

## Detection of *Helicobacter pylori* by Real-Time PCR for 16s rRNA in Stools of Noninfected Healthy Children, Using ELISA Antigen Stool Test as the Gold Standard

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### Keywords

*Helicobacter pylori*, childhood infection, stool PCR, stool antigen detection.

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### Abstract

**Background:** We previously detected *Helicobacter pylori* infection by stool antigen ELISA assay in 33–41% of asymptomatic Chilean children between 2–3 years of age, of which 11–20% had a transient infection and 21–22% a persistent infection. A total of 88% of ELISA-positive samples were also rtPCR positive, while 37/133 (33%) of ELISA-negative stool samples were rtPCR positive. The significance of a ELISA-negative/rtPCR-positive sample requires clarification. We aimed to determine whether rtPCR is able to detect persistent infections not detected by ELISA.

**Materials and Methods:** We selected 36 children with an ELISA-negative/rtPCR-positive stool sample, of which 25 were never *H. pylori* infected according to ELISA, and 11 had a transient infection with an ELISA-positive sample before or after the discordant sample. At least two additional consecutive ELISA-negative samples per child were tested in duplicate by rtPCR for the 16s rRNA gene.

**Results:** A total of 14 of 78 (17.9%) rtPCR reactions were positive, but only 4/78 (5.1%) were positive in both duplicates, representing a total of 3/36 (8.3%) children with an additional rtPCR-positive sample, only one of whom was persistently negative by ELISA. One child with a transient infection had two positive rtPCR reactions despite negative ELISA samples.

**Conclusions:** In *H. pylori* noninfected or transiently infected children, as determined by stool ELISA, additional ELISA-negative/rtPCR-positive stool samples were found in 8.3% of children, but a possible persistent infection was only identified in 2.7% of children. Thus, the characterization of infection dynamics in children is not being misrepresented by application of stool ELISA. Furthermore, rtPCR does not significantly improve dynamic characterization.

The importance of *Helicobacter pylori* (*H. pylori*) infection in gastroduodenal disease is well established [1]. However, most infected individuals never experience clinically relevant signs and/or symptoms [2]. There is evidence that infection is predominantly acquired mostly during the first few years of life, even prior to 12 months of age in low-income populations [3]. Reliable noninvasive diagnostic methods have been developed for *H. pylori* detection, including Urea Breath Test, Immunoassays for *H. pylori* antibodies, stool antigen detection tests for *H. pylori*, and gene amplification in saliva and stools [4].

Antibody-based enzyme-linked immunosorbent assay (ELISA) for *H. pylori* detection in stools has shown high sensitivity (85%) and specificity (95%) compared to invasive tests in adult and pediatric populations [5,6]. Real-time PCR (rt-PCR), meanwhile, is not widely used for stool detection of *H. pylori*, and results have been variable, with some studies, reporting sensitivity levels of up to 65%, and a specificity of 92%. [7,8].

Ongoing studies focused on healthy child cohorts by our research group have used HpSA ELISA (Premier Platinum HpSA; Meridian Diagnostics, Cincinnati, OH) to analyze stool samples obtained every 3 months, in

order to determine the prevalence of pathogen- and host-related factors associated with *H. pylori* infection acquired during the first years of life [9,10]. Based on these studies, infection status in asymptomatic children is currently categorized into two groups: transient infections (1–3 nonconsecutive ELISA positive stool samples, followed by persistently negative stool samples) or persistent (three or more consecutive positive samples). In a first cohort (*Birth cohort*, 96 children followed from birth to age 5), 41% of children were ELISA positive for at least one stool sample, of which 21% of children had a persistent and 20% a transient infection [9]. In a second cohort (*1-year-old cohort*, 228 children in followed from 12 months of life to age 4), 33% of children had at least one ELISA-positive stool sample, of which 22% were persistently and 11% were transiently infected [11].

Of 579 *ELISA-positive* samples, a subset of 138 samples were analyzed by real-time PCR (rtPCR) for the presence of 23S and/or 16S rRNA. Compared to ELISA, rtPCR was highly concordant for positive samples; 88% of persistent and 86% of transient ELISA-positive samples were also rtPCR positive. In addition, 113 *ELISA-negative* samples were tested; however, 37 (33%) were found to be rtPCR positive [10].

The fact that 33% of ELISA-negative samples were rtPCR positive poses an important question. ELISA was chosen as the *H. pylori* detection technique because several studies show that it is the most accurate noninvasive test for both adults and children. Alternatives include histopathology or urease test [11], both of which are invasive tests that require upper endoscopy and biopsy of gastric tissue. Conceivably, infection could be occurring in children despite ELISA negativity in stools, as suggested by these positive rtPCR results. We aimed to determine whether persistent infections could be accurately detected by rtPCR, despite the possibility of false ELISA negative results. For the purpose of this study, and due to an ethical inability to perform upper endoscopy in asymptomatic children, ELISA antigen stool tests were used as our “gold standard”, and rtPCR for 16S rRNA as the test method.

## Methods

### Overall Design, Participants, Samples

This study was based on two previous prospective cohort studies of Chilean children, from whom stool samples were collection every 3 months. For this study, we selected 36 subjects with an *ELISA-negative/rtPCR-positive* stool sample from both the *Birth cohort* and *1-year-old cohort*. Twenty-five of these children had never been infected with *H. pylori* and 11 had a transient

infection, defined by 1–3 nonconsecutive positive ELISA samples (only ELISA negative samples from these children were included in this study). *H. pylori* infection status was based on ELISA results.

To determine the proportion of these children with a possible persistent infection detectable only by rtPCR, two to three ELISA-negative stool samples from each child were tested by rtPCR for 16S rRNA. Samples were selected in consecutive order, *that is*, just prior and/or subsequent to the current *ELISA-negative/rtPCR-positive* sample. A total of 78 samples were analyzed, as described in Fig. 1 (upper section) and Fig. 2A.

To confirm the reproducibility of the rtPCR protocol, five samples from infected children (previously confirmed by ELISA and rtPCR) were reanalyzed by rtPCR (Figs 1 and 2A).

### Procedures

Stool samples were stored at  $-70^{\circ}\text{C}$ . Bacterial DNA was extracted from infant stool samples using the QIAamp DNA stool kit (Qiagen Sciences, Germantown, MD), following the manufacturer’s instructions with a bead-beating step added to increase the yield of purified DNA. Detection of 16S rRNA was performed using a previously described primer [12].

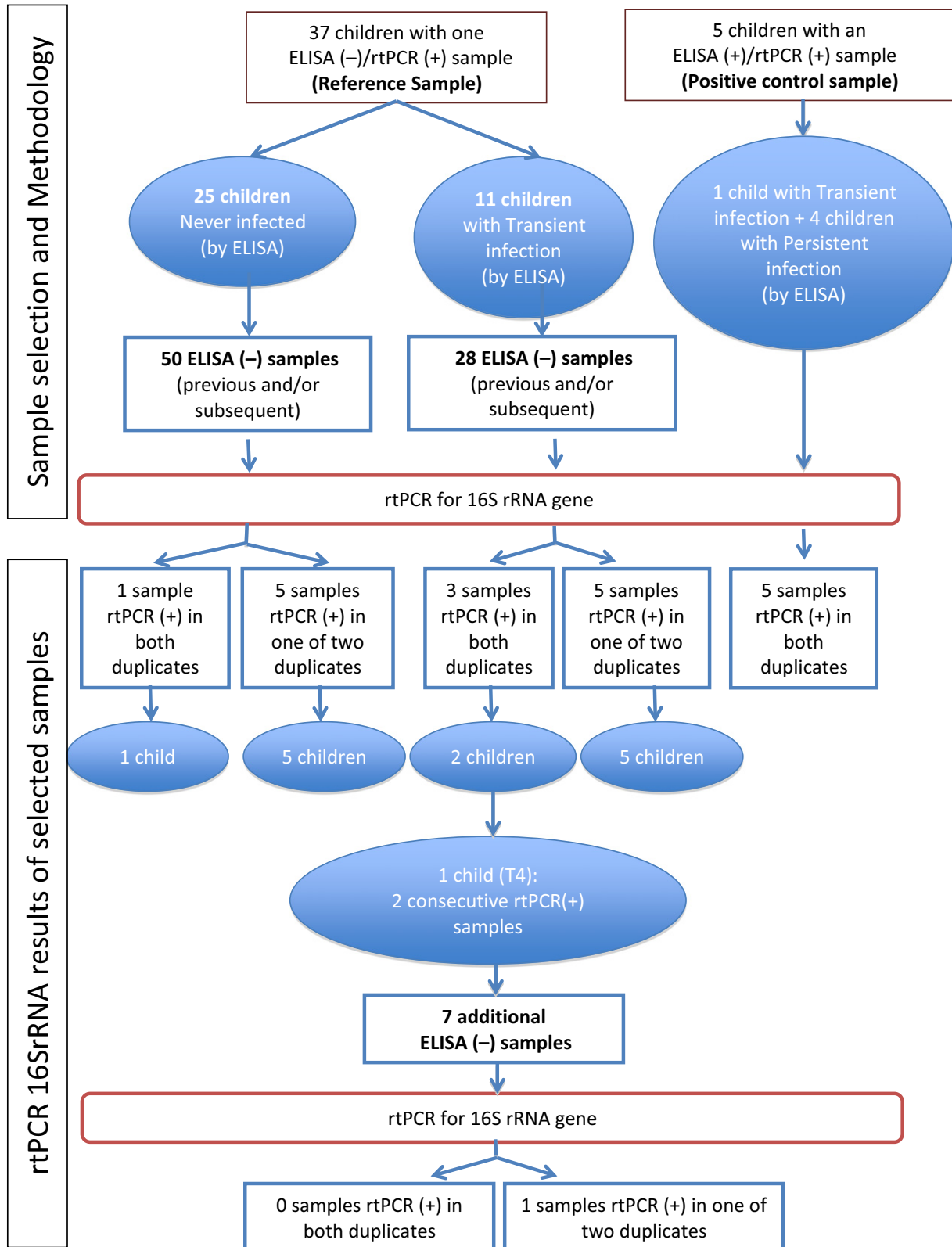
Amplification was performed in a reaction volume of 15  $\mu\text{L}$  with Fast EvaGreen dye qPCR master mix (Biotium, Hayward, CA) with 4  $\mu\text{L}$  of total purified DNA from each stool sample. The PCR was performed using the Roche LightCycler<sup>®</sup> Real-Time PCR System. A three-step PCR was used, followed by melting curve analysis. Strain ATCC 43504 was used as the positive control. Amplification of total bacterial 16S rRNA gene was performed using an in-house PCR in order to identify samples with possible PCR inhibitors. Each sample was analyzed in duplicate. When samples were run in duplicate and resulting in conflicting results (*i.e.*, one positive and one negative), samples were re-run two additional times.

### Statistical Analysis

Comparison of the prevalence of detection by rtPCR between never infected and transiently infected groups was performed using a Z- test (using the software Epi-tools) [13].

### Results

As expected, rtPCR for 16S rRNA was consistently positive in the five control samples previously positive by both ELISA and rtPCR (Figs 1 and 2B).



**Figure 1** Overall study design and rtPCR 16s rRNA results of analyzed samples. The upper part of flowchart details sample selection and methodology; the lower part of flowchart shows rtPCR results of selected samples.





2B). Two of the eleven children with a transient infection by ELISA (18.1%) had one or two additional rtPCR-positive samples in both duplicates, of which one had three positive rtPCR samples, including the reference sample and the two additional samples analyzed in this study (Figs 1 and 2B). There were no significant differences between the two groups ( $p$ -value = .16). In order to corroborate the presence of a persistent infection in the child who had two additional positive samples (Figs 1 and 2B, child T4), we performed rtPCR on every available ELISA-negative stool sample from that child, including two samples prior to and five subsequent to the previously analyzed samples. Only one of seven additional samples had a positive result in one of the duplicates, even after two additional repetitions. The remaining six samples were consistently negative.

Overall, rtPCR testing for 16S rRNA was able to detect a possible persistent infection not detected by ELISA in one of 36 (2.7%) children.

## Discussion

Comprehensively understanding the dynamics of *H. pylori* infection in young children by following cohorts for a prolonged period of time should shed light on the agent–host relationship and the occurrence of disease later in life [9,10]. Reliable noninvasive tests on stool samples as a proxy for an *H. pylori* infection occurring in the stomach are particularly useful for our understanding infection dynamics in apparently healthy children. While antigen detection in stools by ELISA has proven to be reliable, as it is both highly sensitive and specific when compared to urease test and gastric biopsies [5], amplification of bacterial genes in stools by rtPCR has shown variable results. Overall, sensitivity of these tests seems to be lower than ELISA [7,8], although a few studies suggested high specificity, of >90% when compared to invasive tests [8]. Our research group, along with many others [8–10,14], have based our studies in children on ELISA results, and thus, the relatively high rtPCR-positive/ELISA-negative samples detected in samples from our previous study (1/3 of samples tested) required clarification. Importantly, it is conceivable that some children had a persistent infection that was not detected by ELISA. If this is the case, the proportion of persistent infection in the overall characterization of infection dynamics needs to be redefined.

Our results allow us to conclude that both noninvasive tests, ELISA and rtPCR run in duplicate, are highly concordant when defining the infection status of children, not only for persistently infected children, but also for transient or noninfected children, as defined by

ELISA. rtPCR detected only one possible additional “persistent infection” that was not detected by ELISA of 11 transient infections, and none of 25 noninfected children. This finding strengthens our previous results that conclude that 21–22% of Chilean children have a persistent *H. pylori* infection during their first 5 years of life as determined by stool ELISA.

Although scarce, ELISA-negative/rtPCR-positive samples did occur and their significance remains to be elucidated. Importantly, the majority of positive results occurred in only one of the two duplicates, strongly suggesting that these were false positives, which allows us to highlight the importance of performing duplicate reactions if rtPCR is to be used in isolated samples. The relatively higher 16S rRNA detection rate in stools from children with a transient infection compared to those who never had an ELISA-positive sample, suggests that the intermittency of bacterial/antigen load in the stools of these children may be playing a role in determining ELISA versus rtPCR positivity. To confirm this, quantification of bacterial DNA in stools should be performed. The main difficulty of DNA quantification is the quality of DNA extraction and purification from stool, due to the presence of multiple PCR inhibitors such as bile salts, in addition to probable DNA degradation during bacterial transit from the stomach to stools [15]. Future research could aid in answering this pending issue, but in light of our results, which demonstrate that over time the great majority of positive rtPCR/ELISA-negative samples will revert to fully negative samples, stool ELISA is a reliable method in characterizing *H. pylori* dynamics in apparently healthy children.

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**Competing interests:** The authors do not have any disclosure relevant to the manuscript.

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