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ORIGINAL ARTICLE

Helicobacter pylori diagnostic tests in Colombian children

Pruebas diagnósticas de *Helicobacter pylori* en niños colombianos

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What do we know about the subject matter of this study?

Helicobacter pylori infection is acquired in childhood and persists throughout life if untreated, increasing in prevalence with age. The characteristics and consequences of H. pylori infection in children from developing countries and/or countries with a high prevalence of gastric cancer require further study. For this reason, it is important to evaluate the performance of diagnostic methods to detect infection and the clinical management of H. pylori-associated diseases in the population with the characteristics mentioned above.

What does this study contribute to what is already known?

This work contributes to the methodology for the diagnosis of H. pylori in the pediatric population. In this study, the validity of the detection tests in breath, stool, and serum was evaluated through the sensitivity and specificity of the tests compared with the "Gold Standard" which is the histological evaluation. The enzyme immunoassay test for detection of H. pylori antigens in stool showed a higher sensitivity and specificity to distinguish between infected and healthy children.

Abstract

Helicobacter pylori (H. pylori) infection is dynamic in the pediatric population, increasing with age. There are several diagnostic tests available and effective in identifying infected and uninfected people. However, there is still a need for a non-invasive, reliable, and tolerable test for children. **Objective:** To estimate the validity of *H. pylori* testing in children in Cali, Colombia. **Patients and Method:** A total of 236 symptomatic children under 10 years of age referred for clinical evaluation with upper endoscopy due to abdominal pain, gastroesophageal reflux, vomiting, dyspepsia, and diarrhea were included. Three diagnostic tests were used to determine *H. pylori* infection: [13C]-Urea breath test (UBT); *H. pylori* stool antigen (HpSA) test, and serum *H. pylori* antigen by ELISA. The validity was evaluated by sensitivity and specificity analysis, establishing the diagnosis of *H. pylori* infection based on the histological study of gastric mucosal biopsies as the gold standard or reference method. **Results:** The estimated prevalence of *H. pylori* in the three tests ranged from 1.12% to 27.3%, similar to that reported by histological evaluation (21.6%). The sensitivity found through conventional analysis (2x2) for the HpSA test was 97.9%, and for the UBT test and the serum *H. pylori* antigen was 87.5%

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and 88.2%, respectively. Higher sensitivity was found in all three tests when latent class analysis was used, especially in the HpSA test which was equal to the reference histopathological test (100%). **Conclusion:** The HpSA test showed the best discrimination in both infected and healthy children under 10 years of age in Cali, Colombia.

Introduction

Worldwide, *Helicobacter pylori* infection has been associated with several diseases in the adult population[1] and is now recognized as a human carcinogen[2]. This infection is acquired in childhood and persists throughout life if untreated[3].

There are direct and indirect methods for detecting *H. pylori*[4]. Direct methods allow the identification of the bacterium by microscopic visualization in gastric biopsies or by culture. Indirect methods are based on detecting some characteristics of the bacterium, such as the ability to hydrolyze urea or the antigen detection through antibodies from the host immune system; the [^{13}C]-labeled urea breath test, which is based on the ability of *H. pylori* to hydrolyze urea; or the detection of bacterial antigens excreted in stools. Noninvasive tests are reserved especially for certifying eradication at post-treatment follow-up, and not for initial diagnosis (with few exceptions).

As the prevalence of gastric neoplasia is low in the pediatric population, the implementation of digestive endoscopy is often not necessary, especially after the eradication of *H. pylori*[5]. For this reason, there is a need to define a valid noninvasive diagnostic method with high diagnostic sensitivity and specificity, compared to the reference histological test in gastric biopsies. The objective of this research was to estimate the validity of noninvasive tests such as the [^{13}C]-labeled Urea Breath Test (UBT), the HpSA enzyme immunoassay for detection of *H. pylori* antigen in feces, and the enzyme immunoassay for detection of specific IgG antibodies against *H. pylori* in blood, used in the diagnosis of *H. pylori* infection in children under ten years of age in Cali, Colombia.

Patients and Method

Study type and population

Prospective study with a paired design to assess the validity of the tests used in the diagnosis of *H. pylori* infection and to describe the histopathological characteristics associated with infection in children. The study was carried out at the *Hospital Universitario Fundación Valle del Lili*. A total of 236 symptomatic children under 10 years of age residing in Cali were included; 47.9% were female.

Patients were selected from those referred to the outpatient clinic for clinical evaluation by their treating physician who determined that they required esophagogastroduodenoscopy as part of their diagnostic evaluation since they presented symptoms such as abdominal pain, gastroesophageal reflux, vomiting, dyspepsia, or diarrhea. Children whose guardians did not understand the terms of this research, those with advanced diseases such as cancer, heart, respiratory, or renal failure, or those with coagulation disorders were excluded.

This research was approved by the ethics committee of the Faculty of Health of the *Universidad del Valle* (CIREH), under Act 024 of November 13, 2001.

Histopathology procedures

Four gastric mucosal biopsies were obtained from each participant, according to the following protocol: two samples of the lesser and greater curvature of the antrum and two samples of the lesser and greater curvature of the body. Each of the specimens obtained was fixed, processed, and examined separately, following a 2-hour dehydration protocol, embedded in paraffin within the first 24 hours. Histological sections were cut on a microtome (Accu-Cut® SRM) and then stained with hematoxylin-eosin (H&E). To determine the presence of *H. pylori*, the modified Giemsa stain was used to look for curved and spiral-shaped bacteria[5], and negative cases were stained with the modified Steiner stain.

Diagnosis of *H. pylori* infection and validation of diagnostic tests

The diagnosis of *H. pylori* infection based on the histological study of gastric mucosal biopsies was considered the Gold Standard or reference method. In addition, each of the children underwent the tests described below.

[^{13}C]-Urea breath test

Four breath samples were collected in duplicate: basal (t0), 20 minutes (t20), 30 minutes (t30), and 40 minutes (t40) after administering via oral route 2 mg of [^{13}C]-Urea, a stable isotope. Participants were randomly assigned into one of three possible groups to receive [^{13}C]-Urea as a solvent: water or glucose solution or orange juice to compare which of these substances

would increase the discriminatory power between positive and negative values. The urea-derived $^{13}CO_2$ was measured with a mass spectrometer (ABCA, Europa Scientific, Crewe, UK), and three control samples of a reference gas with known CO_2 concentration (5%) were used for each of the 5 patients.

The ratio measurement of $^{13}CO_2/^{12}CO_2$ was compared with a reference gas with a known isotope ratio. The isotope ratio in the baseline sample was compared with the ratio of samples obtained after 20, 30, and 40 minutes of [^{13}C]-Urea ingestion. To determine the result of a test, the difference values of each time (delta) with respect to the baseline [$^{13}C/^{12}C$] were used, considering 5 as the positive cut-off reference point.

Enzyme immunoassay for the detection of *H. pylori* antigens in stool

Stool samples were evaluated by ELISA (Premier Platinum HpSA, Meridian Diagnostics Inc., Cincinnati, OH, U.S.), with the following stool antigen rescue procedure: 100 µl of stool was mixed with 200 µl of diluent. An aliquot of 50 µl was added to each microwell which had a monoclonal capture antibody anti-*H. pylori* adhered to the wall, followed by the addition of one drop of detection reagent (HRP-anti *H. pylori* conjugate), and then it was incubated for one hour at room temperature (25°C). The wells were properly washed 5 times, and the substrate was added to act for 10 minutes at room temperature, followed by one drop of stop solution.

The results were read at dual wavelength (450-630 nm). Positive and negative controls were analyzed at the beginning, middle, and end of the 96-well plate[6]. Negative values were considered < 0.100, ambiguous values > 0.100 and < 0.120, and positive values > 0.120 at a dual spectrophotometric wavelength (450-630 nm).

Enzyme immunoassay for detection of specific IgG antibodies against *H. pylori*

Antibody detection was performed by ELISA (DSL-05-10HPGi Diagnostic Systems Laboratories, Inc.; Webster, TX). The diluted sera from the patients and the calibrators with known absorbance were incubated in wells coated with purified and inactivated *H. pylori* antigens. The wells were treated with a monoclonal IgG antihuman antibody conjugated with peroxidase. To remove nonspecific bindings of the primary antibody and the conjugate, four successive washes were performed after each incubation. Tetramethylbenzidine (TMB) was used as the substrate solution, and 0.2M sulfuric acid was used as the stop solution. The results were interpreted with the absorbance values at dual wavelength (450-630 nm).

The cut-off point for pediatric sera was 10AU. It was considered positive when the absorbance ratio of the calibrator and sample was > 1.1 and negative when the ratio was < 0.9. The absorbance measurement is directly proportional to the existing concentration of anti-*H. pylori* IgG antibodies. For the standardization of the enzyme immunoassay for the detection of specific IgG antibodies against *H. pylori*, sera with known serological status for *H. pylori* were used. The samples were analyzed using a blank and five calibrators. Additionally, the calibrators were dispensed as samples at the beginning, middle, and end of the ELISA plate.

Measurements were performed three times to verify the antibody detection status. The variation of absorbance value readings for the same sample was 2.6% (Intraclass correlation coefficient: 98.4%). The linearity and reproducibility of the measuring instrument were checked before performing the analyses.

The antigen and antibody detection assays were performed by the same operator who did not know the identity of the participants, nor the results obtained in the other diagnostic tests. The linearity and reproducibility of the measuring instrument were verified before the tests were performed.

Statistical analysis

To assess the validity of the tests ([^{13}C]-UBT, serum, and stool ELISA), the following parameters were estimated: sensitivity, specificity, predictive values (positive and negative), and the pre-analytical and post-analytical probabilities of each of these detection methods, using biopsy-based diagnosis as the "gold standard" or reference method. Analyses were conducted using Stata version 9.1. Sensitivity and specificity estimates obtained with the classical 2x2 contingency table analysis were compared with those obtained with a latent class model with four observed variables (histology, HpSA, serum ELISA, and [^{13}C]-UBT) using the LEM software.

Results

236 symptomatic children aged under 10 years underwent esophagogastroduodenoscopy. The prevalence of gastric mucosal colonization by *H. pylori* in children by histological evaluation was 21.6% (95%CI: 16.5-27.4); only in nine cases (3.8%) normal mucosa was observed. The results are similar to those observed with the different diagnostic tests (27.3%; 95%CI: 20.8-33.8), and the difference is explained by the fraction of false positives associated with the different diagnostic techniques used (table 1). The sensitivity achieved with the HpSA test was 97.9% and with the UBT and serum anti-*H. pylori* antibody detection test was 87.5% and 88.2%, respectively. Besides, the speci-

ficity of the HpSA test was 95.3% and the specificity of the UBT was 89.9%, comparable to that obtained with the serum ELISA test, but significantly lower than that estimated with the HpSA test (table 1).

The validity of the UBT varied according to the time of collection of post-basal samples and the liquid used to administer [^{13}C]-Urea. The lowest sensitivity (75%; 95%CI: 42.8-94.5) was observed when water was used and estimates were independent of time (table 2).

Table 3 compares the sensitivity and specificity achieved with the classical 2x2 contingency table analysis and those obtained by latent class analysis, using histology as the gold standard. The sensitivity and specificity estimates of the diagnostic tests compared were similar.

Discussion

Different investigations have described in detail the histopathology of *H. pylori*-associated gastritis in adults, however, the consequences of such infection in children are not well documented in Colombia because the prevalence of gastric neoplasia is low in this population, and the implementation of gastric endoscopy is often not necessary. Additionally, endoscopy is a relatively invasive method that can cause complications or inflict a psychological burden on children and/or their parents; thus, there are limitations to its use in children, especially after *H. pylori* eradication[5]. Therefore, the objective of this research was to estimate the validity of noninvasive tests used in the diagnosis of *H. pylori* infection in children under ten years of age in Cali, Colombia.

The results showed a lower sensitivity and specificity of the tests that detect anti-*H. pylori* antibodies in the serum of children (88.2% and 93.1%), compared to those observed in the HpSA test. Undetected antibody levels may occur when there is recent acquisition of the infection or in those who had the infection in the past but are currently negative. It is also likely that inactive coccoid forms have a variation or degradation of antigenic epitopes inducing a lower immune response and therefore higher false negative rates with anti-*H. pylori* antibody-based tests in these contexts. IgG antibodies develop within weeks of the onset of persistent *H. pylori* infection[13] and titers decline after clearance of infection, often reversing to seronegative within one to two years[14,15]. It can also be considered that the validity of serological assays developed in one population may be considerably diminished in other populations[16]. In addition, the usefulness of serological methods may be limited in studies with younger infants[17] because of the slow production of detectable antibodies in response to *H. pylori* infection[18]. Serology can be used in the diagnosis of *H. pylori* infection even when there are significant changes in the gastric mucosa that may lead to a low bacterial load in the stomach and lower sensitivity of other diagnostic methods[11,19,23]. In the clinical setting, the ESPGHAN/NASPGHAN guidelines for children do not recommend the use of serum, whole blood, urine, and saliva in antibody-based tests (IgG, IgA) for *H. pylori*[24].

With the UBT, the sensitivity and specificity were 87.5% and 89.9%, respectively. The UBT is the most researched and best-recommended noninvasive test for diagnosing *H. pylori* infection in the context of a test-and-treat strategy[11]. The UBT can semi-quantita-

Table 1. Validity of diagnostic tests for *H. pylori* en infection in symptomatic children under 10 years

Reference test		[^{13}C]-Urea		ELISA			
				Serum		Feces	
		(+)	(-)	(+)	(-)	(+)	(-)
Histopathological diagnosis	(+)	42	6	42	6	47	1
	(-)	151	12	53	7	141	17
Prevalence	Pr (A)	27.3%		25.1%		27.3%	
Sensitivity	Pr (+I A)	87.5%		88.2%		97.9%	
Specificity	Pr (-I N)	89.9%		93.1%		95.3%	
LR (+)	Pr (+ I A) / Pr (+ I N)	8.65		12.90		20.70	
LR (-)	Pr (- I A) / Pr (- I N)	0.14		0.13		0.02	
VPP	Pr (A I +)	71.2%		78.9%		87%	
VPN	Pr (N I -)	96.2%		96.4%		99.3%	

A: Probability of infection with *H. pylori*. VPP: Positive predictive value, VPN: Negative predictive value, LR: Likelihood ratio.

Table 2. Validity of the breath test according to the time of collection of the post-basal sample and the solution used to administer the [^{13}C]-Urea

[^{13}C]-Urea form of administration		Parameter	Breath test with [^{13}C]-urea. Sample collection time post - basal		
			20m	30m	40m
Policosa	n = 71	Prevalence	25.4%	23.9%	31.2%
		Sensitivity	94.1%	88.2%	76.5%
		Specificity	96.3%	94.4%	100%
Water	n = 67	Prevalence	25.4%	23.9%	32.5%
		Sensitivity	75%	75%	75%
		Specificity	90.9%	87.3%	87.3%
Orange juice	n = 77	Prevalence	18.3%	23.9%	28.6%
		Sensitivity	94.7%	94.7%	78.9%
		Specificity	89.7%	87.9%	87.9%

Table 3. Sensitivity and specificity of the tests used in the diagnosis of H. pylorien infection in children under 10 years of age according to conventional analysis and latent class analysis

	Sensitivity		Specificity	
Diagnostic method	Analysis 2x2	A.C.L	Analysis 2x2	A.C.L
Histology	1*	100.0%	1*	99.1%
ELISA (serum)	88.2%	91.4%	93.3%	95.0%
[13C]-UBT	87.5%	91.4%	89.9%	92.2%
ELISA (faeces)	97.9%	100.0%	95.3%	94.8%

*Reference test (by definition).

tively assess the *H. pylori* load in the stomach and is an appropriate method for detecting *H. pylori* infection in pediatric patients, with different specificity and sensitivity[11].

False-negative results can originate in patients with clinical conditions that can decrease bacterial loads in the stomach mucosa, such as the use of proton pump inhibitors (PPI), antibiotic use, bleeding peptic ulcer, atrophic gastritis with or without intestinal metaplasia, gastric cancer, MALT lymphoma, and partial gastrectomy[8]. In children under 6 years of age, the clinical application of UBT is also of limited value because the small stomach of children has a smaller volume of distribution of ingested ^{13}C-urea solution and the rate of endogenous $^{12}CO_2$ production is lower in young children than in older children or adults[7]. Another explanation for high false-positive results in young children is the presence of urease-producing microorganisms in the oral cavity, as young children are not willing to swallow ^{13}C-urea during the test procedure[12], or the liquid used to administer the ^{13}C-Urea and the post-basal sample-collection time influence the test result. Results obtained at 40 min and with water produced a higher proportion of false negative results compared to the other solvents.

The results found in this validation study with 236 children under 10 years of age showed an excellent performance with a sensitivity of 97.9% and specificity of 95.3% in the HpSA test for the diagnosis of *H. pylori*, similar to those observed in different investigations carried out in the United States, Europe, and Japan where sensitivity and specificity (97% each) were found[26]. In a Latin American study carried out in 87 children in Brazil[26] the sensitivity and specificity observed were 97% and 100%, respectively. *H. pylori* which is present in mucus and gastric juice is constantly eliminated into the intestine and expelled from the body in stools, the bacterium is found viable in a very low proportion of cases, but proteins and DNA can be detected with enzyme immunoassays or by PCR[25], respectively.

Sensitivity and specificity are considered measures of the performance of a diagnostic test compared to a "gold standard" or reference method. Unlike predictive values, which depend only on the inherent charac-

teristics of the diagnostic method and behave similarly when tests are applied to a group of patients in whom the disease is rare or to a group of patients in whom the disease is frequent, the sensitivity for detecting serum IgG anti-*H. pylori* antibodies and the sensitivity of the UBT were significantly lower in the population, similar to what has been described (6–10), while in the HpSA test, the sensitivity and specificity were the highest for the diagnosis of *H. pylori*.

In conclusion, the sensitivity for detecting serum anti-*H. pylori* IgG antibodies and the sensitivity of the UBT were significantly lower than the HpSA test in the diagnosis of *H. pylori* in children.

Ethical Responsibilities

Human Beings and animals protection: Disclosure the authors state that the procedures were followed according to the Declaration of Helsinki and the World Medical Association regarding human experimentation developed for the medical community.

Data confidentiality: The authors state that they have followed the protocols of their Center and Local regulations on the publication of patient data.

Rights to privacy and informed consent: The authors have obtained the informed consent of the patients and/or subjects referred to in the article. This document is in the possession of the correspondence author.

Conflicts of Interest

Authors declare no conflict of interest regarding the present study.

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