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Significance of Transiently Positive Enzyme-Linked Immunosorbent Assay Results in Detection of *Helicobacter pylori* in Stool Samples from Children

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In young children, the significance of stool samples transiently positive for *Helicobacter pylori* antigen is unknown. As part of a larger prospective study on enteric infections, stool samples were obtained from 323 children at two time points 3 months apart and tested for *H. pylori* antigen using a commercially available enzyme-linked immunosorbent assay (ELISA) test. Seminested PCR for a *Helicobacter*-specific 16S rRNA gene was performed on all 26 pairs reverting from positive to negative (transient positives), all 4 persistent antigen-positive pairs, and 10 randomly selected persistent antigen-negative pairs. *Helicobacter* species were amplified from the first stool samples of 15/26 (58%) of the transient positives and 1 (25%) of 4 persistent positives. No *Helicobacter* species were amplified from the 10 persistent negatives. Among the 15 amplicons from transient-positive stool, *H. pylori* was sequenced and identified from 12 (80%; 95% confidence interval, 52% to 96%) and other *Helicobacter* spp. were identified from three (*Helicobacter canis*, *Helicobacter winghamensis*, and MIT 99-5504). Four of the 15 remained positive by PCR for the second (antigen-negative) stool sample, including all 3 initially identified as non-*H. pylori*. *Helicobacter bilis* was amplified from the second sample of a persistent positive. Two of eight transient positives from whom serum was available had accompanying transient elevations in anti-*H. pylori* antibodies. Transiently positive stool ELISAs for *H. pylori* are common and represent *H. pylori* in the majority of cases where sequences can be obtained. A not-insignificant percentage of antigen-positive stools, however, may represent other *Helicobacter* species.

Helicobacter pylori chronically colonizes the human stomach. Little is know about the transmission of *H. pylori*, although most cases appear to be acquired in childhood. Because the date of acquisition of *H. pylori* is rarely known, most clinical and epidemiological studies have focused on chronic infection. Some data do indicate, however, that transient *H. pylori* infection can occur. For example, among patients who have been infected experimentally (10, 14) or endoscopically (12), the majority with reported follow-up had spontaneous resolution of infection. In children, transiently positive breath, serum, and stool antigen tests for *H. pylori* infections have been reported (17, 21, 24). The validity of these findings, however, has been uncertain due to the lack of standardization of these diagnostic tests in young children.

In a cohort study designed to identify risk factors for *H. pylori* transmission, we collected two sequential stool samples from 323 children. Among these children, 26 reversions (i.e., transient stool positives) were discovered. We conducted a study to determine whether these transiently positive stool samples represent true transient infections or false-positive stool antigen tests.

MATERIALS AND METHODS

Study participants. As part of a large cohort study on transmission of enteric infections (20), households with an index case of gastroenteritis, with at least one additional participating member, were recruited through cooperating community health care settings as well as community outreach. An index case was defined as a case of diarrhea with or without vomiting characterized by at least five stools per day and lasting no more than 14 days. There was no age restriction for index cases. Episodes of possible noninfectious etiology, such as pregnancy, poisoning, or drug effects, were excluded. This source population is predominately low-income and Hispanic, including a large percentage of foreign-born people. A brief telephone interview confirming study eligibility was followed by a home visit within 14 days of the index case of gastroenteritis reported in the home. Visits were conducted by trained research staff fluent in the primary language of the home. After providing informed consent, a structured questionnaire was administered regarding household demographics, socioeconomic markers, risk factors for *H. pylori* infection, household composition, and family relationships. Blood samples were obtained from all consenting household members. Because of concerns about the accuracy of *H. pylori* serologic testing in young children, as well as resistance to phlebotomy in this age group, stool samples were obtained in children 2 years and younger as well as from older children when consent was not provided for a blood draw. Approximately 12 weeks later, a second follow-up visit was conducted to collect samples and collect logs kept by the subjects regarding household episodes of gastroenteritis.

Stool ELISA. Stool samples collected at home visits were transported directly back to the laboratory and stored at −20°C until processed. When samples were not available at the home visits, shipping supplies were left with the subjects and samples were sent by overnight mail. The Meridian (Cincinnati, OH) Premier Platinum HpSA enzyme-linked immunosorbent assay (ELISA) was used to detect the presence of *H. pylori* antigens in stool; the protocol was followed as directed. Briefly, stool samples were diluted, added to antibody-coated microwells, and incubated. *H. pylori*-specific polyclonal antibodies conjugated to horseradish peroxidase were added, incubated, and washed before peroxidase was added; a visible yellow reaction indicated the presence of *H. pylori*.

Stool pairs from children less than 18 years of age were categorized as persistently positive (both stools positive), persistently negative (both stools negative), transiently positive (first positive, second negative), or converting (first negative, second positive). Because no third sample was obtained from this last

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TABLE 1. Characteristics of subjects by classification of stool antigen pairs

Group[a]	HpSA		No. of subjects (no. of sequences analyzed)	Age (median yrs)	Sex (% male)	No. (%) with gastroenteritis
	Visit 1	Visit 2				
Transiently positive	Positive	Negative	26 (26)	0.96	14 (57)	21 (81)
Persistent positive	Positive	Positive	4 (4)	1.4	3 (75)	4 (100)
Persistent negative	Negative	Negative	278 (10)	1.0	157 (56)	239 (86)
Total			308 (30)	1.0	174 (56)	264 (86)

[a] Not shown are 15 subjects converting from negative to positive who are the subject of a separate study.

group, we cannot determine whether these represented true conversions or transiently positive stools. Thus, this group is excluded from further discussion.

DNA isolation, seminested PCR, and sequencing. DNA was isolated from paired stool samples of all persistently and transiently positive stool pairs along with 10 randomly selected persistently negative samples using the QIAGEN (Valencia, CA) QIAmp DNA stool mini kit. The given protocol was followed, and the final elution volume was 200 μl.

PCR confirmation was done with seminested *Helicobacter* species-specific 16S rRNA gene (4), starting with primers C97, 5′-GCT ATG ACG GGT ATC C-3′ (276 to 291 forward), and C05, 5′-ACT TCA CCC CAG TCG CTG-3′ (1478 to 1495 reverse). Ten microliters of the isolated DNA was added to a 100-μl reaction mixture containing 2 U of *Taq* polymerase, a 0.25 μM concentration of each primer, 10 μl of 10× buffer, 0.2 mM deoxynucleotide triphosphates, and water. The second reaction was identical to the first with the exceptions of the primer pair being C97 and C98, 5′-GAT TTT ACC CCT ACA CCA-3′ (381 to 398 reverse), and the template being 2 μl of a 1/100 dilution of the product from the first reaction. After visualization, PCR amplicons were extracted from agarose using the QIAGEN (Valencia, CA) QIAquick gel extraction kit. The protocol was followed as directed, and elution volumes were dependent on DNA concentrations (determined by gel electrophoresis visualization) such that the final volume of 22 μl contained 80 ng. After addition of primer, sequencing was performed using the Amersham (Piscataway, NJ) ET Terminator and ABI (New York, NY) Big Dye sequencing chemistry system and an ABI 3100 sequencer.

Serum ELISA. Blood samples were centrifuged, and serum was stored at 4°C for later analysis. An in-house serum ELISA was used to evaluate *H. pylori* status as previously described (16). Briefly, high-binding microwell plates were coated with antigen isolated from multiple *H. pylori* strains. Dilute serum samples were added to the wells in triplicate and incubated before addition of a peroxidase-bound secondary immunoglobulin G (IgG; Sigma-Aldrich, St. Louis, MO), another incubation, and finally addition of a substrate showing *H. pylori* status. This ELISA has sensitivity and specificity of 86% and 100%, respectively, in children (20); it has not been specifically tested for validity in children less than 2 years old.

IgM titers were determined for nine available serum pairs. To assess the serum IgM responses to *H. pylori* whole-cell antigen preparation, an ELISA based on an extensively characterized pool of sonicates from five U.S. strains was used (18, 19). For the IgM ELISA, sera diluted 1:20 were added in duplicate and incubated before addition of a secondary IgM (Biosource, Camarillo, CA). Positive samples were those with optical densities greater than the mean plus 2 standard deviations of persistently seronegative samples tested.

RESULTS

A total of 323 children provided stool samples at both visits 3 months apart. A total of 75% of these children were index cases for their households. Children (56% male) ranged in age from 10 days to 12 years (median, 1 year), with 84% under age 2. A total of 264 (86%) children had experienced an episode of diarrhea with or without vomiting within 2 weeks of the first home visit. The 323 children resided in 289 homes containing a median of 6 members (range, 2 to 18). In 220 (76%) homes, Spanish was reported as the primary language.

Of the 323 paired stool samples, 26 (8.1%) were transiently positive, 278 (86%) were persistently negative, and 4 pairs (1.2%) were persistently positive (Table 1). Conversions (negative to positive) were found in 15 (4.6%) pairs and are excluded from further consideration here. A total of 73% (22/30) of the positives (18/26 transiently positive and 4/4 persistent positive) resided with at least one other *H. pylori*-positive person; however, this did not differ significantly from persistent negatives (69%). A difference was noticed when comparing the number residing with at least one other person who reported symptoms consistent with infectious gastroenteritis: 20 of 30 (67%) positives (17/26 transiently positive and 3/4 persistent positive) compared to 49% of persistent negatives ($P = 0.06$).

Seminested PCR was conducted on all 26 transiently positive sample pairs, all 4 persistently positive stool antigen pairs, and on a random selection of 10 persistently negative stool antigen pairs. Among the 26 transiently positive stools, sequenced PCR products from the first visit sample confirmed the presence of a *Helicobacter* sp. in 15 (58%), and one sample was indeterminate (Table 2). Twelve of the 15 16S rRNA gene sequences (80%; 95% confidence interval, 52 to 96) were consistent with *H. pylori*; the remaining three were consistent with *Helicobacter canis*, *Helicobacter winghamensis*, and MIT 5504 (2, 5, 11). In 4 of the 15 initially antigen-positive samples, the second stool sample was also positive for *Helicobacter* sp., despite a negative stool antigen test; three of the four second-visit amplicon-positive samples came from subjects with non-pylori *Helicobacter* sp. sequenced from the first visit sample.

Among the four children with persistently positive stool antigen tests, two each yielded a single amplicon at only one of the two visits (25%). On sequencing, one child had *H. pylori* in the first stool sample only and the other child had *Helicobacter bilis* in the second sample only. Thus, among 17 children with positive sequences for *Helicobacter* spp., 4 (23.5%; 95% confidence interval, 6.8 to 49.9%) represented non-pylori species. All 10 pairs of samples from children with persistently negative stools were negative for *Helicobacter* amplicons.

Serum samples were available at both time points for 8 transient positives, 2 persistent positives, and 148 persistent negative pairs (including 5 of the 10 randomly selected for sequence analysis). Of the eight transients with serum, two (25%) also had transiently positive titers of anti-*H. pylori* IgG with a >2-fold reduction in titer between visits. Six of the eight (75%) were consistently seronegative without remarkable change in titer between visits. Both of the persistently positive stool antigen children were persistently seronegative without notable changes in titer, including the child with confirmed *H. bilis*. Of the 148 persistently negative children with serum results, 143 (97%) were seronegative at both visits and 5 had positive or borderline results at one (3 children) or both (2 children) visits.

Paired IgM results were also available for 9 (3%) of the 308 children, including 7 transients, 1 persistent positive, and 1 persistent negative stool antigen pair. Only one transient positive had a positive IgM at the first time point only; this child was positive

TABLE 2. Results of stool PCR and sequencing from 26 transiently positive and 4 persistently positive children

Stool type and subject ID	1st PCR	1st sequence[a]	2nd PCR	2nd sequence[a]
Transiently positive stools				
1-1	+	*H. canis*	+	Indeterminate
52-2	+	*H. pylori*	−	
203-2	+	*H. pylori*	−	
208-1	−		Indeterminate	
208-4	+	*H. pylori*	−	
269-3	−		−	
277-1	+	*H. pylori*	−	
445-3	+	*H. pylori*	−	
691-1	+	*H. pylori*	−	
828-1	+	*H. pylori*	−	
902-4	+	*H. pylori*	−	
1121-4	+	*H. winghamensis*	+	*H. winghamensis*
1850-1	+	*H. pylori*	+	*H. pylori*
1877-1	+	MIT 99-5507	+	Indeterminate
1953-1	−		−	
2077-1	−		−	
2084-5	+	*H. pylori*	−	
2197-3	+	*H. pylori*	−	
2273-1	−		−	
2304-1	+	Indeterminate	−	
2439-1	−		−	
2541-1	−		−	
2724-1	+	*H. pylori*	−	
3012-1	−		−	
3199-1	−		−	
3201-3	−		−	
Persistently positive stools				
407-1	−		+	*H. bilis*
2558-1	−		−	
3003-1	+	*H. pylori*	−	
3279-1	−		−	

[a] Where no sequence result is given, no data were available.

for IgG also only at the first time point. The remaining eight children had negative IgM results at both time points.

DISCUSSION

In this study, we were able to detect *Helicobacter* species in 15 (58%) of 26 antigen-positive stools from children who subsequently had antigen-negative stool samples, including 12 (46%) confirmed for presence of *H. pylori* sp. Given the relatively low sensitivity of stool PCR for *Helicobacter* sp. using our methods (approximately 25% to 50%), we conclude that the great majority of transiently positive stools are likely to represent transient infection with *Helicobacter* sp. (7, 26). The lack of IgG seroconversion or IgM seropositivity in a small subsample supports the transient nature of these infections, although the validity of serology in young children is not well established (8, 15, 25).

Transient *H. pylori* infection may play an important role in the permanent acquisition of *H. pylori*. Transient *H. pylori* infections have previously been reported serologically (i.e., seroreversions), with breath tests, and with stool antigen testing. In our study, 26 (8%) of 323 had a transient *Helicobacter* infection at a relatively random date in their lives. Thus, it seems highly probable that children—particularly from more-crowded households—have frequent and recurrent exposure to *H. pylori* that only occasionally progresses to persistent infection. In our study we have shown a trend ($P = 0.15$) for persistent positives and transients to reside with at least one *H. pylori*-infected symptomatic person. In both experimental (10) and accidental (12, 13) exposures of humans to the organism, *H. pylori* detection and associated inflammation were followed by spontaneous clearance within 14 days in a subset of subjects; in others the infections persisted. Epidemiological studies have also shown evidence of transient *H. pylori* infections in young children (17) and patients previously cured of *H. pylori* (9). In addition to humans, transient infections have also been reported in previously cured macaques (23). We speculate that exposure to *H. pylori* is common and that chronicity is established in the minority of cases. The reasons for persistence or lack thereof are unknown.

A not-insignificant proportion of *Helicobacter* spp. identified in the children's stools were non-pylori *Helicobacter* sp. Among 17 children with amplifiable DNA from stools (15 transient positives and 2 persistent positives), PCR with sequencing indicated infection with non-pylori species, including *H. winghamensis*, *H. bilis*, *H. canis*, and MIT 99-5504. These organisms are typically considered zoonoses (*H. winghamensis* and *H. bilis* in rodents, *H. canis* in dogs and cats, and MIT 99-5507 in rhesus monkeys with colitis), although rare reports of diarrhea (*H. canis* and *H. winghamensis*) or biliary disease (*H. bilis*) in humans have also been made (3, 4, 11). Among the children with non-pylori *Helicobacter* infections in our study, all four suffered from gastroenteritis in the 2 weeks prior to initial stool culture. These symptoms, however, were not appreciably different from those of other children in our study, the majority of

whom were index cases of gastroenteritis. Given the subject selection criteria, the indistinct symptomatology of individual enteric pathogens, and the infrequency of each of the non-pylori species, we can draw no conclusions about these organisms' pathogenicities. It is even uncertain as to whether these organisms were responsible for the positive stool antigen tests. Although none of the stools from 10 children with persistently negative antigen tests yielded non-pylori amplicons, 3 of the children with transient positive antigen tests continued to yield *Helicobacter* even after their stools were no longer antigen positive. Thus, it remains possible that these *Helicobacter* species were coincidental, rather than causal, for initial antigen positivity. In a rodent model, however, Sjunnesson and colleagues found significant cross-reactivity in HpSA between the *H. pylori* and other *Helicobacters*, including *H. bilis* and *H. canis* (*H. winghamensis* and 99-5507 were not tested) (22). Thus, we believe between 6.8% and 50% of stool *Helicobacters* in children represent non-pylori species and that these may create false-positive antigen tests.

The proportion of confirmed *H. pylori* in transient-positive stools (46%) was higher than that observed in persistent-positive stools (12.5%). This would suggest that shedding of the organism diminishes with increasing chronicity of infection. We have previously shown that hypochlorhydria may enhance *H. pylori* shedding in stool (6). Others have shown that acute *H. pylori* induces hypochlorhydria in some subjects (1, 14). Thus, through its physiological effects, acute *H. pylori* infection may perpetuate transmission to other hosts, whether it becomes chronically established or not. Further studies will be needed to understand the discrepancies in these findings, particularly in light of limited data on stool antigen specificity in young children.

The prevalence of *H. pylori* is decreasing over time in the United States and other developed countries. Despite this decline, our data suggest that *H. pylori* is circulating at a high endemic rate among a lower-socioeconomic-status U.S. population. Of 30 children with *H. pylori* infection at first testing, 87% were no longer shedding the organism 3 months later. Thus, most infected hosts eliminate infection, at least some of the time. It is likely that large exposures, recurrent exposure, or exposure with the right confluence of cofactors—i.e., nutritional status, coinfections, and gastric acid level—are required to establish chronicity. Understanding the factors that determine success of *H. pylori* colonization will undoubtedly shed critical insights into prevention and control of this common pathogen.

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