Use of the Noninvasive Entero-test in the Detection of *Helicobacter pylori* in Children in an Endemic Area in Colombia

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ABSTRACT

Background and Objective: Gastric infection with *Helicobacter pylori* (*H pylori*), a strong risk factor for gastric cancer, is highly prevalent in children residing in the Colombian Andes. We aimed to validate the use of the Entero-test to culture and genotype *H pylori* strains from asymptomatic Colombian children.

Methods: Children (ages 10–15 years, n = 110, 80 of which were *H pylori* positive by the urea breath test [UBT]) were subjected to the Entero-test, and strings were cultured and/or used for DNA extraction for polymerase chain reaction (PCR). These children had been treated for *H pylori* in 2007. A second population of children (ages 10–15 years, n = 95),which had not been previously treated, was also subjected to the Entero-test.

Results: Of UBT-positive children in the treated group, 29 of 80 (36%) Entero-test samples were *H pylori* culture positive; 29 additional string extracts were tested by PCR for the *H pylori* virulence factors *cagA* and *vacA*. PCR from cultures and extracts yielded a sensitivity of 74% and specificity of 87%. In the untreated group, 16 of 94 UBT-positive children (17%) produced Entero-tests that were culture positive. Fifty-eight of 94 (62%) string extracts were PCR positive for *cagA* and/or *vacA*. In previously treated children, *H pylori* strains were more often the less virulent *vacA* s2 ($P = 0.001$), m2 ($P = 0.006$), and i2 genotypes ($P = 0.039$).

Conclusions: The Entero-test may be used as a noninvasive test to detect *H pylori* in asymptomatic children residing in high-risk areas for gastric cancer. Treatment of *H pylori* in children was associated with less virulent genotypes.

Key Words: cagA, gastric infection, string test, vacA, virulence

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Gastric cancer is the fourth most commonly diagnosed cancer and second leading cause of cancer deaths worldwide, responsible for 738,000 deaths in 2008 (1). In many parts of the world, such as the Andean regions of Latin America, this disease is the most common cause of cancer-related death. Identification of *Helicobacter pylori* (*H pylori*) as a risk factor for gastric cancer (2) has revealed a new set of challenges for reducing the mortality of the disease. As part of ongoing studies in the natural history of infection, a cohort of children was previously established in the Andean region of Colombia, where *H pylori* has a high prevalence in untreated children (3), and the proportion of infected children increased with age (53% for 2-year-olds and 87% for 9-year-old asymptomatic children (4)). Large-scale investigations of *H pylori* in asymptomatic children in endemic areas have been limited because of the inability to obtain biopsies for ethical reasons. The Entero-test has been validated as a noninvasive procedure to obtain *H pylori* from gastric samples, with a variable diagnostic efficacy of culture and/or polymerase chain reaction (PCR) ranging from 37% to 97% (5,6); however, these studies have been performed in adults, and none have included children in an endemic area.

Previous studies have shown a difference in the virulence of strains in children depending on age and history of treatment. For example, some reports indicate that genotypes recovered from children are less virulent than those of adults (7). The virulence-associated genes *cagA* and *vacA* play a role in the pathogenesis of *H pylori*. The *cagA* gene, which encodes a 120- to 145-kDa protein, is a marker for the *cag* pathogenicity island and is found in >50% of *H pylori* strains (8). The *cagA* gene, closely associated with the *vacA s1* genotype, is considered an inducer of proinflammatory cytokines in the gastric mucosa and is a risk factor for disease (9,10). The *vacA* gene encodes an 88-kDa vacuolating cytotoxin (VacA) that forms pores in eukaryotic cell membranes (11). Within the *vacA* gene, 3 regions of marked sequence diversity can be distinguished: the s region (encoding the signal peptide) is present as either an s1 or an s2 allele, the intermediate region is present as either i1 or i2, and the m region (the middle region of the toxin) can be either m1 or m2 (12–14). The mosaic combination of s and m region alleles determines the production of the vacuolating cytotoxin and is associated with the pathogenicity of the bacterium. *VacA* s1m1 strains have been associated with increased virulence and greater gastric epithelial damage and ulceration than s2m2 strains (12,15). Our aim was to validate the use of the Entero-test as a noninvasive test to identify the presence of *H pylori* and evaluate the prevalence of *H pylori*–specific virulence genotypes in an endemic area.

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METHODS

Subjects

All subjects' parents provided informed consent for participation in accordance with the standard of the ethics review boards that approved this study: The Vanderbilt University institutional review board and the research ethics committee of the Universidad of Nariño. Two rural communities of children living in close proximity to each other with similar demographics in the endemic region of Nariño, Colombia, were selected. As part of an earlier study evaluating the effect of *H pylori* infection on growth velocity, a cohort of asymptomatic children was established in both communities in which 1 group of children (from towns of Nariño and Genoy) received a course of anti-*H pylori* treatment in 2007with poor results and the other (from towns of Laguna and Cabrera) was observed without treatment (3). The 2 regions have predominantly rural populations of similar ancestry and socioeconomic status. Both groups continue to be monitored by urea breath test (UBT). Within 4 to 8 weeks of a UBT (delta over baseline >5 parts per thousand was considered a positive test), subjects were offered the opportunity to participate in evaluation using the Entero-test. Children were recruited from both communities independent of UBT status. From the treated population, 110 children participated (80 UBT positive and 30 UBT negative); from the untreated population, 95 participated (94 UBT positive and 1 UBT negative).

String Samples (Entero-test)

The pediatric Entero-test consists of a small plastic capsule attached to a 90-cm string made of absorbent material. The capsule is swallowed with water and dissolves in the stomach lumen, leaving a string that collects gastric juices that may harbor *H pylori*. Before testing, to adjust the device for the individual, length measurements from the nose to laryngeal prominence to xyphoid process of each subject were recorded. Recorded measurements were subtracted from the total length of the Entero-test (90 cm) and the difference was excluded from the amount of string that could be swallowed, to reduce the risk of having the string enter the duodenum. Collection of samples in both populations was done by the same group of trained nurses who followed a uniform protocol for collection of samples, with the exception of the time the strings were left in the stomach, which was 30 minutes in the treated group. This initial time period was based on the study in adults by Yoshida et al (16), who reported comparable sensitivity with studies using longer incubation times (1 hour) (17–20). Following our evaluation of results from this protocol, and in an attempt to increase the percentage of successful cultures, we used an incubation time of 45 minutes in the untreated group. After removing the strings, pH of fluid from the string was checked using pH paper strips. The distal portion (approximately 5 cm) of each string was placed in transport media (thioglycolate + 20% glycerol) under sterile conditions, frozen, and transferred in dry ice to Vanderbilt University for culture and PCR (17,21). Frozen samples were thawed, plated on antibiotic plates (trypticase soy agar + 5% sheep blood, vancomycin 20 μg/mL, nalidixic acid 10 μg/mL, bacitracin 30 μg/ml, amphotericin 2 μg/mL), and placed in microaerobic conditions, using Campy Pak Plus envelopes (Difco/BBL, Lawrence, KS) at 37°C for 5 to 7 days. *H pylori* colonies were identified on the basis of morphology and positive tests for urease, oxidase, and gram stain. Single colonies were then frozen until removed for DNA extraction.

Preparation of Genomic DNA from Entero-test Samples

DNA was extracted from single colony cultures of *H pylori* using proteinase K (1 mg/mL in 50 mmol/L Tris/HCl buffer, pH 8.0, with 1 mmol/L ethylene diamine tetraacetate and 0.45% Tween 20) at 52°C overnight, followed by heating at 95°C for 15 minutes to denature the proteinase K. DNA was extracted with phenol/chloroform and precipitated with ethanol. DNA pellets were washed with 70% ethanol and resuspended in Tris-ethylene diamine tetraacetate buffer. For those subjects from whose Entero-tests no single colony cultures could be obtained, duplicate string segments were digested in 150 μL of proteinase K at 52°C overnight. Digested material was heated at 95°C for 15 minutes to denature the proteinase K, and the supernatant from this preparation was used as a template for PCR (22).

PCR Amplification and Genotyping of Isolated Strains

To reduce the risk of reaction contamination, PCR assembly was performed in a dedicated area, filtered pipette tips were used, and all handling of PCR products was performed in a different laboratory. String extracts and DNA isolated from single colonies underwent PCR for virulence factors (*cagA* and *vacA* s, m, and i regions). The *cagA* gene was detected with primers (CagAF, CagAR) yielding amplimers of 183 bp (23). To amplify *vacA* s regions, primers VA1F and VA1R were selected, resulting in generation of fragments of 259 bp for type s1 variants or fragments of 286 bp for type s2 variants (12). For the detection of the m region of the *vacA* gene, a mixture of 2 forward primers (M2F1, M1F2) and 2 reverse primers (M2R1, M1R2) was used in a simplex PCR, resulting in amplification of fragments of 300 bp for m1 or 200 bp for m2 strains (24). For the i1 region of the *vacA* gene, primers V335F and C1R were used; V335 and C2R were used for the i2 region resulting in amplification of fragments of 426 bp and 432 bp, respectively (11).

H pylori strain PZ5081 (genotype *cagA*+, *vacA* s1/m1) was used as a control for the presence of the *cagA* gene. Strains PZ5106 (genotype *cagA*+, *vacA s1/m2/i1*) and PZ5081 were used as controls for *vacA* genotypes. These positive controls for *cagA a*nd *vacA* genotypes had been validated by Sanger sequencing. Additionally, no-template controls were used in each experiment.

All PCR mixtures consisted of 0.2 mmol/L of each dNTP, 0.2 μmol of each forward and reverse primer, 1.0 U of Amplitaq Gold DNA polymerase (Invitrogen, Carlsbad, CA) in a final volume of 40 μL. Five microliters of DNA (20–50 ng) extracted from *H pylori* cultures was added to each reaction mixture. The PCR programs for *cagA* and *vacA*i amplification consisted of 15 minutes at 95°C, followed by 40 cycles of 1 minute at 94°C, 1 minute at 55°C, 1 minute at 72°C, and a final incubation at 72°C for 3 minutes. The amplification programs for *cagA* and *vacA* i were as follows: 15 minutes at 95°C, then 40 cycles consisting of 1 minute at 94°C, 1 minute annealing at 55°C, and 1 minute at 72°C, followed by a final incubation for 3 minutes at 72°C. The amplification program for *vacA* s was identical except that 45 cycles were used instead of 40, the annealing temperature was 52°C, and the final extension was 5 minutes at 72°C. For *vacA* m, 40 cycles were used, and the annealing temperature was 58°C. PCR products (10 μL of each sample) were electrophoresed in 2% agarose gels stained with ethidium bromide for 1 hour at 100 V.

Statistical Analysis

Sensitivity, specificity, and their 95% confidence intervals (CIs) for PCR using Entero-test culture or string extracts versus UBT were calculated with standard epidemiologic methods. A χ^2 test was used to study associations between categorical variables such as genotypes (*cagA*+, *vacA* s1/s2, m1/m2, i1/i2*)*, demographic

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parameters (sex), and outcomes of attempts at culture. A *t* test was used to compare age distributions between the 2 populations. The Fisher exact test was used for comparing numbers of mixed infections in the 2 populations. We considered $P \leq 0.05$ statistically significant. Calculations were performed using STATA statistical software version 12 (StataCorp, College Station, TX).

RESULTS

Patient Characteristics

In the treated group, the sex proportions were 55.5% male and 44.5% female; in the untreated group, the proportions were 49.5% male and 50.5% female. The average age was 12.50 years (95% CI 12.27–12.74) in the treated group and 12.21 years (95% CI 11.96–12.46) in the untreated group. Sex and age distributions between the treated and untreated groups were not significantly different. Prevalence of *H pylori* infection, as measured by UBT, was 73% (95% CI 63%–81%) in the treated group and 99% (95% CI 93%–100%) in the untreated group.

Entero-test Culture Yield

Treated Population

Culture yield for the Entero-test from UBT-positive subjects was 36% (29/80). Extracts of Entero-test strings failing to produce cultures underwent PCR for virulence factors (*cagA, vacA*), with detection of either the *cagA* or vacA genes from string samples considered a positive result. Yield of PCR for UBT-positive but culture-negative samples was 59% (30/51). Analysis of combined culture and string extracts provided an Entero-test yield of 74% (59/80). Of tests from 30 UBT-negative subjects, 3 nevertheless yielded cultures, and another produced positive PCR results from its string extract.

Untreated Population

A total of 95 Entero-test strings were collected. Culture yield for the Entero-test using the longer incubation time was 17% (16/94 UBT-positive children). Yield of PCR from string extracts from Entero-tests that did not produce single colony cultures was 74% (58/78 UBT-positive children). Analysis of combined culture and string extracts provided an Entero-test yield of 79% (74/94 UBT-positive children).

Culture yield was greater in the treated group (36%) when the Entero-test was left in the stomach for 30 minutes versus 45 minutes in the untreated group (17%). The longer time period was associated with an increase in contamination by other bacteria ($P = 0.0043$); however, the *H pylori* detection rate among UBT-positive children was not significantly different in the 2 groups (79% in the untreated group with 17% cultures vs 74% in the treated group with 36% cultures, $P = 0.48$)

Entero-test Sensitivity and Specificity

Treated Population

In addition to the 80 Entero-test samples collected from UBT-positive children, 30 Entero-test samples were obtained from UBT-negative subjects. From those 30 samples, we cultured *H pylori* from 3 samples and amplified *H pylori* DNA *(cagA)* from an additional sample (Table 1). Using UBT as the criterion standard, the Entero-test had a sensitivity of 74% (95% CI 63–83), specificity of 87% (95% CI 69–96), positive predictive value (PPV) of 94% (95% CI 85–98), and negative predictive value (NPV) of 55% (95% CI 40–70); however, if the 3 samples from UBT-negative subjects that produced *H pylori* cultures are reclassified as true-positives, the sensitivity, specificity, PPV, and NPV are 75% (95% CI 64–84), 96% (95% CI 81–99), 98% (95% CI 92–100), and 55% (95% CI 40–70). If culture of *H pylori* colonies alone (without analysis of string extracts) is compared with the UBT results, we obtain a sensitivity of 37% (95% CI 26–48), specificity of 90% (95% CI 73–98), PPV of 91% (95% CI 75–98), and NPV of 35% (95% CI 24–46). All colonies produced genotyping results for at least 1 amplicon.

Untreated Population

H pylori prevalence rates were naturally so high in these communities that insufficient numbers of UBT-negative children were available in the untreated community for calculating sensitivity, specificity, PPVs, and NPVs.

Genotyping of *H pylori* Strains

Primers for *cagA* and *vacA* amplified the expected fragments of 183 bp for *cagA*, 259 bp for *vacA s1*, and 286 bp for *vacA s2*. Primers for *vacA* m1/m2 and i1/i2 amplified the expected fragments of 300 bp/200 bp and 426 bp/432 bp, respectively.

As shown in Table 2, in samples from UBT-positive children in both groups, most samples were *cagA* positive (78% in the treated group and 80% in the untreated group). To eliminate strains that were false-negatives because of lack of amplifiable DNA, we removed those from the group of *cagA*-negative strains that had failed to provide a detectable signal for any amplicon. The majority of the strains in both groups were *vacA* s1 and/or m1, but the previously treated group had a significantly lower proportion of virulent s1 ($P = 0.001$), m1 ($P = 0.006$), and i1 ($P = 0.039$) strains compared with the untreated group. To examine the evidence for mixed infections, we evaluated PCR results from string extracts, using *vacA* s, m, and i assays, and counted the presence of any of the following: both s1 and s2, m1 and m2, or i1 and i2 for any extract, as a positive indicator for mixed infection. We found 14% (8/59) for the treated group and 1% (1/74) for the untreated group ($P = 0.01$).

TABLE 1. Diagnostic efficacy of the Entero-test

	Urea breath test						
Treated group Entero-test results	Positive	Negative	Total	Sensitivity, % (95% CI)	Specificity, % (95% CI)	PPV, % (95% CI)	NPV, % (95% CI)
Culture/extract positive	59	4	63	74 (63–83)	87 (69–96)	94 (85–98)	55 (40–70)
Culture/extract negative	21	26	47				
Total	80	30	110				

CI = Confidence interval; NPV = negative predictive value; PPV = positive predictive value.

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TABLE 2. Genotypes of *H pylori* strains from UBT-positive subjects

Gene (*cagA*)	Treated N (%)	Untreated N (%)	χ^2 *P* value
Positive	46 (78)	59 (80)	0.80
Negative	13*(22)	15* (20)	
Total	59	74	
Gene (*vacA*)			
s1	32 (59)	62 (91)	0.001†
s2	15 (28)	6 (9)	
s1/s2	7 (13)	0 (0)	
Total	54	68	
m1	38 (60)	54 (90)	0.006†
m2	17 (31)	5 (8)	
m1/m2	0 (0)	1 (2)	
Total	55	60	
i1	18 (72)	28 (97)	0.039‡
i2	6 (24)	1 (3)	
i1/i2	1 (4)	0 (0)	
Total	25	29	

H pylori = *Helicobacter pylori*; UBT = urea breath test.
* Analysis excludes samples providing no signal for any virulence gene, as those are likely to be negative for technical reasons related to insufficient amplifiable DNA.
† Statistical significance.
‡ Statistical significance if i2 and i1/i2 are combined.

DISCUSSION

The most widely used methods for the diagnosis of *H pylori*, such as culture and histology, are sensitive and specific but require invasive procedures and gastric biopsies. Among noninvasive procedures, the UBT is considered both sensitive and specific in the detection of *H pylori*, but it is not useful in the culture and characterization of the organism. The use of the Entero-test in adults has had a variable diagnostic use for culture and/or PCR ranging from 37% to 97% (6), but no published studies have included children. Our study is the first to evaluate the use of the Entero-test in a large cohort of asymptomatic children living in an endemic area at high risk for gastric cancer. When we combined results obtained from both culture and string extracts, we obtained diagnostic efficacy comparable (74% and 79% for treated and untreated populations, respectively) with that of adult studies (5,6).

The Entero-test allows an evaluation of *H pylori* genotypes in children, without the selection bias inherent in the requirement for endoscopy. Although bias may never be completely excluded, we think that it is likely that our study provides a less biased evaluation of *H pylori* genotypes in asymptomatic children compared with methods using endoscopy, which require that a child be symptomatic. Our Entero-test protocols differed in the length of time that the string was left in the stomach (30 minutes in the treated population vs 45 minutes in the untreated population). Assuming that this difference is unlikely to introduce a bias that will affect genotypes observed, we can evaluate genotypes observed in the treated and untreated populations. In both groups, the prevalence of markers of virulence in asymptomatic children was high. The percentage of *cagA*-positive samples from infected children was similar in both populations (78% in the treated group and 80% in the untreated group), whereas the percentage of *vacA* s1 alleles was 59% (treated group) and 91% (untreated group). Past studies in adults in Nariño, Colombia, have analyzed genotypes in infected patients for whom *H pylori* therapy failed to eradicate the infection and found an increased proportion of low virulence strains, namely *cagA* negative and *vacA* s2/m2 (25). Our findings in children are consistent with those in adults, supporting the idea that treatment, even if it fails to eradicate the infection, may select for less virulent strains. Since the antibiotic treatment 3 years before the present study, children had not been tested for antibiotic resistance in their infections, nor were infections classified as reinfections or recrudescence. Therefore, selection of less virulent strains by treatment of children should be confirmed by following subjects at more frequent time points. Strains obtained by culture of Entero-tests may allow the possibility of addressing such questions in future studies in asymptomatic children.

Our method of evaluating mixed infections from the string extracts may be considered a conservative estimate, given that we are examining polymorphisms in only 1 gene, *vacA*, and that a high proportion of strains in these populations are of s1/m1/i1 genotypes. Nevertheless, it is interesting that the treated population of children shows significantly more mixed infections, consistent with the effect of treatment favoring the less virulent strains over the originally predominant high virulence *vacA* s1/m1/i1 strains.

The fraction of children in our untreated cohort that was *H pylori* positive by UBT was high (99%), so that we were unable to calculate sensitivity, specificity, PPV, or NPV with the untreated subjects. As we reported earlier, the proportion of infected children in these mountain populations increases with age (4). Our study suggests that this increase continues into the early teenage years. In developed countries, recent birth cohorts are increasingly failing to develop persistent *H pylori* infections (26,27), with sequelae that may predispose to asthma and esophageal diseases (28–32); however, we see no evidence that *H pylori* prevalence is decreasing in the rural populations that we have studied here, suggesting that the decreases in incidence rates of gastric cancer seen in developed countries are unlikely to be duplicated soon in these populations.

The percentages of *cagA* and *vacA* s1 strains are higher than those reported from children in other countries, even including symptomatic children. In gastric biopsies from symptomatic Portuguese children, *cagA* was detected in 36.1%, and *vacA* s1 was detected in 32.7% of children (33). Lower frequencies of both *cagA* (47%) and *vacA* s1 (58%) have been reported in gastric biopsy isolates from Mexican children with recurrent abdominal pain but no peptic ulceration (34). The findings in our study indicate the presence of virulent strains in asymptomatic children in a population with a high incidence of gastric cancer in adults (35). High prevalence of virulent genes in asymptomatic children may contribute to the high incidence of gastric cancer in adulthood and indicates the importance of early detection of *H pylori*, larger epidemiological studies, and continued surveillance. In conclusion, the Entero-test may be used as a noninvasive test to detect *H pylori* in asymptomatic children residing in areas of high incidence of gastric cancer. Technical improvements to increase the sensitivity and NPV of the Entero-test would be beneficial to increase its use for detecting *H pylori* in these high-risk populations.

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