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Quantitative detection and genotyping of *Helicobacter pylori* from stool using droplet digital PCR reveals variation in bacterial loads that correlates with *cagA* virulence gene carriage

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Abstract

Background—Epidemiologic studies of the carcinogenic stomach bacterium *Helicobacter pylori* have been limited by the lack of non-invasive detection and genotyping methods. We developed a new stool-based method for detection, quantification, and partial genotyping of *H. pylori* using droplet digital PCR (ddPCR), which allows for increased sensitivity and absolute quantification by PCR partitioning.

Materials and Methods—Stool-based ddPCR assays for *H. pylori* 16S gene detection and *cagA* virulence gene typing were tested using a collection of 50 matched stool and serum samples from Costa Rican volunteers and 29 *H. pylori* stool antigen-tested stool samples collected at a U.S. hospital.

Results—The stool-based *H. pylori* 16S ddPCR assay had a sensitivity of 84% and 100% and a specificity of 100% and 71% compared to serology and stool antigen tests, respectively. The stool-based *cagA* genotyping assay detected *cagA* in 22 (88%) of 25 stools from CagA antibody-positive individuals and 4 (16%) of 25 stools from CagA antibody-negative individuals from Costa Rica. All 26 of these samples had a Western-type *cagA* allele. Presence of serum CagA antibodies was correlated with a significantly higher load of *H. pylori* in the stool.

Conclusions—The stool-based ddPCR assays are a sensitive, non-invasive method for detection, quantification, and partial genotyping of *H. pylori*. The quantitative nature of ddPCR-

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based *H. pylori* detection revealed significant variation in bacterial load among individuals that correlates with presence of the *cagA* virulence gene. These stool-based ddPCR assays will facilitate future population-based epidemiologic studies of this important human pathogen.

INTRODUCTION

Helicobacter pylori chronically infects over half the world's population and is the only bacterium to be classified as a carcinogen by the WHO because of its role in gastric cancer development. Gastric cancer is the third leading cause of cancer deaths worldwide, and 89% of gastric cancer cases are attributable to *H. pylori* infection [1, 2]. *H. pylori* is in fact responsible for a range of disease outcomes from asymptomatic gastritis (inflammation of the gastric mucosa) to peptic ulcer and gastric cancers, but may be protective against other diseases including esophageal cancer [3, 4] and asthma [5, 6]. Differences in disease outcome are due in part to genetic differences among *H. pylori* strains. *H. pylori* exhibits extensive inter-strain genetic diversity as well as intra-strain genetic diversification during the course of infection [7].

Presence of the strain-variable *cagA* gene and specific *cagA* allelic variations are associated with increased risk of peptic ulcers and gastric cancer [8–11]. The *cagA* gene is contained within the *cag* pathogenicity island that encodes a Type IV secretion system that delivers the CagA protein into host gastric epithelial cells [12]. Inside the host cell, the CagA protein is tyrosine-phosphorylated at EPIYA (Glu-Pro-Ile-Tyr-Ala) sites and deregulates host SHP-2 tyrosine phosphatase, a *bona fide* oncoprotein, resulting in changes to the host cell morphology [13–16]. The *cagA* gene is grouped into two different allele types based on whether it encodes an EPIYA-C or EPIYA-D motif. Phosphorylated EPIYA-D motifs interact more strongly with host SHP-2 than phosphorylated EPIYA-C motifs [17]. *cagA* alleles encoding an EPIYA-D motif are predominantly found in *H. pylori* strains circulating in East Asian countries and are associated with an increased risk of gastric cancer development [11].

Molecular epidemiologic studies of *H. pylori* investigating the role of bacterial genetic diversity in disease outcome have been limited by the lack of non-invasive methods for detection and genotyping of *H. pylori*. Current *H. pylori* genotyping methods to assess the presence of known virulence genes or alleles rely on upper gastrointestinal endoscopy for subsequent culturing from a gastric biopsy, which precludes sampling from the majority of *H. pylori*-infected individuals who are asymptomatic. Non-invasive tests for *H. pylori* infection, including the urea breath test and stool antigen test, detect the presence of *H. pylori* infection but do not differentiate between strains or provide *H. pylori* genetic information. For this reason, there has been great interest in development of non-invasive stool-based tests for detecting and genotyping *H. pylori*. *H. pylori* is present in stool at low abundance and is generally not culturable. PCR-based methods (either multiple rounds of conventional PCR or real-time PCR) for detecting *H. pylori* DNA in stool have been reported with sensitivities varying between 25% and 92% [18–22]. However, PCR-based tests for detecting and genotyping *H. pylori* from stool have not been adopted for use in epidemiologic studies or diagnostics likely because of issues with false positives from contamination and low sensitivity and reproducibility.

A recent development in PCR technology, droplet digital PCR (ddPCR), addresses many challenges of detection and genotyping of *H. pylori* in stool. ddPCR partitions a single PCR reaction into 20,000 droplets, allowing increased sensitivity for detection of rare targets and absolute quantitation by analysis of the frequency of positive droplets [23]. Reaction partitioning also increases tolerance of PCR inhibitors, further improving assay sensitivity for inhibition-prone samples such as stool [24, 25]. ddPCR assays can include fluorescent hydrolysis probes to increase the specificity of the assay, and multiple probes can be used to differentiate between different alleles of a gene. Because of the unique advantages that ddPCR offers, this new technology has been adopted for use in detection of infectious agents in a variety of samples types [26, 27] as well as a method for detection of known cancer biomarkers [28–30].

We have developed a new, non-invasive method for detection, quantification, and *cagA* genotyping of *H. pylori* from stool samples that uses ddPCR. We tested this method using a collection of matched serum and stool samples from volunteers in Costa Rica, a country with an *H. pylori* prevalence of 78% [31] and one of the highest incidence and mortality rates of stomach cancer [32]. Development of non-invasive methods to genotype *H. pylori* will facilitate challenging molecular epidemiology studies to investigate prevalence and track transmission of specific *H. pylori* strains in populations and to examine *H. pylori*-host genetic interactions in disease progression.

METHODS

Study populations and specimen collection

Samples from Costa Rica—Stool and serum samples were collected from 50 volunteers as part of a larger population-based study of the human microbiome that included 150 adult volunteers from Hojancha, Guanacaste, chosen by random selection of household number using census data. The 50 volunteers whose samples were analyzed in this study were selected to include 25 men and 25 women under the age of 60 years to minimize the likelihood of *H. pylori* clearance due to *H. pylori*-related disease progression. The 50 volunteers had a median age of 35 years (range 19 to 56 years). Stool samples were collected at home and placed by participants into a vial containing 5 ml RNAlater nucleic acid preservative (Ambion) and immediately stored in a cooler with dry ice prior to delivery to the study clinic. At the clinic, samples were transferred to liquid nitrogen for storage and shipment to a biorepository in the U.S. Blood was collected at the study clinic and the serum was separated and frozen in aliquots. Samples were collected within 6 days of each other and only from individuals who had not been on antibiotic treatment for at least six weeks. The study protocols and procedures for the protection of human subjects were approved by the Institutional Review Boards of the U.S. National Cancer Institute and the National University of Costa Rica. Informed consent was obtained from each participant.

Samples from the United States—Stool samples were collected from 29 individuals seen at outpatient clinics at Harborview Medical Center in Seattle, Washington for a variety of medical reasons. Stool samples were collected at home by the patient and brought to the clinic on the same day. Stools were tested for *H. pylori* antigen because of clinical

indications and deidentified leftover specimens were provided for this study. Patients ranged in age from 1 to 68 years (median 27 years). Specimens were held at 4°C before division into ~1ml aliquots and frozen at -75°C. Formed stools were emulsified in a sufficient quantity of molecular grade water to facilitate aliquot distribution.

***H. pylori* and CagA IgG ELISA**

Serum samples from the Costa Rican participants were analyzed for *H. pylori* antibodies using the Wampole *Helicobacter pylori* IgG ELISA II Test System and for CagA antibodies using the CagA IgG ELISA Kit (Alpco). Individuals were considered *H. pylori*-positive by serum test if they were positive for *H. pylori* antibodies and/or CagA antibodies.

Stool DNA extraction

Effects of stool sample storage conditions and DNA extraction methods on yield of *H. pylori* DNA was tested using a stool sample from one *H. pylori*-positive volunteer (Supplementary Materials and Methods, Supplementary Table S4). Based on the results, stool DNA of all other samples were extracted using the QIAamp Stool DNA Mini Kit (Qiagen) according to the manufacturer's instructions, with the lysis step performed at 95°C. For samples stored in RNeasy, the sample was first transferred to a microcentrifuge tube and centrifuged to remove the RNeasy.

Primer and probe design

The primer and probe sequences for all assays are listed in Table 1 and the design and testing of the primers and probes are described in the Supplementary Materials and Methods.

Droplet Digital PCR

Droplet digital PCR was performed according to the manufacturer's instructions with each 20 µl reaction containing 1x ddPCR Supermix for Probes (BioRad), 900 nM of each primer (Table 1), 250 nM of each probe (Table 1), and 10 µl stool DNA. For the Costa Rican stool DNA samples, the concentration was adjusted to 100 ng/µl and 1 µg stool DNA was analyzed per reaction. Droplets were generated using the QX200 Droplet Generator (BioRad). Reactions were subject to thermal cycling with the following conditions: 95°C for 10 minutes, 45 cycles of 94°C for 30 seconds and 55°C for 1 minute, and 98°C for 10 minutes. Droplets were then analyzed for fluorescent amplitude using the QX200 Droplet Reader (BioRad). Data was analyzed using the QuantaSoft software version 1.6.6 (BioRad) and thresholds were set manually. Placement of the threshold was set for each assay by visually inspecting the amplitude plot for a positive control sample and placing the threshold between the cluster of positive droplets and the cluster of negative droplets. The thresholds were set at 4500 for the *H. pylori* 16S assay, 6500 for the *cagA* assay, and 2000 for the EPIYA assay. To increase the total amount of stool DNA being assayed, two reactions were run for each stool sample and the results were combined by averaging the number of copies per µl. A positive control (stool DNA from an *H. pylori*-positive volunteer) and negative control (molecular grade water) were included in each batch of samples analyzed.

Statistical Analysis

Differences in *H. pylori* load as measured by the *H. pylori* 16S ddPCR assay were compared using the Wilcoxon rank-sum test. Statistical analyses were performed using SAS version 9.4 (SAS Institute Inc.)

RESULTS

Development and optimization of stool-based ddPCR assay to detect *H. pylori*

To first test the performance of the ddPCR assay to detect the *H. pylori* 16S gene amongst a complex background of stool DNA, we spiked 10-fold dilutions of known quantities of *H. pylori* strain G27 genomic DNA, as determined by a NanoDrop spectrophotometer (Thermo Scientific), into stool DNA from an *H. pylori*-negative volunteer. As shown in Figure 1, we observed a clear separation between droplets containing *H. pylori* 16S and droplets negative for *H. pylori* 16S, and the assay correctly quantified the concentration of *H. pylori* genomes spiked down to the lowest amount tested (one *H. pylori* genome per µg stool DNA).

Development of stool-based ddPCR assay to detect and EPIYA type the *H. pylori* *cagA* gene

Unlike the *H. pylori* 16S gene, the *cagA* gene exhibits extensive nucleotide diversity among *H. pylori* strains making the design of universal primers and probes for this gene more challenging. To assess the compatibility of the *cagA* ddPCR primers and probes with a globally representative sample of *H. pylori* strains, we examined the polymorphisms present within the primer and probe regions for 37 *H. pylori* strains described in Olbermann et al. [33] and tested the ability of the *cagA* ddPCR assays to detect *cagA* and distinguish between EPIYA types in strains having some of these polymorphisms (Supplementary Materials and Methods, Supplementary Tables S1–S3). We spiked genomic DNA of six North American strains (EPIYA-C) [34] and eight Japanese *H. pylori* strains (EPIYA-D) [11], as well as two fully sequenced European strains (EPIYA-C), 26695 [35] and G27 [36], into *H. pylori*-negative stool DNA. The *cagA* detection ddPCR assay detected the *cagA* gene for 15 (94%) of 16 strains tested. The *cagA* gene was not detected for strain Em7-1 even though this strain has the same sequence at the *cagA* primer and probe sites as strain 26695, which was positive by the *cagA* ddPCR assay (Supplementary Table S3). The *cagA* EPIYA type was correctly determined for 15 (94%) of 16 strains tested. Neither EPIYA motif was detected for Japanese strain Oki633, which had two polymorphisms within the EPIYA-D probe site (Supplementary Table S3). The ddPCR amplitude plots for the *cagA* detection and EPIYA typing assays for two of the spiked strains, Em47-1 (EPIYA-C) and Oki573 (EPIYA-D), are shown in Supplementary Figure S1.

Validation of ddPCR assays using population-based and clinical samples

The stool-based ddPCR assays were tested using a collection of 50 matched serum and stool samples from Costa Rican volunteers. Of these 50, 32 (64%) were *H. pylori*-positive based on the *H. pylori* serum test and 25 (50%) were positive for *H. pylori* having a *cagA* gene based on the serum test to detect CagA antibodies. Of the 25 volunteers positive for CagA antibodies, 20 were also positive for *H. pylori* antibodies and five were negative for *H. pylori*

antibodies. The stool-based *H. pylori* 16S ddPCR assay detected *H. pylori* in the stool of 27 (84%) of the 32 participants positive for *H. pylori* serum antibodies and 31 (84%) of the 37 participants positive for *H. pylori* or CagA antibodies by the serum test. The *H. pylori* 16S and *cagA* ddPCR assays were positive for four of the five participants who were positive for only CagA antibodies and the *H. pylori* loads in the stool were relatively high in these samples (17, 34, 48, and 452 *H. pylori* 16S copies per μg stool DNA). Of the 13 participants negative for both *H. pylori* and CagA antibodies, all 13 (100%) were negative for the *H. pylori* 16S ddPCR assay (Table 2).

The stool-based *H. pylori* 16S ddPCR assay was further tested using 29 stool samples from patients at a U.S. hospital for which the *H. pylori* stool antigen test was performed. Of the 12 that were *H. pylori*-positive, all 12 (100%) were positive for the *H. pylori* 16S ddPCR assay. Of the 17 samples that were *H. pylori* negative by stool antigen test, 12 (71%) were negative by *H. pylori* 16S ddPCR assay and five were positive (Table 2). The five samples that were positive for the *H. pylori* 16S ddPCR assay but negative for the *H. pylori* stool antigen assay had *H. pylori* loads in the lower half of the range, which may have been below the detection limit for the stool antigen assay. Furthermore, three of these five patients had a previous diagnosis of *H. pylori* infection indicated in their medical records.

The stool-based *cagA* ddPCR assays were compared to the CagA serum antibody test using the samples from 50 Costa Rican volunteers. Of the 25 participants serum positive for CagA antibodies, 21 (84%) were positive for the *cagA* detection ddPCR assay and 22 (88%) were positive for the *cagA* EPIYA typing ddPCR assay, with all samples having a *cagA* gene encoding the EPIYA-C motif. Of the 25 participants serum negative for CagA antibodies, 16 (64%) were negative for the *cagA* detection ddPCR assay and 21 (84%) were negative for the *cagA* EPIYA typing ddPCR assay (Table 3).

Load of *H. pylori* in the stool varies and is correlated with serum CagA antibody status

For the 31 Costa Rican stool samples positive for *H. pylori* by ddPCR, there was a two log range of *H. pylori* load in the stool (median 8.4, range 1 – 452 *H. pylori* copies per μg stool DNA). Of the 17 U.S. stool samples that were positive by *H. pylori* 16S ddPCR assay, the load of *H. pylori* in the stool varied between 0.5 to 120.9 copies *H. pylori* 16S per μg stool DNA, with a median of 13.6 (Table 2). The *H. pylori* 16S ddPCR assay was performed a second time for the 50 Costa Rican samples, and the copy number data was highly reproducible, with a correlation coefficient of 0.98 (Figure 2A). For 35 of the 50 samples having 10 or fewer *H. pylori* 16S copies per μg stool DNA, the correlation coefficient was 0.74 (Figure 2B).

It is not known whether the load in the stool is correlated with the load in the stomach or whether other factors such as level of inflammation, disease state, or bacterial genetic factors are involved. To assess the potential role of the *cagA* virulence gene in load of *H. pylori* in the stool, the *H. pylori* 16S copy number per μg stool DNA was compared between Costa Rican volunteers positive for CagA antibodies and negative for CagA antibodies. There was a statistically significant increase in *H. pylori* load in the stool of volunteers positive for CagA antibodies compared with volunteers negative for CagA antibodies [median *H. pylori*

16S copy number per μg stool DNA (range) CagA positive: 11.2 (0 – 443), CagA negative: 1.6 (0 – 30); Wilcoxon rank-sum test p-value: 0.009] (Figure 2C).

DISCUSSION

The interplay between environment, host genotype, and *H. pylori* strain differences that lead to a wide range of disease outcomes (gastritis, peptic ulcers, and gastric cancer) as well as possible protective effects against esophageal cancer and asthma are not well understood. Non-invasive tests to detect and genotype *H. pylori* will facilitate molecular epidemiology studies to examine the distribution of specific *H. pylori* genes and alleles in populations and their role in disease outcome. While there are well established non-invasive tests for detecting *H. pylori* infection (urea breath test, serology test, stool antigen test), these tests do not provide information about the genotype of the *H. pylori* strain or the bacterial load. *H. pylori* genotype can be determined by first obtaining a culture from a gastric biopsy, but this involves an expensive and invasive procedure. We have developed a new non-invasive method for detection, quantification, and genotyping of *H. pylori* from stool samples using ddPCR that is sensitive and reproducible.

This study takes advantage of two different collections of samples to compare these new, non-invasive ddPCR assays to two different *H. pylori* diagnostic tests, the serum test and stool antigen test, which are widely used for epidemiologic studies and clinical diagnosis. The sensitivity and specificity of the stool-based ddPCR assay to detect *H. pylori* infection depended on the *H. pylori* test that was used for the comparison, either serology or stool antigen test. The serology test has a sensitivity of 85% and specificity of 79% and the stool antigen test has a sensitivity of 94% and specificity of 92% [37]. These two tests detect different biological processes of *H. pylori* infection (antibody response to *H. pylori* and *H. pylori* shedding into the stool) so it is not surprising that the stool-based *H. pylori* ddPCR assay would compare differently to these two tests. Serology tests for *H. pylori* can produce false positives due to circulating antibodies still present after clearance of infection and false negatives due to low antibody response to the *H. pylori* antigen employed by the test. Of the six individuals who were positive for either *H. pylori* or CagA antibodies but were negative for *H. pylori* 16S ddPCR stool assay, two were positive by ddPCR stool assay upon repeat testing and both had low copy numbers of *H. pylori* 16S, indicating that they were near the limit of detection for this assay. For the samples from the U.S. hospital, the higher sensitivity is likely due to both tests detecting *H. pylori* shed into the stool. The five samples that were positive for the *H. pylori* 16S ddPCR assay but negative for the *H. pylori* stool antigen assay had low *H. pylori* loads, which may have been below the detection limit for the stool antigen assay. Three of these five patients also had a previous diagnosis of *H. pylori* infection indicated in their medical records, supporting the positive results by *H. pylori* 16S ddPCR assay.

It is also possible that differences in storage conditions between the Costa Rican and the U.S. stool samples could have contributed to differences in sensitivity of the assay. We used the ddPCR assays to quantitatively assess different stool DNA extraction methods and stool sample storage conditions (see Supplementary Materials and Methods and Supplementary Table S4). We found that the *H. pylori* DNA was robust to cycles of freezing and thawing

and storage at ambient temperatures in RNAlater preservative, making it possible to conduct epidemiologic studies in which immediate freezing of the sample is not feasible. We also found that stool DNA extraction methods that employ bead beating to lyse bacterial cells yielded less *H. pylori* 16S copies per µg stool DNA than those without bead beating. *H. pylori* lyses fairly easily, so the decrease in the relative proportion of *H. pylori* in samples that underwent bead beating was likely due to an increase in the yield of DNA from difficult to lyse bacterial species in the stool. Stool samples were collected from one *H. pylori*-positive volunteer at two different time points that were 20 months apart. *H. pylori* 16S was detected by the stool-based ddPCR assay at both time points although the load was slightly higher at the second time point (81 vs. 49 16S copies per µg stool DNA, Supplementary Table S4). Future studies that include more *H. pylori*-positive individuals would be needed to further examine fluctuations in *H. pylori* load in the stool over time.

Aside from detection of *H. pylori* infection, we have also developed ddPCR assays to detect the *H. pylori cagA* gene and distinguish between different alleles of this gene, which are associated with differences in gastric cancer risk. The *cagA* detection ddPCR assay and the *cagA* EPIYA typing ddPCR assay have similar sensitivities for detection of the *cagA* gene (84% and 88%, respectively), but the specificity is higher for the *cagA* EPIYA typing ddPCR assay (84% compared to 64% for the *cagA* detection ddPCR assay). The *cagA* EPIYA typing ddPCR assay can be used for both detection and allele typing of *cagA* although it will not detect *cagA* allele types that encode neither an EPIYA-C nor an EPIYA-D motif. However, these allele types make up less than 3% of *cagA* allele types [38].

All 26 of the Costa Rican stool samples that were positive for the *cagA* gene by the *cagA* EPIYA typing ddPCR assay had the Western type of the gene encoding an EPIYA-C motif. While this result cannot be confirmed by the serum CagA antibody test, which does not distinguish between *cagA* allele types, this result is expected based on analysis of the *cagA* allele types of 33 Costa Rican *H. pylori* strains, all of which had Western type *cagA* gene [38]. Furthermore, an analysis of the geographical origin of 24 Costa Rican *H. pylori* strains characterized 21 as belonging to the hpEurope group and three as belonging to the hspWAfrica group [39], which would be expected to have *cagA* genes encoding an EPIYA-C motif. *H. pylori* strains of Amerindian origin have an EPIYA-DC motif with characteristics of both EPIYA-C and EPIYA-D motifs [40]. The *cagA* EPIYA typing assay would characterize the *cagA* gene of these strains as encoding an EPIYA-C motif.

Using ddPCR also allows for absolute quantitation of *H. pylori* load in the stool, information not obtained by other stool-based methods. We observed a two log range of *H. pylori* loads in the stool and the load is significantly higher in those with a *cagA*-positive strain. Density of *H. pylori* in the stomach also varies among infected individuals, and a higher *H. pylori* load in the stomach among individuals infected with a *cagA*-positive strain has been previously reported [41]. The relationship between *H. pylori* load in the stool and density of infection in the stomach, as well as other possible factors such as level of inflammation, disease state, or bacterial genetic factors, is not presently understood. Future studies that use matched gastric biopsy and stool samples will need to be conducted to address these questions.

It is a limitation of this study that the stool-based ddPCR assays were not compared to histology and culturing from gastric biopsy, which are considered as the gold standard for *H. pylori* diagnosis. Further validation of the stool-based ddPCR assays should be conducted on matched gastric biopsy and stool samples from various populations. These studies could also include comparison of the *H. pylori* genotypic information obtained from stomach samples to that from stool samples since individuals can have a mixture of *H. pylori* genotypes due to co-infecting strains of *H. pylori* or within-strain genotypic variation.

In conclusion, we have developed a sensitive and reproducible method to non-invasively detect and genotype *H. pylori* and measure bacterial load from stool samples. This non-invasive method to genotype *H. pylori* will allow future studies of *H. pylori* biomarkers of transmission and pathogenesis. Further understanding of the host and bacterial factors involved in *H. pylori*-associated disease development can inform strategies for tailored treatment decisions to reduce disease burden. This is particularly relevant to *H. pylori* since infection confers risk for some diseases and possible protection against others.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

H. pylori 16S ddPCR amplitude plot for *H. pylori*-negative stool DNA spiked with three different biologically relevant concentrations of *H. pylori* strain G27 genomic DNA. Each dot represents one droplet. Dots above the threshold (black line set at fluorescence amplitude of 4500) are positive for the *H. pylori* 16S gene and dots below are negative. Expected number of *H. pylori* genome copies and measured number of *H. pylori* 16S gene copies with Poisson 95% confidence intervals in parentheses are shown above the amplitude plots. Two 20 μ l reactions were run for each sample and the results were combined.

Figure 2.

Analysis of *H. pylori* load in the stool by quantification of *H. pylori* 16S gene copy number per μ g stool DNA using ddPCR. A–B) Comparison of *H. pylori* 16S copy number of 50 Costa Rican stool samples (A) and 35 of the 50 Costa Rican stool samples having *H. pylori* 16S copy numbers ≥ 10 (B) for two separate ddPCR runs. A trend line and the corresponding linear equation and correlation coefficient (R^2) are indicated. C) Boxplots (box indicates 25th–75th percentile and median, whiskers indicate the minimum and maximum data points) of the *H. pylori* 16S copy number of stool samples from *H. pylori*-positive Costa Rican volunteers with positive ($n=25$) and negative ($n=12$) serum CagA antibody test. The *H. pylori* 16S copy number per μ g stool DNA was compared using the Wilcoxon rank-sum test ($p=0.009$).

Table 1

Primers and probes used for droplet digital PCR (ddPCR) assays to detect and genotype *H. pylori* from stool

ddPCR assay	Primer or probe name	Sequence and chemical modifications ^I	Reference
<i>H. pylori</i> 16S	HPF	5' – GCGACCTGCTGGAACATTAC – 3'	Gramley, 1999
	HPR	5' – CGTTAGCTGCATTACTGGAGA – 3'	Gramley, 1999
	Hp16S_HEX	5' HEX - AAGCCCTCCAACAACACTAGCATCCAT - BHQ1 3'	this study
<i>cagA</i> detection	cagA_F	5' – TGGCTCAAGCTCGTGAAT – 3'	this study
	cagA_R	5' – TGGAAAACCTGAACGAATCAGA – 3'	this study
	cagA_FAM	5' FAM – CTTCYACATTATGYGCAACKATC – BHQ1 3'	this study
<i>cagA</i> EPIYA typing	EPIYA_F1	5' – TCAGTTAGCCCTGAACC – 3'	this study
	EPIYA_F2	5' – TCAACTAGCCCTGAACC – 3'	this study
	EPIYA_R1	5' – GCCCTACCTTACTGAGAT – 3'	this study
	EPIYA_R2	5' – GAAAGCCCTACTTTACTGAG – 3'	this study
	EPIYA-C_HEX	5' HEX – TCCGCCGAGATCATCAATCGTAGC - BHQ1 3'	this study
	EPIYA-D_FAM	5' FAM – AAGCCTGCTTGATTTGCCTCATCAAA – BHQ1 3'	this study

^I HEX: hexachloro-fluorescein, FAM: 6-carboxyfluorescein, BHQ: black hole quencher

Table 2

Comparison of results of stool-based *H. pylori* ddPCR assay detection and *H. pylori* load with results of *H. pylori* serum test (samples from 50 Costa Rican volunteers) and *H. pylori* stool antigen test (samples from 29 patients at a U.S. hospital)

<i>H. pylori</i> 16S ddPCR	Costa Rican Samples <i>H. pylori</i> or CagA serum test ^a		U.S. Samples <i>H. pylori</i> stool antigen test	
	positive n=37	negative n=13	positive n=12	negative n=17
positive	31 (84%)	0	12 (100%)	5 (29%)
median copies <i>H. pylori</i> 16S per µg stool DNA (range)	8.4 (1–452)	N/A	22.3 (1.1–121)	1.1 (0.5–3.5)
negative	6 (16%)	13 (100%)	0	12 (71%)

^aPositive= positive for *H. pylori* or CagA serum antibody test, Negative=negative for both

Table 3Comparison of stool-based *cagA* gene ddPCR and CagA serum antibody assays for 50 Costa Rican volunteers

<i>cagA</i> ddPCR assay	<u>CagA serum test</u>	
	positive n=25	negative n=25
<i>cagA</i> gene ddPCR		
positive	21 (84%)	9 (36%)
negative	4 (16%)	16 (64%)
<i>cagA</i> EPIYA typing ddPCR		
positive	22 (88%)	4 (16%)
negative	3 (12%)	21 (84%)
both <i>cagA</i> ddPCR assays		
positive for both	20 (80%)	3 (12%)
positive for <i>cagA</i> gene ddPCR, negative for EPIYA typing ddPCR	1 (4%)	6 (24%)
negative for <i>cagA</i> gene ddPCR, positive for EPIYA typing ddPCR	2 (8%)	1 (4%)
negative for both	2 (8%)	15 (60%)