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Original Article

Helicobacter pylori culture as a key tool for diagnosis in Colombia

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Abstract

Introduction: The presence of *H. pylori* in the stomach is associated with gastric pathologies. However, its diagnosis through culture methods is challenging because of its complex nutritional requirements and microaerophilic conditions for optimal growth. The preferred method for rapid diagnosis of *H. pylori* is the Rapid Urease Test (RUT) from human biopsies, which relies on the high activity of the urease enzyme present in *H. pylori.* However, RUT cannot say much more information about *H. pylori*. This makes evident the need for bacterial culture to know essential information such as the strain type, the kind of infection present and the bacteria's antibiotic susceptibility.

Methodology: Gastric biopsies from 347 patients were used for *H. pylori* isolation. We correlated the culture results with the RUT and histological grading used at Hospital Universitario Fundación SantaFe de Bogotá (HU-FSFB), Colombia. The concordance between techniques was determined by the Cohen's Kappa coefficient (*K*). The sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) were also calculated.

Results: The culture standardization was successful, and it could be applied for diagnosis in the clinical practice. *H. pylori* was positive by culture in 88 (26.34%) patients. The concordance of RUT and culture was strong (*K*= 0.805), and between histology and culture was moderate (*K*= 0.763) as well as for the gold standard defined and culture (*K*= 0.80).

Conclusions: We present evidence that RUT and histological methods will be better interpreted for diagnosis of *H. pylori* if combined with bacterial isolation in cholesterol enriched culture.

Key words: *Helicobacter pylori*; diagnosis; cholesterol enriched culture; rapid urease test; human gastric biopsies.

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Introduction

Helicobacter pylori (H. pylori) is a Gram-negative, microaerophilic bacterium that colonizes the human gastric mucosa persistently, which can lead to an acute inflammatory response and damage of gastric epithelium. Inflammation can then progress to several disease states, ranging in severity from superficial gastritis, chronic atrophic gastritis, peptic ulceration, to mucosa associated lymphoma and gastric cancer [1].

Approximately 50% of the world population is chronically infected with *H. pylori*. The prevalence of infection varies immensely between countries and is inversely related to socioeconomic status [2], defined by occupation, family income level, and living conditions [3]. While the infection rate in many developing countries is over 80%, the prevalence in industrialized countries ranges between 20 to 40% [3,4]. It is assumed that *H. pylori* is transmitted via the oral-oral or fecal-oral route during early childhood and persists without antibiotic treatment for a life-time in its gastric niche [3,4].

H. pylori is a fastidious microorganism, and its culture is challenging because of its complex nutritional requirements and microaerophilic conditions for optimal growth. Many laboratories use rich growth media complemented with animal serum which presents variability between lots and manufacturing companies. With the discovery of a cholesterol-trans-glucosidase protein (Ctg) in *H. pylori*, which allows it to use cholesterol from eukaryotic cells [5], a new media was developed, solid and liquid, which is free of serum and complemented with cholesterol and fatty acids [6]. The validation of this medium demonstrated that most of *H. pylori* strains respond positively to the

cholesterol supplement, and the lack of serum proteins facilitated extraction of bacterial secreted proteins for other studies [6].

One of the preferred methods for the rapid diagnosis of *H. pylori* is the rapid urease test (RUT) from human biopsies. This test relies on the high activity of the urease enzyme present in *H. pylori*. After a positive urease test of the extracted tissue, *H. pylori* is diagnosed and the subsequent standard procedure includes eradication therapy using a combination of a proton pump inhibitor and three to four antibiotics [7]. In Colombia, the RUT is regularly used in the clinical diagnosis of *H. pylori*, which is performed on tissue obtained during an endoscopy. However, this test gives information only about the presence or absence of the microorganism in the stomach. Like all other tests based on urease activity, RUT does not provide any information about the *H. pylori* strain type, the kind of *H. pylori* infection present in the patient nor the susceptibility of the bacteria to antibiotics.

In the last 10 years, new evidence on *H. pylori* and its relationship with humans has indicated that this bacterium is part of the human microbiota. One study has shown that *H. pylori's* eradication is linked to the development of gastroesophageal reflux disease (GERD) [8,9]. The raising concern of complications related with *H. pylori's* eradication, the need for reliable confirmation of its presence in patients, and the interest of obtaining additional useful data from biopsies motivated us to establish the first isolation and culture of *H. pylori* from human biopsies in cholesterol-complemented media in Bogotá (Colombia), with the objective of complementing the diagnostic methods available for gastric pathologies. To achieve this, we correlated microbiological results with routine diagnostics methods for the diagnosis of *H. pylori* used by the Gastroenterology Department of the Fundación Santa Fe de Bogotá, Colombia, which until now included the RUT and histopathological analysis.

Methodology

Samples

One *antrum* and one *corpus* stomach biopsy from 334 symptomatic voluntary patients older than 18 years who attend the digestive endoscopy service at HU-FSFB, Colombia having an endoscopy indication from June 2014 to August 2016 were used.

Inclusion / exclusion criteria

Patients were included only if written informed consent was obtained. Exclusion criteria were as follows: Patients with cardiovascular and respiratory diseases, cancer patients undergoing radiation or chemotherapy treatments during the 6 months prior to sampling, patients who had antibiotic therapy, bismuth treatment, proton pump inhibitors (PPI), or H2-blockers within the previous month, patients with coagulopathy and amyloidosis.

Samples excluded from the study were those in which only one area of the stomach mucosa was sampled.

Ethics statements

Ethical approval of this study was obtained from Hospital Universitario Fundación Santa Fe de Bogotá ethics committee and Los Andes University ethics committee.

Rapid Urease Test (RUT)

At the time of sampling, the RUT was instantaneously conducted from an additional antrum biopsy with the Sensibacter pylori-Test® (Laboratorio Microanálisis Ltda, Bogotá, Colombia) according to the manufacturer's instructions. This test is based on the pH change in urea solutions when they are exposed to the patients' biopsies upon urease activity of the bacteria present in the tissue, which changes the medium's color from yellow (negative) to magenta (positive).

Culture

Biopsy samples were stored and transported to the laboratory in 300 µL of Brucella Broth (BB) containing 5 µg/mL trimethoprim (TMP). Then, the samples were manually macerated using mini grinders and 100 µL of 10^{-1} dilution from the macerated sample was used for the isolation of single colonies on petri dishes with cholesterol-complemented GC agar.

A *H. pylori* GC agar plates were made using 36 g⁄L GC agar (Oxoid, Wesel, Germany), 1% vitamin mix (100 g ⁄ L d-glucose, 10 g ⁄ L l-glutamine, 26 g ⁄ L l-cysteine, 0.1 g⁄L cocarboxylase, 20 g⁄L Fe(III) nitrate, 3 g ⁄ L thiamin, 13 mg⁄L p-aminobenzoic acid, 250 mg ⁄L nicotinamide-adenine dinucleotide, 10 mg ⁄L guanine, 0.15 g⁄L l-arginine, uracil 5 mg/L, 1 mg ⁄ mL nystatin, 5 mg ⁄ L trimethoprim, and 10 mg ⁄ L vancomycin), and a final solution of 1X cholesterol (Gibco, Munich, Germany), as reported by Jimenez-Soto *et al* [6]. All bacteria were incubated at 37 °C in a controlled atmosphere containing 100 mL/L CO2.

Histopathological analysis

Gastric biopsy specimens were immersed in 10% formalin and embedded in paraffin. Sections were stained with hematoxylin and eosin. The following

histopathological parameters were evaluated: 1) Presence of lymphoplasmacytic inflammatory infiltrate (chronic inflammation); 2) polymorphonuclear activity; 3) *H. pylori* presence; 4) glandular atrophy and 5) intestinal metaplasia. These parameters were scored on an ordinal scale 0-3 corresponding to absent, mild, moderate or severe, according to the Sidney scale. *H. pylori* infection was defined as the morphological identification of any amount of *H. pylori* regardless of the score of the other parameters.

DNA extraction and Polymerase Chain Reaction (PCR) for 23S rRNA

This technique was used as a *H. pylori* culture-presence confirmatory procedure. To verify the presence and identity of the bacteria in the culture, DNA was extracted. This was done using a Quick-gDNA Miniprep kit (Zymo Research, Orange County, CA, USA) according to the manufacturer's instructions. Conventional PCR using primers HPYS (5'- AGG TTA AGA GGA TGC GTC AGT C -3') and HPYA (5'- CGC ATG ATA TTC CCA TTA GCA GT -3') and cycling conditions according to Ménard *et al*. [10] were performed for the amplification of a 267 bp fragment of 23S rDNA gene. Reactions were completed in 25 µL of 1X GoTaq® Green Master Mix (Promega, Fitchburg, WI, USA), 10 pmol/µL of each primer and 2 µL of genomic DNA. PCR products were separated in 2% (w/v) agarose gel in TAE 0.5X (Tris/Acetate/EDTA) buffer under 80 V for 80 min. Bands were visualized with a ChemiDoc™ XRS system (Bio-Rad, Hercules, CA, USA) using GelRed™ Nucleic Acid Gel Stain (Biotium, Fremont, CA, USA).

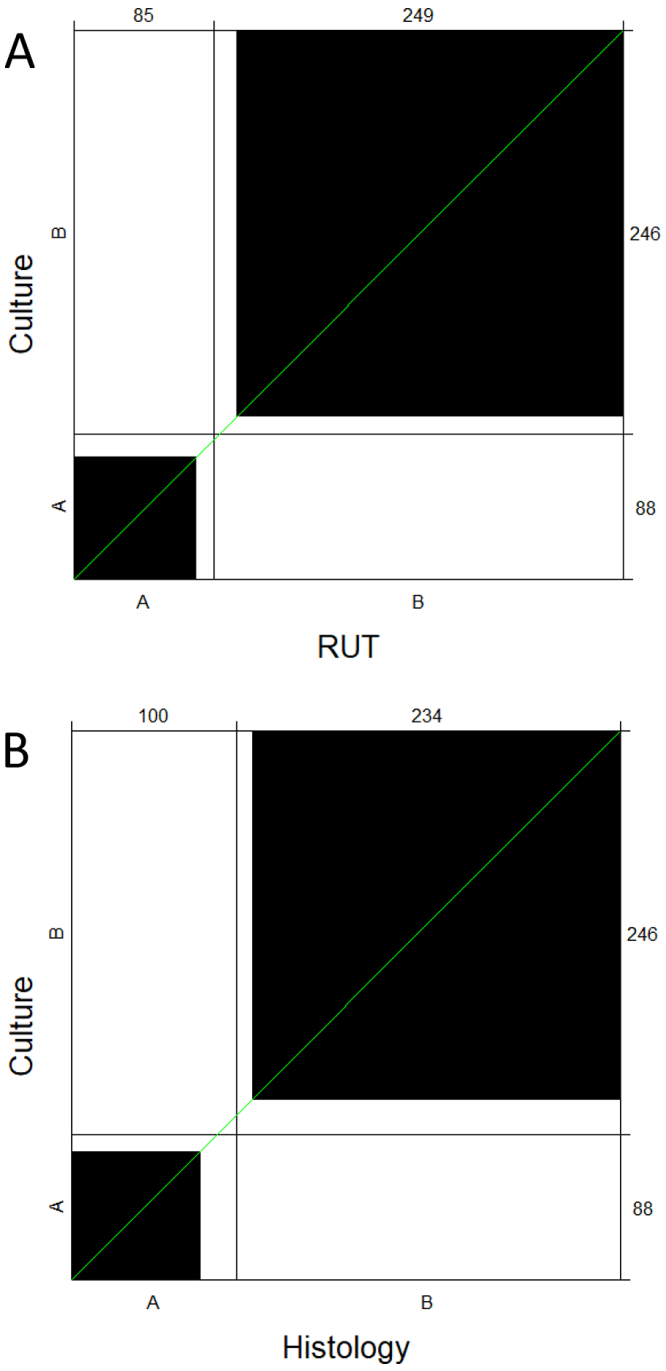
Gold standard for H. pylori infection

Based on the techniques used in the HU-FSFB for *H. pylori* diagnosis, a patient is considered infected with *H. pylori* when both biopsy RUT and Histopathology were positive.

Statistical Analysis

To estimate the recovery rate of the *H. pylori* culture, which is defined by the concordance between the RUT results and culture, histology and culture results, and the defined gold standard (RUT and histology) and culture was calculated through Cohen's Kappa coefficient [11]. Additionally, the sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) for the diagnostic performance of the culture method were calculated using the package "epiR" from R. Statistical analyses were performed using R Studio version 1.0.153 and R version 3.4.1.55. A probability (p-value) less than 0.05 was considered significant.

Figure 1. Agreement charts for comparing H. pylori recovery by culture with (A) rapid urease test (RUT) and (B) histology.



The observed (black) and expected (white) diagonal elements are represented by superposed rectangles. The extent at which the rectangles are above or below the line indicates the extent of any disagreement. The line above and/or below the intersection of the rectangles indicates the direction of the disagreement.

Results

It was necessary to determine the effectiveness of the isolation, especially for a study of this magnitude, where multiple researchers are involved in the process of RUT evaluation, histopathology and culture of the microorganisms.

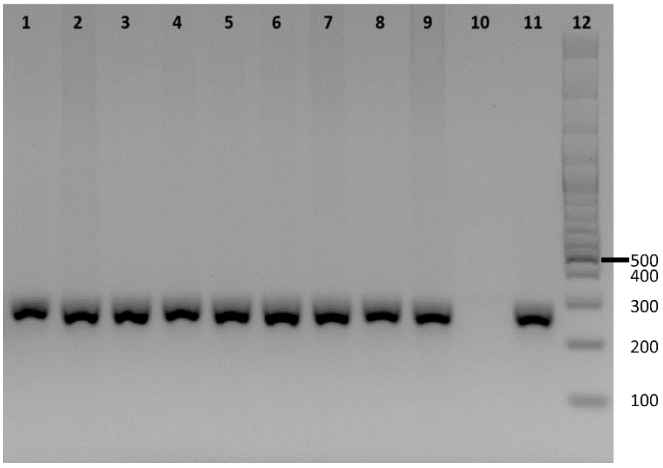
H. pylori was found positive by culture in 88 (26.34%) patients from the 334 enlisted in this study. Based on the RUT and culture data, almost all of them, 74 patients (84.09%), were both RUT and *H. pylori* culture positive (Urease+/ Culture+), whereas only some, 14 patients (15.90%), were RUT negative and *H. pylori* culture positive (Urease - /Culture +). Additionally, 11 patients were RUT positive and *H. pylori* culture negative (Urease +, Culture -) (Table 1).

The concordance of the RUT and culture for the definition of *H. pylori* infection was strong [11], with a Cohen's Kappa coefficient of 0.805 (p-value= 5.0839e-49) (Figure 1). The sensitivity of the culture, which is the proportion of true positives that were correctly identified by the test compared with those results of RUT, was 0.87. The specificity, which is the proportion of true negatives that were correctly identified by the test, was 0.94, the PPV was 0.84 and the NPV was 0.96. Based on the results, we would expect 87% of patients with *H. pylori* infection to have a positive *H. pylori*'s culture, whereas 94% of those without a *H. pylori* infection would have negative culture results. This only regarding the compare results of Culture and RUT.

Furthermore, including the histopathology and culture data, 78 patients (88.63%) were positive for both histopathology and *H. pylori* culture (His+ / Culture+), whereas only 10 patients (11.36%) were histopathology negative and *H. pylori* culture positive (His- /Culture+). Finally, 22 patients were histopathology positive and *H. pylori* culture negative (His+/Culture -) (Table 1).

The results of the isolation by culture and histopathology indicated that the concordance between both techniques was moderate, with a Cohen's Kappa coefficient of 0.763 (p-value= 1.3802e-44) (Figure 1). The sensitivity of the culture compared with histopathology was 0.78 whereas the specificity was 0.96. The PPV was 0.88 and the NPV was 0.91.

Figure 2. PCR products for a 267 bp region of 23S rRNA of Helicobacter pylori.



2% (w/v) Agarose gel. Line 1-9: Samples. Line 10: Blank of reaction. Line 11: Positive control, Helicobacter pylori NCTC 11637. Line 12: 100bp DNA ladder.

Additionally, the concordance between the two standard methods used at HU-FSFB was calculated; a moderate concordance with a Cohen's Kappa coefficient of 0.754 (p-value= 1.02693e-43) was obtained.

Finally, it was defined a Gold standard with the two methods used at HU-FSFB (RUT and Histology), and based on this, the culture results were evaluated. Cohen's Kappa coefficient was 0.80 (p-value= 1.1448e-48) showing a strong concordance. The sensitivity for the culture was 0.91 and specificity was 0.93.

As for the confirmation with molecular biology methods, all samples from the antrum and corpus of the 88 patients with positive *H. pylori* culture showed the expected band (267 bp) after the PCR protocol for the amplification of 23S rRNA (Figure 2).

Discussion

In Colombia *H. pylori* prevalence in patients from stomach biopsies has been reported in some studies

Table 1. Diagnostic performance for the culture test, rapid urease test (RUT) and histopathology.

		Culture		**Total**
		+ n (%)	**- n (%)**	
RUT	+	74 (84.09)	11 (12.5)	85
	-	14 (15.90)	235 (87.5)	249
Histopathology	+	78 (88.63)	22 (25)	100
	-	10 (11.36)	224 (75)	234
Total		**88**	**246**	**334**

[12,13]. These studies showed a prevalence of 69% based on histopathological analysis, and 64% based on RUT, which is based on the activity of urease generated by the presence of *H. pylori* in the stomach. Although effective, a urease-based method as the only diagnostic method may cause false positive and false negative results, considering that *H. pylori* is not the only one *Helicobacter spp*. and urease-positive bacteria in human mucosa associated with gastric pathologies development [14]. Additionally, the activity of the enzyme can be compromised by external factors, like antibiotic or PPI treatment [15]. Given the afore mentioned, the isolation by culture is accepted as the Gold standard for determining a pathology caused by a microorganism according to the Maastricht V Consensus Report [16]

However, although the isolation of *H. pylori* from gastric tissues by culture was accepted as a reference method, it has not been used in the routine diagnosis. Different factors have made it difficult to stablish its culture in labs across the world, such as nutritional requirements, and consequently the slow growth of the bacteria, and the specific controlled microaerophilic atmosphere requirements.

Nowadays, *H. pylori's* growing resistance to antibiotics makes its treatment difficult, including in Colombia. This highlights the importance of performing isolation by a culture for antimicrobial susceptibility test not only as a key tool in decision making after the failure of a second-choice treatment but as a complement in the initial treatment decision to get an appropriate use of antibiotics.

We were successful in standardize, for the first time, the culture of *H. pylori* from stomach biopsies in the clinical setup of the HU-FSFB and applied it for standard diagnostics of *H. pylori* in this clinic. An important factor for this success was the transportation and sample processing time. First, the transportation and processing of the sample should be performed on the same day as the sampling; otherwise, *H. pylori*'s recoverability (viability) will be compromised. This bacterium cannot survive in the transportation media at room temperature for more than 6-8 hours in biopsies after extraction (data not shown). To preserve the microorganism for more days, a sophisticated and more expensive media is required. Second, in a regular incubator with controlled CO_2 concentration, after the fourth day of incubation, it is important to check the growth aspect every single day until the tenth day. This is the time required for the bacteria to grow (4 to 10 days). If the petri dish was clear after the 10th day, that sample was interpreted as negative. For some samples, we obtained growth on the ninth day, whereas for other on the fourth day. The cause for the different growth times is still unknown. Third, when growth was evident, it was important to expand the culture as soon as possible as preparation for subsequent procedures, such as susceptibility tests and/or *H. pylori* type classification. The importance of rapid expansion is due to the difficulties encountered while recovering bacteria from single colonies after 24 hours of colony detection.

The best working dilution was a dilution of 10^{-1} from a macerated biopsy sample, achieving isolation of single and countable colonies on a petri dish with negligible contamination.

To discard the possibility of an over- or under-estimation of *H. pylori* prevalence owing to a poor recovery rate in the bacterial culture stage, we calculated the concordance between the result of RUT, the preferred method for the rapid diagnosis of *H. pylori*, and histopathology with the growth of the bacteria in the culture from the gastric biopsy of 334 patients.

The results showed a strong concordance [11] between the RUT and culture for the determination of *H. pylori* infection, with a *k* value of 0.805, which indicated that in addition to the RUT, the culture of the bacteria can be consider a reliable tool for the diagnosis. As seen in the results for RUT and culture (Table 1), 14 patients were negative for the RUT but positive for *H. pylori* culture. Because some of these patients were positive only for the corpus biopsy culture and not for the antrum biopsy, the gastroenterology team at the HU-FSFB modified their diagnostic protocols, by taking two biopsies (one from antrum and one from corpus) for the RUT. This was implemented to avoid false negatives caused by patchy distribution of *H. pylori* in the stomach.

For histopathology and culture results, we obtained a moderate concordance with a *k* value of 0.763. This result was proof that a combination of tests performed on multiple biopsies is more sensitive and specific for the diagnosis of *H. pylori* infection than any single test.

Additionally, we observed that the use of the culture as an additional diagnostic test improved the concordance than when only the two standard methods were used. This was evident by comparing the *k* values between the culture and defined gold standard, and the HU-FSFB's methodologies without the culture, because the *k* value for the RUT and histopathology was 0.754, whereas the *k* value for the culture and gold standard was 0.80, i.e., it changed from a moderate concordance to a strong concordance.

With the validation of the culture, we can conclude that the prevalence of *H. pylori* infection in these voluntary patients who attend the digestive endoscopy service at HU-FSFB was 26,34%**.** This seems to be quite low for a developing country such as Colombia if we take into consideration that different studies have reported that the infection rate for this kind of countries to be approximately 80% [3,4]. The prevalence was in the range observed for most industrialized countries. Considering that *H. pylori* prevalence is inversely related to socioeconomic status [17,18], we must consider that the hospital at which the investigation was carried out is an institution that provides services mainly to people of medium to high socioeconomic status. The prevalence determined was in the range reported for industrialized countries [3,12], but the patients' socioeconomic level could have influenced these results.

In summary, we successfully standardized *H. pylori* culture from biopsies. The isolation methodology can be applied for diagnosis in addition to the RUT and histopathology, without any concern about the recovery rate of bacteria in the culture and making it an efficient method for diagnosis in Colombia. The culture of the microorganism will allow us to know the genotypic and phenotypic data, additionally, it will allow us to carry out a susceptibility test and contribute to a decrease, or at least control, the resistance rates in Colombia, which seem to be high for antibiotics such as clarithromycin (2-63.1%) [19-21] and metronidazole (70-82%) [19-22].

It is important to highlight that in a country such as Colombia, where gastric cancer is one of the leading cause of death related to cancer [23], the culture as a routine technique is a key tool to improving the diagnosis of *H. pylori*, and allows a more cost- effective treatments in the eradication of *H.pylori* as proved by Cosme *et al*. [24] considering the high rates of resistance to clarithromycin and the socioeconomic status present.

To determine the prevalence of *H. pylori* in the general Colombian population, it will be important to carry out studies in hospitals that provides services including different socioeconomic levels, or at least, include patients from different institutions with the objective of truly represent the Colombian population.

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