professionals. However, PCR is gradually gaining recognition as the preferred diagnostic method in many sectors, especially after its importance was highlighted during the COVID-19 pandemic.

Thus, this study aimed to evaluate the performance of three biopsy-based methods (RUT, histopathology - HIST, and PCR) in diagnosing *H. pylori* and to assess their combined effect in confirming infection in dyspeptic patients at the Gastroenterology Unit of Maputo Central Hospital (HCM) in Mozambique.

Methodology

Patient characteristics

This cross-sectional study was conducted at the Gastroenterology Unit of HCM, the largest quaternary-level teaching hospital and national reference center, between 2017 and 2020. The study population consisted of patients who attended gastroenterology consultations during the study period, with inclusion based on a non-probability sampling method. A total of 80 adult patients (aged ≥ 18 years) with symptoms of dyspepsia and clinical indications for upper digestive endoscopy were included. Furthermore, these patients had to meet the following criteria: not had been treated with antibiotics, proton pump inhibitors, or non-steroidal anti-inflammatory drugs for at least 4 weeks before endoscopy; absence of concomitant diseases; and a positive RUT (a rapid method used to screen for *H. pylori* infection).

Laboratory analysis

Clinical specimens' collection by endoscopy and execution of the rapid urease test

All patients were examined by a gastroenterologist, and during upper digestive endoscopy, four biopsies were taken from each patient: two from the antrum region and two from the body region. During this clinical procedure, endoscopic findings were recorded, followed by the performance of the RUT. This involved placing the biopsy in a test medium containing urea and phenol red as a pH indicator. Due to the high production of the urease enzyme by *H. pylori*, urea is broken down into carbon dioxide and ammonia, increasing the pH and causing a color change in the test. The RUT was performed using a commercially available and validated PYLO DRY™ Kit, following the manufacturer's instructions, and the result was considered positive when the color changed from yellow to orange/red.

After obtaining the RUT result, gastric biopsies from patients with RUT-positive results were sent to the Department of Pathology at HCM for histopathological diagnosis, and to the Microbiology Laboratory of the Faculty of Medicine at Universidade Eduardo Mondlane (UEM) for molecular confirmation of the presence of *H. pylori* through PCR. Gastric biopsies sent for histopathological analysis were preserved in 10% buffered formalin, while those sent for molecular analysis were preserved in saline solution.

Histopathological analysis

This analysis was performed to confirm the presence of *H. pylori* in biopsies and to characterize the

morphology of gastric lesions in patients. Histopathological samples were embedded in paraffin, then cut into 4 micro sections and stained with Hematoxylin and Eosin to determine the type of lesion, and with modified Giemsa to identify *H. pylori*. Histopathological evaluation was conducted according to the recommendations of the Sydney system [24].

Molecular analysis

Molecular analysis began with extracting bacterial genomic DNA from gastric biopsies using the QIAamp DNA Mini Kit (Qiagen, USA) according to the manufacturer's instructions, followed by DNA purity assessment using a Nanodrop. All DNA samples were then stored at -80 °C for subsequent molecular confirmation of the presence of *H. pylori*. To confirm the presence of *H. pylori* in the extracted DNA, two conserved genes (*16S rRNA* and *23S rRN*A) were amplified by PCR. The *23S rRNA* gene, in addition to detecting *H. pylori*, can provide information about its resistance to macrolides [25,26], however, in this case, it was used solely for detection purposes to prevent false-positive or false-negative results. The amplification of these genes was performed using the following primer sets: for *16S rRNA*: *forward* - GCGCAATCAGCGTCAGGTAAT and *reverse*-GCTAAGAGAGCAGCCTATGTCC [27]; for *23S rRNA*: *forward*-AGGTTAAGAGGATGCGTCAGTC and *reverse*-CGCATGATATTCCCATTAGCAGT [28].

Each PCR reaction was carried out in a 25 µL volume containing 1× Flexi Buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 1 µM of each forward and reverse primer, 0.1 U/µL of GoTaq® G2 Flexi DNA Polymerase (Promega), 50 ng/ µL genomic DNA, and nuclease-free water to reach the final volume. Genomic DNA from the *H. pylori* strain 26,695, provided by the Pathogen Genome Bioinformatics and Computational Biology Lab, iMed-ULisboa.

PCR amplification was performed in a thermocycler (Bio-Rad C1000™ Thermal Cycler) under the following conditions: initial denaturation at 94 °C for 5 minutes, followed by 35 cycles of denaturation at 94 °C for 1 minute, annealing at 60 °C for 30 seconds, extension at 72 °C for 45 seconds, and a final extension at 72 °C for 7 minutes.

Following amplification, the PCR products were analyzed on a 2% agarose gel stained with ethidium bromide (500 ng/ml) from Sigma-Aldrich (U.S.A.). The products were visualized and analyzed using an ultraviolet transillumination system (VWR®-CC003531, USA), considering the expected product sizes for the *16S rRNA* gene (522 bp) and the *23S rRNA* gene (267 bp). A 100 bp ladder (New England BioLabs®, Inc. - USA) was used as the molecular weight standard. A PCR result was considered positive when both target genes (*16S rRNA* and *23S rRNA*) were detected.

Confirmation of H. pylori infection

The criteria to confirm infection required that the patient had a positive test using at least two diagnostic methods, rather than just one. Therefore, all patients with positive results from TRU + HIST, TRU + PCR, or TRU + HIST + PCR were classified as confirmed cases. Patients with positive results from only one method were considered undetermined.

Ethical approval

The study was approved by Mozambique´s National Bioethics Committee for Health (CNBS) with the following registration: IRB00002657, ref.224/CNBS / 2017 & ref.411/CNBS/2020.

Statistical analysis

Statistical analysis was performed using Microsoft® Excel version 2019 and SPSS version 20 (SPSS Inc., Chicago, IL, USA). Results were expressed as absolute (n) and relative frequencies (%). The chi-square test was employed to evaluate the relationship between *H. pylori* infection and demographic variables (sex and age) as well as clinical outcomes (endoscopic findings and histopathological characteristics). The significance level adopted was set at a p of < 0.05.

Results

Data from 80 dyspeptic patients included in the study, comprising 58 females (72.5%) and 22 males (27.5%), were analyzed. The patients' ages ranged from 18 to 79 years, with a mean age of 40 and a standard deviation of 13 years. A positive result from the rapid urease test (RUT) was one of the inclusion criteria; thus, 100% (80/80) of the analyzed samples were positive for *H. pylori* by RUT. Among these, the histopathological method showed a positivity rate of 35% (28/80), and the molecular method (PCR) showed a positivity rate of 65% (52/80), as shown in Table 1. The infection was

Table 1. Diagnosis of *H. pylori* by using three biopsy-based methods.

Diagnostic method	Results	
	Positive, n = 80 (%)	Negative, n = 80 (%)
RUT*	80 (100.0)	0 (0.0)
PCR	52 (65.0)	28 (35.0)
HIST	28 (35.0)	52 (65.0)

RUT: Rapid urease; HIST: Histopathological method; PCR: Polymerase Chain Reaction; *Performed when recruiting patients.

confirmed when results from all diagnostic methods were combined. Accordingly, 75% (60/80) were confirmed as cases (infected with *H. pylori*), and 25% (20/80) were considered undetermined (unconfirmed) since they were positive only by a single method (RUT), as shown in Table 2.

The analysis of confirmed cases revealed a significant relationship between *H. pylori* infection and the patient's gender (p = 0.044), with females being more frequently infected (78.3%, 47/60) compared to males (21.7%, 13/60). In contrast, no significant association was observed between infection and age or clinical outcomes (endoscopic and histological findings), as shown in Table 3.

Table 2. Combination of diagnostic methods for confirmation of infection.

Combined diagnostic methods	Frequency n = 80 (%)	Confirmation of infection** n = 80 (%)
RUT + HIST	8 (10.0)	
RUT + HIST + PCR	20 (25.0)	Confirmed, 60 (75.0)
RUT + PCR	32 (40.0)	
RUT (single method)	20 (25.0)	Unconfirmed, 20 (25.0)

RUT: Rapid urease; HIST: Histopathological method; PCR: Polymerase Chain Reaction; ** Confirmation of infection was based on the definition in point 2.3.

Discussion

Several methods have been developed for diagnosing *H. pylori* infection; however, none can be considered the definitive gold standard for detecting this pathogen [29,30]. In this study, three biopsy-based methods were employed to diagnose *H. pylori* and evaluate their combined effect in confirming the infection in dyspeptic patients. Based on the results, the performance of these diagnostic methods can be ranked as follows: RUT > PCR > HIST, as shown in Table 1. These findings are consistent with those of a similar study conducted in Iran [31]. For accurate diagnosis of *H. pylori*, many studies recommend combining at least two of the three biopsy-based methods, which allows for precise determination of infected cases [29,30]. A similar observation was made in our study, where the combination of the three biopsy-based methods (RUT + PCR + HIST) enhanced the ability to identify cases of infection. However, when comparing the results of this combined approach with those of the single RUT method, there appeared to be a decrease in the number of confirmed infection cases, raising suspicions of false-positive results from the RUT method.

RUT is a fast, inexpensive, and simple method that provides results in a short period, with sensitivity and specificity reaching up to 95% [32,33]. However, this method can occasionally produce false-positive results, particularly in the presence of other urease-producing bacterial species such as *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Enterobacter cloacae*, and *Citrobacter freundii*, which can be isolated from the oral cavity and stomach of patients with hypochlorhydria [34,35]. This may be a plausible explanation supporting our suspicion of false-positive results from the RUT method, although it is unlikely that these bacteria are present in sufficient concentrations to produce a positive result [36]. However, to exclude this suspicion, it would be necessary to retest these samples using PCR, targeting genes specific to each bacterial species. Despite the possibility of false-positive results in some cases, the RUT method remains recommended as the most effective screening tool for the infection, though not as a gold standard method [36].

In our study, the histopathological method demonstrated lower performance compared to the other methods (RUT and PCR), as shown in Table 1. A similar trend was observed in studies conducted on Sudanese

Table 3. *H. pylori* confirmation concerning gender, age, endoscopic, and histological findings.

Characteristic	Confirmed n = 60 (%)	Unconfirmed n = 20 (%)	Total n = 80 (%)	*p*
Gender				
Female	47 (78.3)	11 (55.0)	58 (72.5)	**0.044*** **
Male	13 (21.7)	9 (45.0)	22 (27.5)	
Age-group¶				0.260
≤ 40	29(48.3)	12 (60.0)	41 (51.3)	
≥ 40	31 (51.7)	8 (40.0)	39 (48.8)	
Endoscopic findings				
Normal Mucosa	10 (16.6)	4 (20.0)	14 (17.5)	0.866
Erythematous Gastritis	28 (46.7)	8 (40.0)	36 (45.0)	
Erosive Gastritis	22 (36.7)	8 (40.0)	30 (37.5)	
Histopathological features				
Normal Mucosa	7 (11.7)	4 (20.0)	11 (13.8)	0.607
Chronic Gastritis	48 (80.0)	14 (70.0)	62 (77.5)	
Gastric Ulcer	5 (8.3)	2 (10.0)	7 (8.8)	

***Statistically significant; ¶Minimum age =18, maximum age =79, mean ± SD (40± 13); Age groups were created based on the mean age.

and Iraqi patients, although their findings differed slightly from ours [19,37]. Historically, histopathology has been considered the first diagnostic method for detecting *H. pylori* and continues to be the primary tool for diagnosing patients with severe gastrointestinal symptoms. However, its accuracy is influenced by various factors, such as the expertise of the pathologist, the precision of the staining technique, and the careful observation of the gastric biopsy [19,20,38]. Some of these factors may have influenced the histopathological results obtained; however, it is difficult to pinpoint specific causes, as two pathologists were involved in the diagnosis to minimize inter-observer variation. Additionally, we suspect that the lower density of *H. pylori* colonization may have impacted the performance of the histopathological method in our study. Reduced *H. pylori* colonization density was also identified as a factor influencing the diagnostic results in a previous study comparing the prevalence of *H. pylori* between Mozambican and Portuguese dyspeptic patients [39,40].

Numerous studies suggest that histopathology is a reliable method for the accurate diagnosis of *H. pylori* infection, particularly in dyspeptic patients. However, it should always be combined with another method, such as PCR, to enhance diagnostic accuracy and detect the coccoid form of this pathogen, which complicates histopathological diagnosis [11,18,35]. PCR is the only method that can accurately detect *H. pylori* strains in this form [19,35,41]. The strategy of combining diagnostic methods (HIST + PCR) has been widely adopted in several African countries, not only because it enhances the detection of infection[42,43], but also, because it provides further insights into mucosal status and allows for the genotyping of virulence and resistance factors, facilitating effective eradication of the infection [39].

Following RUT, the PCR method showed the second-highest diagnostic performance, detecting *H. pylori* in 65% (52/80) of cases. Although the difference was small, our findings were consistent with studies conducted in Saudi Arabia [44] and Iraq [45]. The lower-than-expected performance of PCR in this study was likely due to several factors, including the presence of polymerase inhibitors and storage conditions (such as repeated thawing and freezing), which are known to hinder PCR performance [25,46,47]. While sample storage issues are sometimes unavoidable, PCR inhibition could have been mitigated by using commercial kits designed to remove these inhibitors during DNA preparation for PCR amplification [25,48], however, this would have increased the overall cost of the method..

The analysis of confirmed cases revealed a significant relationship between gender and infection ($p = 0.044$), with females being more frequently infected than males. This could be attributed to the gender distribution in the studied population, where females were the predominant group. A similar pattern was observed in a study conducted in Yemen [49]. Some studies suggest that gender should not be considered a risk factor, as both females and males are equally likely to be infected with *H. pylori*. Therefore, this topic remains controversial, as the relationship between gender and infection may vary depending on geographic location and other specific factors [50-53].

Conclusions

Based on the results, we concluded that RUT and PCR showed high performance in diagnosing *H. pylori* compared to the histopathological method (HIST). Although HIST showed lower performance, its combination with other methods (RUT and PCR) improved the accuracy of *H. pylori* diagnosis and infection confirmation. Therefore, we recommend the implementation of a combined biopsy-based approach in routine clinical practice to enhance diagnostic precision and the clinical management of gastrointestinal diseases associated with *H. pylori*.

Authors' contributions

Conception and design: NDM, MI, EB, CC, ET, JMBV, FFV, and JS. Administrative support: LM and JS. Endoscopic and urease test analysis: RV, LD, SM, LM, and MI. Histopathological analysis: EB and CC. Molecular analysis by PCR, collection, interpretation of data, and drafting of the manuscript: NDM. Revision of the manuscript: MI, EB, CC, FFV and JS. All the authors read and approved the submitted version of the manuscript.

Acknowledgments

We thank the entire team of Departments of Gastroenterology and Pathology of the Hospital Central de Maputo, and the Microbiology Lab of the Faculty of Medicine-UEM (in particular to Fabião Mausse and Darlenne B. Kenga for having helped with the reception and storage of samples).

Funding

Fundo Nacional de Investigação (FNI – ref.299) and Agência Italiana de Cooperação para o Desenvolvimento (ref. AID11096) through Biotechnology Center-UEM, Mozambique. NDM was the beneficiary of a master's fellowship from the Instituto Gulbenkian de Ciência of the Fundação Calouste Gulbenkian, Portugal (ref. 6/BI-M/21), which partially supported the study. FFV is funded by Fundação para a Ciência e a Tecnologia (FCT) by projects

PTDC/BTM-TEC/3238/2020, UIDB/04138/2020, UIDP/04138/2020, UIDB/00211/2020 and UIDB/04046/2020 (DOI 10.54499/UIDB/04046/2020).

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Conflict of interests: No conflict of interests is declared.