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

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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/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-negative results. The HP64-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-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, 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 (10⁹ CFU/ml), and serial 10-fold dilutions were performed until reaching ~10⁸ CFU/ml. A 1-ml portion of bacterial suspension was used to extract genomic DNA using QIAamp tissue kits (Qiagen, Inc., Valencia, CA) ac-
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

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Primer pair</th>
<th>Detection limit (CFU)</th>
</tr>
</thead>
<tbody>
<tr>
<td>16S rRNA</td>
<td>Hp1/Hp2</td>
<td>1–10</td>
</tr>
<tr>
<td></td>
<td>VAG-F/VAG-R</td>
<td>1–10</td>
</tr>
<tr>
<td></td>
<td>ICT37/ICT38</td>
<td>10–100</td>
</tr>
<tr>
<td></td>
<td>H. pylori</td>
<td>DNA only</td>
</tr>
<tr>
<td></td>
<td></td>
<td>H. pylori DNA + gastric tissues</td>
</tr>
<tr>
<td></td>
<td></td>
<td>H. pylori DNA + saliva tissues</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Parameter</th>
<th>H. pylori DNA</th>
<th>HP64-f/HP64-b</th>
<th>VAG-F/VAG-R</th>
<th>EHC-U/EHC-L</th>
<th>ICT37/ICT38</th>
</tr>
</thead>
<tbody>
<tr>
<td>True positive (n)</td>
<td>47</td>
<td>45</td>
<td>47</td>
<td>46</td>
<td>48</td>
</tr>
<tr>
<td>False negative (n)</td>
<td>3</td>
<td>5</td>
<td>3</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>False positive (n)</td>
<td>3</td>
<td>1</td>
<td>4</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Sensitivity (%)</td>
<td>94</td>
<td>90</td>
<td>94</td>
<td>92</td>
<td>96</td>
</tr>
<tr>
<td>Specificity (%)</td>
<td>94</td>
<td>98</td>
<td>92</td>
<td>98</td>
<td>98</td>
</tr>
<tr>
<td>Accuracy (%)</td>
<td>94</td>
<td>94</td>
<td>93</td>
<td>95</td>
<td>97</td>
</tr>
<tr>
<td>PPV (%)</td>
<td>94</td>
<td>98</td>
<td>92</td>
<td>98</td>
<td>98</td>
</tr>
<tr>
<td>NPV (%)</td>
<td>94</td>
<td>91</td>
<td>94</td>
<td>92</td>
<td>96</td>
</tr>
</tbody>
</table>

"n", Number of samples; PPV, positive predicator value; NPV, negative predicator value.

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-b, 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).
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 predictive value, and negative predictive 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 *H. pylori* infection as determined by histology, culture, 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).

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.
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-negative 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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