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Unreliability of Results of PCR Detection of *Helicobacter pylori* in Clinical or Environmental Samples▽†

Mitsushige Sugimoto, Jeng-Yih Wu, Suhaib Abudayyeh, Jill Hoffman, Hajer Brahem, Khaldun Al-Khatib, Yoshio Yamaoka, and David Y. Graham*

Department of Medicine, Michael E. DeBakey Veterans Affairs Medical Center and Baylor College of Medicine, Houston, Texas

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The aim of this study was to compare published *Helicobacter pylori* primer pairs for their ability to reliably detect *H. pylori* in gastric biopsy specimens and salivary samples. Detection limits of the 26 PCR primer pairs previously described for detection of *H. pylori* DNA in clinical samples were determined. Sensitivity and specificity were determined using primers with detection limits of <100 CFU/ml using 50 *H. pylori*-positive and -negative (by concordance by culture and histology) coded gastric biopsy specimens. These results were then confirmed with gastric biopsy specimens and saliva from patients with confirmed *H. pylori* status. Five of the twenty-six previously reported primer pairs (HP64-f/HP64-r, HP1/HP2, EHC-U/EHC-L, VAG-F/VAG-R, and ICT37/ICT38) had detection limits of <100 CFU/ml in the presence of gastric tissue. None had 100% specificity or sensitivity; all produced false-positive results. The HP64-f/HP64-r for *ureA* and HP1/HP2 for 16S rRNA individually had sensitivities and specificities of >90% with gastric biopsy specimens. No combinations of primer pairs improved the results. Using these five primer pairs, 54% of the positive saliva samples were determined to be false positive; both the HP64-f/HP64-r and the HP1/HP2 sets produced false positives with saliva. We conclude that clinicians should not rely on results using current PCR primers alone to decide the *H. pylori* status of an individual patient or as a basis for treatment decisions. The results of studies based on PCR identification of *H. pylori* in environmental samples should be viewed with caution. Possibly, specific primers sets can be identified based on the presence of multiple putative virulence factor genes.

Many detection methods for the presence of *Helicobacter pylori* infection have been described, each with advantages and disadvantages such that the choice is dependent on the application (e.g., for clinical diagnosis versus an epidemiology study) and the amount of error acceptable (8, 11, 17). Clinically, noninvasive methods are preferred, with urea breath testing and stool antigen testing being the current tests of choice (6). Culture is particularly important for susceptibility testing, although molecular methods applied to biopsy specimens or stools provide an alternative for detecting clarithromycin resistance (35, 44, 56).

PCR methods are used for the detection of *H. pylori* DNA in gastric mucosa and gastric juice, as well as in feces, saliva, dental plaque, and environmental samples. Limitations of PCR methods include the propensity for false-positive results in part due to the detection of cDNA from non-*H. pylori* organisms. This is particularly important in environmental samples which may contain previously uncultured organisms or non-*H. pylori Helicobacter* spp. False-negative results may also occur due to a low number of organisms or to the presence of inhibitors in the sample. This is especially important in stools and environmental samples.

A number of target genes have been proposed as candidates for the PCR detection of *H. pylori*, including the 16S rRNA gene, the 26K species-specific antigen gene, the *glmM* gene, the *ureA* gene, the *ureB* gene, the *vacA* gene, and the *cagA* gene (see Table S1 in the supplemental material) (7, 16, 20, 26, 27, 29, 32, 34, 40, 43, 47, 48, 55). Although previous reports generally report good sensitivity and/or specificity of the primer pairs used, systematic studies comparing different PCR primer pairs are rare using very well-characterized cases (e.g., negative or positive by multiple tests) (12, 45). Thus, controversy remains regarding which primer pair or sets of primers is the potential "gold standard" for gastric and nongastric clinical samples such as saliva or for environmental samples. A number of primers have been suggested for detection of *H. pylori* DNA in saliva (see Table S2 in the supplemental material) (2, 5, 9, 10, 14, 16, 18, 22, 24, 25, 28, 29, 32–34, 42, 48, 49, 53–55) with detection rates ranging from 5 to 100%. Because *H. pylori* in saliva generally reflects the reflux of organisms from the stomach, detection rates vary (4, 37, 38). There is also the possibility of cross-reactivity with spiral urease-containing organisms normally present in the mouth, especially if primer pairs are not carefully selected.

The aims of the present study were to compare the accuracy of the reported PCR primer pairs using gastric mucosal biopsy specimens known to either contain *H. pylori* or to be *H. pylori* negative by multiple tests. We also examined their accuracy in saliva from patients whose *H. pylori* status was known.

MATERIALS AND METHODS

Detection limits of PCR primer pairs. We selected 26 PCR primers from those previously reported to have been used for detection of *H. pylori* (see Table S1 in the supplemental material). *H. pylori* strain 26695 (ATCC 700392) was used as the standard strain. In brief, the bacterial concentration was adjusted to an optical density of 0.9 at 625 nm (10^9 CFU/ml), and serial 10-fold dilutions were performed until reaching ~10^0 CFU/ml. A 1-ml portion of bacterial suspension was used to extract genomic DNA using QIAamp tissue kits (Qiagen, Inc., Valencia, CA) ac-

* Corresponding author. Mailing address: Michael E. DeBakey Veterans Affairs Medical Center, RM 3A-320 (111D), 2002 Holcombe Blvd., Houston, TX 77030. Phone: (713) 795-0232. Fax: (713) 790-1040. E-mail: dgraham@bcm.tmc.edu.

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TABLE 1. Primer pairs with low detection limit of <100 CFU/ml for *H. pylori* only, a mixture of *H. pylori* plus gastric tissues, and a mixture of *H. pylori* plus saliva

Target gene	Primer pair	Detection limit (CFU)		
		H. pylori DNA only	*H. pylori* DNA + gastric tissues	*H. pylori* DNA+ saliva tissues
16S rRNA	Hp1/Hp2	1–10	1–10	1–10
HP0075-0076	EHC-U/EHC-L	1–10	1–10	1–10
ureA	HP64-f/HP64-r	1–10	1–10	10–100
vacA	VAG-F/VAG-R	1–10	10–100	10–100
glmM	ICT37/ICT38	10–100	10–100	1–10

cording to the manufacturer's instructions. DNA was eluted with 100 μl of the elution buffer provided, and 1 μl of DNA sample was used for each reaction.

Since experiments using pure *H. pylori* DNA might not represent the actual conditions when testing clinical samples (e.g., presence of inhibitors), we also "spiked" samples of gastric tissue. In brief, 1-ml serial dilutions of *H. pylori* (from 10^9 to $\sim 10^0$ CFU/ml) were added to a biopsy specimen ($\sim$8 mm^3) or to 1 ml of saliva proven to be *H. pylori* negative and, after centrifugation at 12,000 rpm for 2 min, the pellets were used to extract genomic DNA as described above.

We defined primers whose low detection limit by PCR was <100 CFU/ml as high-quality primer pairs, and these were used in experiments with gastric biopsy specimens.

PCR primers and conditions. We evaluated the 26 primer pairs previously reported for detection of *H. pylori* in clinical samples and included 2 primer pairs for the *ureA* gene, 2 for the 860-bp DNA gene, 3 for the16S rRNA gene, 1 for the 26K species-specific antigen gene, 8 for the *vacA* gene, 6 for the *cagA* gene, 3 for the *glmM* gene, and 1 for the adhesin gene. We used PCR conditions exactly matching those described by the authors reporting their use (7, 10, 16, 20, 26, 27, 29, 32, 34, 40, 43, 47, 48, 55).

Detection of *H. pylori* in gastric mucosal biopsy samples. Gastric mucosal biopsy samples were obtained from 100 patients undergoing upper gastrointestinal endoscopy at the Michael E. DeBakey VA Medical Center. *H. pylori* status was defined by culture, histopathologic staining (Genta or El-Zimaity triple stains), and rapid urease tests. *H. pylori*-positive cases were defined as being positive by all three tests and negative as negative by all three tests. The biopsy sample from which *H. pylori* had been cultured was chosen for evaluation for those deemed *H. pylori* positive. The biopsy specimens were randomized and coded, and the results remained blinded to all involved in the PCR studies and analyses until the study was completed.

The five primer pairs chosen for the study were defined as high quality based on the detection limit experiments described above. Each was used by two investigators separately and completely independently to assess *H. pylori* status. One investigator (A) had more than 10 years of experience with PCR, whereas investigator "B" had $\sim$3 years of experience.

Specimen collection for saliva study. Saliva samples were collected from a second group of 37 patients undergoing upper gastrointestinal endoscopy by using a dental type aspirator (Yankauer suction tube; Tyco Healthcare, Mansfield, MA) attached to a specimen collection device (40-ml specimen trap; Tyco Healthcare). Saliva was aspirated after the endoscope was swallowed; the aspirator was removed prior to withdrawal of the endoscope, and the samples were frozen at −80°C until utilized.

Biopsy samples for culture and histology and rapid urease testing were also prepared as described above. The saliva samples were randomized separately from the biopsy samples, and their relationship remained blinded until the PCR analyses were completed.

Data analysis. Written informed consent was obtained from all patients using protocols approved by the Baylor College of Medicine's Institutional Review Board. Statistical differences in detection rate of *H. pylori* infection among the different primer pairs were determined by using the chi-square test. A P value of <0.05 was accepted as statistically significant. Calculations were carried out by using the statistical software StatView 5.0 (SAS Institute, Inc., Cary, NC).

RESULTS

Detection limit experiments. We evaluated 26 primer pairs for the detection of DNA from *H. pylori* DNA alone, *H. pylori* plus gastric tissue samples, and *H. pylori* plus saliva samples. Of these, primer pairs HP64-f/HP64-r, Hp1/Hp2, EHC-U/EHC-L, VAG-F/VAG-R, and ICT37/ICT38 revealed low *H. pylori* detection limits of <100 CFU/ml using all of three different methods (Table 1). Four primer pairs designed for the 16S rRNA, *ureA*, HP0075-0076, and *vacA* gene loci yielded detection limits of 1 to 10 CFU per PCR with DNA extracted from pure *H. pylori* cultures. Detection limits were independent of the presence of gastric tissues or saliva sample, except for the primer pairs VAG-F/VAG-R for *vacA* and HP64-f/HP64-b for *ureA*. VAG-F/VAG-R and HP64-f/HP64-b decreased the detection limit from 1 to 10 CFU/ml to 10 to 100 CFU/ml.

Taken together, there were two candidates defined as excellent for the detection of <10 CFU of *H. pylori* DNA with or without gastric and saliva samples: the PCR primers HP64-f/HP64-b for *ureA* and Hp1/Hp2 for 16S rRNA. Three primer pairs were defined as good candidates, defined as the detection of <100 CFU of *H. pylori* DNA; these were VAG-F/VAG-R for *vacA*, EHC-U/EHC-L for HP0075/HP0076, and ICT37/ICT38 for *glmM*.

Sensitivity and specificity of different PCR primers using gastric biopsy specimens. We examined 100 biopsy specimens whose *H. pylori* status was defined based on three separate tests, including positive samples from which *H. pylori* had been cultured. We used the five primer pairs defined as excellent or good above (i.e., detection limits of <100 CFU/ml) (Table 1).

TABLE 2. Detection with five primer pairs of *H. pylori* in 100 gastric biopsy samples by two investigators (A and B)

Parameter[a]	Primer pair and investigator									
	Hp1/HP2		HP64-f/HP64-r		VAG-F/VAG-R		EHC-U/EHC-L		ICT37/ICT38	
	A	B	A	B	A	B	A	B	A	B
True positive (*n*)	47	45	47	46	48	33	42	40	45	44
False negative (*n*)	3	5	3	4	2	17	8	10	5	6
False positive (*n*)	3	1	4	1	1	0	2	1	2	1
True negative (*n*)	47	49	46	49	49	50	48	49	48	49
Sensitivity (%)	94	90	94	92	96	66	84	80	90	88
Specificity (%)	94	98	92	98	98	100	96	98	96	98
Accuracy (%)	94	94	93	95	97	83	90	89	93	93
PPV (%)	94	98	92	98	98	100	95	98	96	98
NPV (%)	94	91	94	92	96	75	86	83	91	89

[a] *n*, Number of samples; PPV, positive predicative value; NPV, negative predicative value.

TABLE 3. Detection with five primer pairs of *H. pylori* in saliva and gastric samples

Parameter[a]	Primer pair and sample type[b]									
	Hp1/HP2		HP64-f/HP64-r		VAG-F/VAG-R		EHC-U/EHC-L		ICT37/ICT38	
	G	S	G	S	G	S	G	S	G	S
True positive (*n*)	16	4	15	4	15	5	8	3	11	4
False negative (*n*)	1	13	2	13	2	12	9	14	6	13
False positive (*n*)	4	5	4	1	4	4	1	0	0	3
True negative (*n*)	16	15	16	19	16	16	19	20	20	17
Sensitivity (%)	94	24	88	24*	88	29	47	18	65	24
Specificity (%)	80	75	80	95	80	80	95	100	100	85
PPV (%)	80	44	79	80	79	56	89	100	100	57

[a] *n*, Number of samples; PPV, positive predictive value.
[b] G, gastric biopsy sample; S, saliva sample. *, The sensitivity is provided but is not an representative in this case because the finding of *H. pylori* is dependent on whether it refluxed from the stomach and thus is a random event.

The two investigators separately produced almost identical results (Table 2). No primer pair produced 100% specificity and sensitivity, since all of them had various numbers of false-negative and, more importantly, false-positive results. Two primer pairs (HP64-f/HP64-r and Hp1/Hp2) produced sensitivity, specificity, positive predicative, and negative predicative values of >90%.

Two of the fifty positive samples (4%) were negative with all five primer pairs, and one sample was false positive with four primer pairs. However, there was no statistically significant difference with regard to *H. pylori* detection rate among the different five primer pairs. We searched for combinations of primer pairs that would improve the results, but none were evident.

***H. pylori* detection in saliva.** Saliva samples ranging from 10 to ~30 ml were obtained from 37 patients. DNA was extracted from the saliva samples and gastric biopsy samples; 17 patients had proven *H. pylori* infection as determined by histology, culture, and rapid urease testing, and 20 were determined to be *H. pylori* negative by all tests (Table 3). This experiment also served to confirm the results of the original study with 100 patients and to evaluate the primer pairs using non-gastric biopsy samples. With saliva, a variable proportion of cases with gastric *H. pylori* infection are expected to be negative on a single examination (4), such that we provide only the calculated specificity and positive predictive value results in Table 3.

Overall, 13 of the 37 salivary samples were positive with at least one primer pair. Seven (54%) of the positive saliva samples were false-positive results, defined as a positive result in a patient whose gastric mucosa was negative for *H. pylori* by multiple tests; four of the seven false-positive results had negative results with all five primer pairs in the stomach. Thus, if one had evaluated 20 *H. pylori*-uninfected patients with these primer pairs, 35% (95% confidence interval [CI] = 15 to 59%) would have yielded false-positive results. The results varied depending on the primer set utilized; only primer set EHC-U/EHC-L had no false-positive salivary samples, and the number of false-positive gastric biopsy samples was also low (i.e., 5.8% [1/17]) in the present study and only 3% rate in the larger study. The relatively high specificity was offset by low sensitivity (84% in the initial study and 47% in the gastric biopsies done as part of the salivary study).

DISCUSSION

Although PCR methods are widely used for clinical and research studies, comparative studies providing specific details about the controls and blinding of the data analyses are few. Our comparison study used five primer pairs chosen based on their ability to detect low numbers of organisms in the presence of tissue DNA. The gastric biopsy samples evaluated were definitely positive (based on culture, histology, and rapid urease tests) or negative; no primer set yielded 100% specificity and sensitivity. Two primer pairs (HP64-f/HP64-r for *ureA* and Hp1/Hp2 for 16S rRNA) yielded sensitivities and specificities of >90% but had false-negative results in specimens from which *H. pylori* had previously been cultured. Both also produced false-positive results, with the Hp1/Hp2 primer set having a false-positive rate of 25% with saliva samples.

The EHC-U/EHC-L primer set had a relatively low sensitivity (84%), but the false-positive rate with gastric biopsy samples was only 3% (95% CI = 0.5 to 14%) and 0% (95% CI = 0 to 19%). While this result suggested that EHC-U/EHC-L set might be the best choice clinically, Song et al. evaluated oral samples from 20 individuals, of whom only 8 had *H. pylori* gastric infections (based on urea breath tests results) using nested PCR with the EHC-U/EHC-L set, and all oral cavity samples were determined to be positive, including those from patients judged to be *H. pylori* negative (50). Their false-positive rate was therefore 100%, due possibly to the presence of cross-reacting DNAs. Another possibility is that *H. pylori* can selectively colonize the mouth and not the stomach. There are few data to support that hypothesis. For example, Wang et al. reported 64 to 78% homology of DNA, presumably originating from *H. pylori*, in saliva samples compared to *H. pylori* isolated from gastric mucosal biopsy samples (55). Song et al. also reported that DNA sequences differed between oral locations and gastric biopsy samples within the same individual (50). A recent study reported that 40% of 39 patients had viable *H. pylori* in their oral cavities despite *H. pylori* eradication. In addition, 56% of those without detectable *H. pylori* in the mouth before treatment had "*H. pylori*" detected in the mouth when reexamined after *H. pylori* eradication (9). When the isolates from oral and gastric isolates were compared by using RAPD [random(ly) amplified polymorphic DNA] anal-

ysis of the DNA, they were typically different, suggesting that either different *H. pylori* strains exist in the stomach and saliva in same patient or that there was misidentification of the spiral-shaped, urease-positive organisms obtained from the mouth (10). It seems likely, given that more than 300 recognized taxa have been identified in the oral cavity, many spiral bacteria are as yet uncultivated. There is also known antigenic cross-reactivity between *H. pylori* and mouth organisms such as *Campylobacter rectus* (21, 30, 51, 52).

There are numerous studies from Western countries showing that *H. pylori* eradication from the stomach results in lifelong cure of the infection, making separate oral infections unlikely (3, 15, 41). Finally, in the era before universal precautions, dentists and dental workers were shown not to share the increased risks of acquiring *H. pylori* infections with those working with gastric contents such as gastroenterologists (31). Overall, it seems most likely that there are non-*H. pylori* stains that commonly colonize the mouth and whose DNA cross-reacts with that *H. pylori* DNA, especially primer pairs based on urease (i.e., many oral flora have urease activity) or 16S rRNA. A number of studies using DNA fingerprinting methods have also shown that salivary and gastric samples have identical patterns for the same individual (34, 36, 39, 42). Our data showing infrequent detection of *H. pylori* in saliva are consistent with those of Berloco et al., who repeatedly sampled saliva for 2 weeks from known *H. pylori*-positive patients and found that to obtain a 50% probability of a positive result required testing for 3 consecutive days (4). We conclude that the data are most consistent with the notion of transient presence of gastric *H. pylori* in the mouth due to reflux from the stomach. It is possible that the organisms may be retained in dental plaque by attachment to *Fusobacterium* spp., despite the fact that the acidic milieu is not conducive to their growth there (1, 57).

Noninvasive testing with the urea breath test and stool antigen test has repeatedly been shown to provide reliable diagnostic information for clinicians both before and after eradication therapy. Overall, these studies suggest that it would not be prudent for clinicians to rely on PCR data alone to decide the *H. pylori* status of an individual patient or as a basis for treatment decisions. In addition, results of studies using PCR based on the primers studied here reporting the detection of *H. pylori* in environmental samples should be viewed with caution since even multiples of these primer sets produced a very high rate of false-positive results (i.e., 54% in saliva samples). Possibly, the accuracy and specificity could be improved by using magnetic beads or other techniques to separate the organism coupled with primers designed to detect *H. pylori*-specific proteins unrelated to urease (13, 19, 23) or possibly to have positive results with multiple potentially *H. pylori*-specific virulence genes (e.g., *cagA* or *vacA*) (40, 46, 54). The Human Microbiome Project should enrich the GenBank with many as-yet-uncultured human microbes such that searches should help eliminate primer pairs that cross-react with non-*H. pylori* bacteria and cause false-positive PCR results. Currently, researchers should at least demonstrate that the primer pairs used in such analyses are not positive in patients without gastric *H. pylori* infections.

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