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Original

Primary culture of *Helicobacter pylori* from gastric biopsies obtained by endoscopy

Silvia Molina-Castro[1,2], Christian Campos-Núñez[3,4], Sundry Durán-Bermúdez[5], Manuel Chaves-Cervantes[4], Vanessa Ramírez-Mayorga[1,6]

Authors' affiliation:
[1] Universidad de Costa Rica, Programa de Epidemiología de Cáncer, Instituto de Investigaciones en Salud. San José, Costa Rica; Universidad de Costa Rica, Escuela de Medicina, Departamento de Bioquímica. San José, Costa Rica
0000-0002-7523-9919
[2] Caja Costarricense de Seguro Social, Hospital San Francisco de Asís, Servicio de Gastroenterología. Grecia, Alajuela, Costa Rica; Hospital Clínica Bíblica, Centro Integrado del Aparato Digestivo. San José, Costa Rica.
0000-0003-2790-0378
[3] Clínica Burstin, LABGIPAT S. A., Laboratorio de Patología. San José, Costa Rica.
0000-0001-5921-2057
[4] Hospital Clínica Bíblica, Centro Integrado del Aparato Digestivo. San José, Costa Rica.
0000-0003-0285-6243
[5] Universidad de Costa Rica, Programa de Epidemiología de Cáncer, Instituto de Investigaciones en Salud; San José, Costa Rica; Universidad de Costa Rica, Escuela de Nutrición, Sección de Nutrición Pública. San José, Costa Rica.
0000-0003-4104-3261

Abbreviations:
ACG, Adenocarcinoma gástrico
CG, Cáncer gástrico
IBP, Inhibidor de bomba de protones
MI, Metaplasia intestinal
PSA, Prueba de sensibilidad a antimicrobianos
RUT, Prueba de ureasa rápida, por sus siglas en inglés
H. pylori: Helicobacter pylori

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✉silvia.molinacastro@ucr.ac.cr

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Abstract

Objetive: To determine the feasibility of Helicobacter pylori bacteria culture in Costa Rica through documentation of sample collection, comparison of histopathological diagnosis, and description of diagnoses associated with isolates obtained with rapid urease results.

Methods: Descriptive investigation involving patients between the ages of 35 and 70 years, of both sexes, who attended the Digestive Endoscopy Service of the Hospital Clínica Bíblica between February and June 2019 for a gastroscopic study. Gastric biopsies were obtained for histopathological diagnosis, rapid urease test, and Helicobacter pylori culture. For the latter, biopsies were transported in a semisolid transport medium, the tissue was macerated and cultured on Skirrow agar and Helicobacter selective agar; one plate of each medium was incubated at 37 °C in microaerophilia for 48 hours to 10 days. Culture positivity was determined by observation of colonial morphology and the bacteria were identified by the microscopic new analysis, Gram staining, and biochemical tests (catalase, urease, and oxidase).

Results: Forty-four patients were included (age: 50.6 ± 10.0, 54.5% male). Helicobacter pylori were recovered in biopsies from 27 patients (61.4% success rate). Bacterial recovery was similar in Skirrow and Helicobacter selective media. The weekly recovery success rate increased during the study to reach 100% success at week 11. The culture was compared with rapid urease in 27 patients and the agreement between the two methods was Cohen's kappa coefficient of 0.48. Culture detected the bacteria in 56% of patients, rapid urease in 37%, and the combination of both techniques allowed detection in 60%. The most frequent endoscopic diagnosis in patients with positive culture was erythematous gastritis and superficial chronic gastritis and the predominant histopathological diagnosis was chronic gastritis with gastric atrophy. The diagnosis by culture coincided with the detection of toluidine blue in 80.4% of the cases.

Conclusions: Helicobacter pylori culture can be implemented in Costa Rica. This study had a bacterial recovery rate of 61.4%. Combining the culture method with the rapid urease test and histological detection contributes to an accurate and timely diagnosis. Based on the protocols described in this research, we recommend that each laboratory standardize the conditions that allow a good recovery percentage and an implementation adapted to their routine activities.

Keywords: Gastric atrophy, gastric cancer, bacteriology, diagnosis.

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Worldwide, gastric cancer (GC) is the sixth in incidence and the third in cancer mortality. In Costa Rica, it is the fourth most frequent cancer (age-adjusted rate of 12.8/100,000 inhabitants) and the first in mortality (age-adjusted rate of 10.3/100,000 inhabitants). In addition, by 2020, a prevalence of 27 cases/100,000 inhabitants was reported, and projections by the International Agency for Research on Cancer indicate that the number of GC in Costa Rica is expected to increase by 2040.[1] The overall 5-year survival rate for GC patients in Costa Rica is 41%.[2] Most stomach tumors are gastric adenocarcinomas (GAC), histologically classified (Lauren classification) into two types: diffuse and intestinal. Diffuse GCA is mainly related to mutations in CDH1 (E-cadherin).[3] Intestinal-type GCA is the result of a series of precancerous lesions known as the Correa cascade, which describes the transformation from normal gastric mucosa to carcinoma *in situ*.[4] The main risk factor associated with intestinal-type GCA is infection with the bacterium *Helicobacter pylori (H. pylori)*.[5] However, only a small percentage of individuals infected with *H. pylori* develop malignancy,[6] so infection is not a predictive factor, but a variable that combines with genetic and environmental factors to favor the appearance of lesions. International guidelines indicate the eradication of *H. pylori* when dyspepsia, precancerous lesions, ulcers, and a family history of GC are present.[7]

If this method is chosen, two biopsies from the antrum, two from the body, and one from the incisura are recommended for the detection of precancerous lesions.[8] Detection of *H. pylori* is performed by conventional histochemical staining. The use of immunohistochemistry is recommended when there is a lot of active chronic gastritis, as higher sensitivity is required. Similarly, a rapid urease test (RUT) can also be used as a first-line diagnosis, as it is a test with high sensitivity and specificity. The sensitivity of the test increases if two biopsies are taken, one from the body and one from the antrum.[9]

Before invasive or non-invasive *H. pylori* testing, treatment with proton pump inhibitors (PPIs) should be withheld for at least two weeks, and antibiotics and bismuth compounds for at least four weeks.[7]

H. pylori culture may be necessary when patients are resistant to treatment and antibiotic sensitivity testing is required, as well as to obtain population data on resistance levels that define empiric treatment selection. This method is not routinely performed in bacteriology laboratories in Costa Rica, probably because it presents several challenges. The first of these is the sampling itself, which must be done by endoscopy since a biopsy of the gastric mucosa is required. Endoscopy, although a minimally risky process, is invasive. During the sampling process, microorganisms from the upper digestive tract, respiratory tract, and oral cavity can be introduced, so there is a high probability of contamination. *H. pylori* is a microaerophilic bacterium that requires oxygen concentrations of less than 5% to survive, which complicates its culture and makes it not a methodology that any bacteriology laboratory can implement.

This research was carried out to document the experiences of the implementation of sampling and culture of *H. pylori* bacteria in a small sample of patients in Costa Rica, to compare it with other tests currently used in clinical routine (RUT and histopathological diagnosis) and to describe the diagnoses associated with the isolates obtained.

Methods

Patient selection. Costa Rican patients between 35 and 70 years of age, of both sexes and without distinction of ethnicity, who attended the Digestive Endoscopy Service of the Hospital Clínica Bíblica between February and June 2019 for the clinical need of upper gastrointestinal tract endoscopy and who agreed to participate in the study through informed consent were included. The project was approved by the Scientific Ethical Committee of the Universidad de Costa Rica (UCR).

Those who presented any of the following diseases or characteristics were excluded: MALT lymphoma, duodenal ulcer, operations for remaining disease (previous surgery for GC or secondary gastric tumors), having taken treatments against *H. pylori* in the last 30 days or PPIs in the last 14 days.

Endoscopy of the upper digestive tract. Patients were sedated with intravenous propofol at standard doses. A diagnostic upper GI endoscopy procedure was performed with Pentax 7010 endoscopy equipment (Pentax Medical, Tokyo, Japan).

Tissue sampling and endoscopic diagnosis. Six fresh gastric mucosal tissue samples of approximately 0.5 cm3 and distributed as follows:

two from the antrum, two from the body, and one from the angular incisura, were taken for histological evaluation according to the Sydney protocol.[8] Endoscopic diagnosis was assigned according to the most severe lesion detected. Biopsy for *H. pylori* culture was taken from the antrum in areas where chronic inflammatory changes, full crypt pattern changes, or areas with gastritis in the nodular pattern were observed and areas with intestinal metaplasia (IM) were avoided.

Histopathologic diagnosis. The biopsies were embedded in kerosene and two slides per area were prepared, of which one was stained with hematoxylin-eosin, according to routine methods for histological evaluation, and the other with toluidine blue for the diagnosis of *H. pylori*.

Rapid urease test. The RUT result was queried from the endoscopic diagnostic report of patients whose specimens yielded a positive culture for *H. pylori*. It was only performed for patients who had the test indicated by the treating physician. The result reported for the test is either positive or negative.

Biopsy transport. A semisolid transport medium was made with 7.5 g Brucella agar (Oxoid, Hampshire, UK), 6.3 g brain-heart infusion broth (BHI, Oxoid, Hampshire, UK), and 1 vial of *H. pylori* selective supplement (Oxoid, Hampshire, UK), added after sterilization. Samples were kept refrigerated (4-8°C), transported at room temperature (approximately 25°C), and processed within 8 hours of collection.

Culture. The biopsy was macerated with 0.4 mL of BHI broth in a 1.5 mL conical tube with a sterile plastic pistil. 0.1 mL of the macerate was spread on four plates: two of Skirrow agar[10] and two of *Helicobacter* selective medium (anaerobic Wilkins-Chalgren base supplemented with *H. pylori* selective supplement and 10% human blood).[11] The composition of both media is shown comparatively in Table 1. The human blood used was obtained from blood banks, from expired units of packed red blood cells. One plate of each medium was incubated in a 2.5 L jar and placed in sachets generating two different atmospheres: microaerophilia with CampyGen and anaerobiosis generated with AnaeroGen, at 37°C and for at least 48 hours and up to 10 days. All culture media as well as the atmosphere-generating sachets were Oxoid brands (Hampshire, UK). Culture positivity and the presence of contaminants were qualitatively assessed by observing colonial morphology. *H. pylori* had punctate colonies approximately 1 mm in diameter, colorless and smooth, with no hemolysis present. The percentage of culture recovery was obtained by dividing the number of *H. pylori*-positive cultures by the total number of samples processed.

Table 1. Detailed composition of the culture media used in the study to grow *Helicobacter pylori*

Medium	Skirrow	Homemade medium for *Helicobacter pylori*
Base	Columbia Agar	Chalgren Wilkins Agar
Components (g/mL concentration)	Special peptone (23) Agar (10) NaCl (5) Starch (1)	Enzymatic casein hydrolysate (10) Meat peptone (10) Agar (10) Yeast extract (5) NaCl (5) Dextrose (1) L-Arginine (1) Sodium pyruvate (1) Hemin (0.005) Menadione (0.0005)
pH	7.3	7.1
Supplementary blood	10% of lysed horse blood	10% of human blood
Antimicrobials (concentration in mg/L)	Vancomycin (10) Trimethoprim (5) Polymyxin B (2500*)	Vancomycin (10) Trimethoprim (5) Cefsulodin (5) Amphotericin B (5)

*IU/L: International units per liter.

Identification of *H. pylori*. The bacterial growths obtained were identified with the usual tests for *H. pylori,*[11] fresh morphological observation in physiological solution (motility and morphology), with Gram staining (helicoidal bacillary morphology and Gram-negative staining) and biochemical tests: positive urease activity in Steward's urea broth, positive catalase with hydrogen peroxide and positive oxidase in strips (Oxoid, Hampshire, United Kingdom).

Statistical analyses. All analyses included in the study were performed using GraphPad Prism software version 9.4.0 for Windows (GraphPad Software LLC, San Diego, CA). The Shapiro-Wilks test was used for the normality analysis of the demographic data. For the analysis of dichotomous variables, unpaired t-tests by the Holm-Šídák method were used. Comparison between the culture and the rapid urease test was performed using a contingency table and concordance was calculated with Cohen's kappa coefficient.

Results

Demographic characteristics of the patients. Forty-four patients were included, of whom 24 (54.5%) were men. The participants' ages ranged from 36 to 70 years, with a mean of 50.6 ± 10.0. Stratified by sex, in women the mean age was 50.7 ± 10.4 (range: 36-70), and in men, 50.5 ± 9.9 (range: 36-68). Both total ages and those stratified by sex presented a normal distribution (p>0.05 in the Shapiro-Wilks test).

***Helicobacter pylori* culture.** Of the 44 biopsies cultured, H. pylori were recovered in 27, representing a 61.4% success rate. During the first two months of the study, the biopsies were cultured in both microaerophilic and anaerobic atmospheres and, although no difference in culture positivity was observed between the two; however, weaker growth and a suboptimal physiological state of the bacteria was observed in the anaerobic atmosphere, so it was decided to continue the culture in microaerophilic conditions. There was no difference in culture positivity between culture media, Skirrow, and selective medium for Helicobacter; however, the growth of H. pylori in the Skirrow medium was less abundant, but so was the growth of contaminants. Upon analysis, it was observed that the was the growth of contaminants. Upon analysis, it was observed that the weekly recovery success rate was increasing during the first 5 weeks, with a small decrease in weeks 6 and 7, and then reached a plateau with 100% success after week 11 (Figure 1).

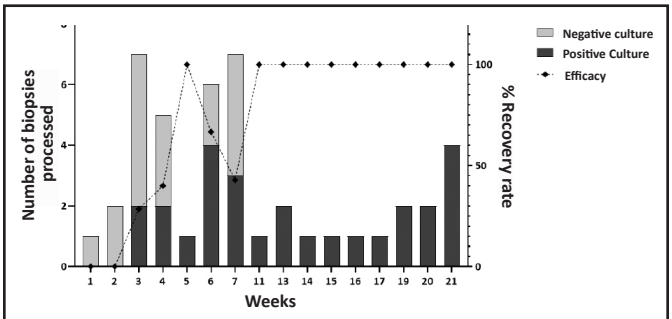

Figure 1. Temporal evolution of the number of biopsies processed and the percentage of *Helicobacter pylori* culture positivity from gastric biopsies. The percentage of culture effectiveness increased to 100% after week 11 due to a better selection of the patients sampled.

Comparison of culture with the rapid urease test. RUT results were available for 27 of the subjects with culture-positive biopsy. The bacterial culture was positive in 9 of the 10 RUT-positive patients. In the patients with negative RUT, the bacteria were recovered in 6 of 17. Similar results were obtained in both tests in 74% (Table 2).

Table 2. Contingency table for the results obtained when analyzing biopsies by two methods of H. pylori detection on biopsy: rapid urease test and culture

Rapid urease test	*H. pylori* culture (%)*		*Total*
	Positive	**Negative**	
Positive	9 (33.3)	1 (3.7)	*10 (37.0)*
Negative	6 (22.2)	11 (40.7)	*17 (63.0)*
Total	*15 (55.5)*	*12 (44.4)*	***27 (100.0)***

*Percentage of the total samples.

The combination of both methods, i.e., classifying a patient as positive with any of the positive tests, allowed the detection of the bacterium in 60% of the cases, while culture alone was detected in 56% and RUT alone in 37%. The concordance between both methods was calculated using Cohen's kappa coefficient, which yielded a result of 0.48.

Endoscopic diagnosis. The endoscopic diagnosis was performed on all the subjects included in the study. The most frequent lesions were erythematous gastritis, superficial chronic gastritis, erosive gastritis, and esophageal lesions. A higher number of positive cultures for *H. pylori* were observed in samples from patients diagnosed with erythematous gastritis (p=0.007) and superficial chronic gastritis (p=0.008).

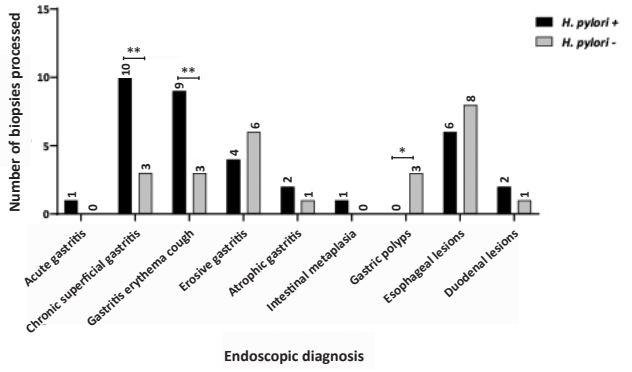

Figure 2. Result of *Helicobacter pylori* culture according to endoscopic diagnosis. Most positive cultures were obtained in patients with superficial chronic gastritis and erythematous gastritis. *p < 0.05, **p < 0.01

Histopathological diagnoses. The histopathological diagnosis was performed in the body, incisura, and antrum for 25 of the 27 biopsies with positive culture for *H. pylori*. Two samples were excluded because it was not possible to make a histopathological diagnosis. All culture-positive biopsies showed chronic gastritis, while 80% showed atrophy in the antrum and 92% in the incisura. Only 12% of the culture-positive patients had IM, mostly in the incisura.

Of the 25 biopsies with positive culture for *H. pylori* and histopathological analysis, 21 were positive by both methods. There were four cases in which toluidine blue staining failed to detect the presence of the bacterium, whereas it was detected by the culture method. All these cases presented a histological diagnosis of chronic gastritis, three of them with atrophy. There were no cases in which the bacterium was detected by the staining method and could not be cultured.

Discussion

This study was performed on biopsies obtained from a sample of adult patients living in Costa Rica, with a balanced distribution between men and women and with similar average ages between both groups. This is a small group of patients who come to the endoscopy service of a private hospital by choice or by indication of their primary care physician. The aim was to determine the feasibility of culturing *H. pylori* bacteria in Costa Rica through documentation of sample collection, comparison of the histopathological diagnosis, and description of the diagnoses associated with the isolates obtained with rapid urease results. The protocols and results are intended to be available for other laboratories at national and regional levels to venture into the primary culture of *H. pylori* from gastric biopsies obtained by endoscopy. Increasing levels of antimicrobial resistance by *H. pylori* result in the decreased success of eradication therapy, which consists of a combination of two or three antimicrobials, along with a PPI, for periods of 10 to 14 days, which is a difficult regimen for the patient.[7] *H. pylori* culture allows for antimicrobial susceptibility testing (AST) and additional information about the bacteria, such as virulence characteristics.[12-15]

The expertise of the endoscopist in sampling is critical since the distribution of *H. pylori* in the stomach mucosa is not homogeneous, so biopsies should be taken from sites with the highest probability of bacterial load, and sampling from sites of atrophy or IM should be avoided for culture purposes. The collection from sites that show inflammatory data suggestive of *H. pylori* infection such as the full crypt pattern, for example, should be favored. Some authors suggest that two biopsies from the antrum and two from the body should be taken for culture.[11]

In the recovery of bacterial isolates from clinical samples, the transport of the sample must be taken into account.[16] There are commercial methods for the transport of biopsies; however, they are difficult to obtain in Costa Rica because they are not routinely used, in addition to being expensive. Therefore, we developed a semisolid formulation, described as suitable for transporting samples of *H. pylori* and other bacteria.[17] It was supplemented with an antibiotic cocktail selective for *Helicobacter* since the sample is usually highly contaminated with other bacteria and yeasts from the gastrointestinal tract, which is one of the factors that most frequently compromise the success of the isolation. Antibiotics used for both transport and culture media should include at least one substance specific for gram-positive bacteria, one for gram-negative bacteria to which *H. pylori* is resistant, and one with antifungal activity.[18]

The culture was carried out using two different media, the Skirrow and the homemade medium prepared in our laboratory. The differences in the growth and physiological state of the bacteria may be since the anaerobic Wilkins Chalgren base is richer than the base used for the Skirrow since it is supplemented with hemin, L-arginine, and glucose. Similarly, the Skirrow medium does not contain an antifungal agent, so it may have a higher risk of fungal contamination. However, we believe that its use is desirable because one of the most common contaminants of *H. pylori* culture samples is lactobacilli. These bacteria are naturally resistant to all antibiotics present in both media; however, poor growth was observed in Skirrow, as it is a less nutritious medium. Based on these observations, our recommendation is to use both media during isolation and only the homemade medium for amplification of the bacteria needed for PSA, DNA extraction, or other applications. Although the literature agrees that the ideal atmosphere for culturing *H. pylori* is microaerophilic,[19] one study reported that culturing under anaerobic conditions increased the percentage of the recovery.[20] However, this was not the case in this work, so it was decided to leave this alternative aside and focus on microaerophilic culture.

In this study a recovery of 61.4% was obtained; however, the recovery percentages reported in the experiences of other laboratories are very heterogeneous and depend on the variables mentioned above (composition of transport and culture media, experience of the endoscopist in sample collection, processing time) and on the experience of the laboratory in general. Based on previously described protocols, we recommend that each laboratory standardize the conditions that allow a good recovery rate and an implementation adapted to its routine activities.

Among the different methods of diagnosis from biopsies, i.e., RUT, histology, and culture, there is no consensus as to which of them is the gold standard and each has advantages and disadvantages. RUT is characterized by being quick, inexpensive, and easy to interpret, while histology allows the detection of not only the presence but also the extent of damage and the type of lesion associated with the infection. However, it is more costly in time and money and requires a laboratory specialized in tissue processing. In addition, some experts question the species-level identification of a bacterium by morphology alone in simple staining. On the other hand, culture theoretically has 100% specificity and once isolation is obtained, additional characterization tests such as PSA or the presence of certain virulence factors are possible. The disadvantages are the variability of the result, its difficulty due to the metabolic requirements of the bacteria, the cost, and the time to obtain a result (approximately 10 days).

Due to the small sample size and the fact that there is no gold standard among the three methods, neither positive nor negative predictive values nor sensitivity and specificity values were calculated, but the level of concordance between culture and RUT was calculated using Cohen's kappa coefficient and was found to be within the range of moderate concordance (0.41- 0.60). Similarly, it was observed that combining the results of both tests increased the percentage of infection detection by 4%. In only one of the cases with positive RUT, it was not possible to obtain the culture. Among the reasons that could cause a false negative culture is excessive contamination that does not allow successful isolation of *H. pylori* colonies, an altered physiological state of the bacteria, and a low bacterial load, recent treatment with antibiotics or PPIs, among others. On the other hand, negative RUTs were also present in cases where culture was obtained. RUT can give false negative results for several causes other than prior antibiotic use, PPI, or the presence of extended IM. These include heterogeneous distribution of bacteria in the mucosa or the presence of upper GI bleeding.[21 22] Some considerations should be considered to increase the sensitivity of the test. For example, sampling the body of the stomach when the patient has a gastric ulcer because in these cases, there is a high probability of glandular atrophy and IM in the antrum that decreases the presence of the bacteria.[23] On the other hand, formalin contamination of the forceps with which the biopsy is taken is a factor that has been mentioned as a cause of false negatives, but the results have not been conclusive.[24, 25] False positives are rare and occur due to the overgrowth of urease-positive contaminants and the way to avoid them is the addition of some antibacterial agent in the reagent.[26]

Concerning the endoscopic diagnoses and their relationship with the culture result, the highest recovery was obtained in patients with erythematous and chronic superficial gastritis. These are the pathologies in which one would

expect to find a higher bacterial load, compared to more advanced stages of the Correa cascade such as atrophy and IM, where the habitat of the bacteria is altered, especially concerning acid secretion.

In conclusion, this work demonstrates that *H. pylori* culture in Costa Rica is feasible under the conditions of our country and compiles the basic indications so that other laboratories can implement the technique. *H. pylori* culture from gastric biopsies is not necessary in all cases as a diagnostic method, since other alternatives are more convenient in terms of time, cost, and invasiveness. However, as a complement to other methods, and in particular cases such as patients with antibiotic-resistant strains, it is a fundamental tool for an optimal approach to the patient resulting in the best possible care.

Declaration of ethics: the authors declare that they all agree with this publication and that they have made contributions that justify their authorship; that there is no conflict of interest of any kind and that they have complied with all the pertinent ethical and legal requirements and procedures. All sources of funding are fully detailed in the acknowledgments section.

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