ORAL Helicobacter pylori, Its Relationship to Successful Eradication of Gastric H. pylori and Saliva Culture Confirmation

The present study was designed to explore the existence of oral Helicobacter pylori (H. pylori), its relationship in the oral cavity to the success rate of eradication of the gastric H. pylori infection, and to determine if the mouthwash solution contained lysine (0.4%) and glycerol monolaurate (0.2%) (LGM) could eliminate oral H. pylori, as well as using the saliva H. pylori culture to confirm the existence of oral H. pylori. A total of 159 symptomatic individuals with stomach pain and 118 asymptomatic individuals with no stomach complaints, were recruited and tested using the saliva H. pylori antigen test (HPS), the H. pylori flagellin test (HPF), the urea breath test (UBT C\(^13\)) and the polymerase chain reaction (PCR) test, which tests were also confirmed by saliva culture. The test subjects also received various treatments. It was found that the H. pylori antigen exists in the oral cavity in UBT C\(^13\) negative individuals. Traditional treatment for gastric eradication had only a 10.67% effectiveness rate on the oral H. pylori infection. In groups of patients with the oral H. pylori infection, but with negative UBT C\(^13\), a mouthwash solution provided a 72.58% effectiveness rate in the 95% of the confidence interval (CI) ranges on the oral H. pylori infection. Traditional drug gastric eradication and teeth cleaning (TC) had less than a 10% effectiveness rate. Treatment of the oral infection increased the success rate of eradication of the stomach infection from 61.33% to 82.26% in the 95% CI ranges. We concluded that the successful rate of eradication of gastric H. pylori bears a significant relationship to the oral infection from H. pylori.

Key words: Helicobacter pylori, rapid saliva diagnostic test, urea breath test, LGM mouthwash solution, saliva H. pylori culture

INTRODUCTION

An article published in The Lancet in 1983 by J.R. Warren and B. Marshall entitled, “Unidentified Carved Bacilli on Gastric Epithelium in Active Chronic Gastritis”, indicated that Helicobacter pylori (H. pylori) contributes to ulcers, gastritis, and gastric adenocarcinoma (1). Warren and Marshall shared the Nobel Prize in Physiology and Medicine in 2005 for this discovery. Despite Warren and Marshall’s discovery of a gastric etiological basis for H. pylori, there is a secondary issue that has not been well-addressed: 1) Why is the recurrence rate, after the successful eradication of H. pylori in a patient’s stomach approximately 13% per year, and 2) Is oral H. pylori involved in the recurrence of gastric infection?

Our research hypothesizes and the clinical trials have indicated that there are oral infections of H. pylori that may be a source of infection, independent of the stomach H. pylori infection, or which may be part of an infection that was not effectively treated by traditional eradication in the stomach (3-9). In the current literature, there is no definite conclusion as to whether or not oral H. pylori exists (10). It is proposed that no living H. pylori exists in the oral cavity, and that the positive results detected by PCR in the oral cavity may be a fragment of H. pylori, instead of living bacteria, or part of a reflex from the stomach. The H. pylori comes from the stomach reflex only to survive in the oral cavity for a few hours because of the high oxygen concentration in the oral cavity. If the proposed idea is correct, then the fragment or dead H. pylori should not have any negative effect on the drug eradication of the stomach H. pylori infection. However, we found H. pylori in the oral cavity with the urea breath test (UBT C\(^13\)) negative patients, who had no reflex of H. pylori from the stomach. Also, in our clinical trial (3, 6) we found there is a close relationship between oral and stomach H. pylori infections. Obviously, if living oral H. pylori does exist in the oral cavity, and exists either before or after the stomach drug treatment, it raises significant issues regarding the treatment protocols (7).

Krajden et al. first reported successfully cultured H. pylori from dental plaque, but all 71 patients’ saliva culture failed to show any positives (11). Since then, many researchers and scientists attempted saliva H. pylori cultures (S-Hp-C), but are without success (12). Why is it so difficult for S-Hp-C One of the possible reasons is that there are only small numbers of H. pylori in the oral cavity and they cannot successfully compete with the large number of other oral bacteria in a conventional culture process. It seems that the conventional culturing technique for S-Hp-C has been stretched to the limit. If we do not fundamentally change the conventional culturing technique, the likelihood of obtaining positive S-Hp-C is low. A key issue in conventional culturing technique is that antibiotics are always used. Antibiotics can inhibit oral bacterial growth, but also kill H. pylori in saliva. In our proposed culturing technique, we use...
H. pylori treatment on saliva samples. The urea produces an ammonia cloud forming a protective surrounding for H. pylori, and the strong acidic HCL eliminates other oral bacterial.

There are reports that indicate drug eradication on stomach H. pylori with no effect on oral H. pylori (5). In the food industry ε-polysine (L) and the Glycerol Monolaurate (GM) are used in preserving meat products. The L is typically produced as a homo-polypeptide of approximately 25–30 L-lysine residues. The epsilon (ε) refers to the linkage of the lysine molecules. In contrast to a normal peptide bond that is linked by an alpha-carbon group, the lysine amino acids are molecularly linked by the epsilon amino group and the carboxyl group. L belongs to the group of cationic polymers (13). In water, L contains a positively charged hydrophilic amino group. It is adsorbed electrostatically to the cell surface of the bacteria, followed by a stripping of the outer membrane. This eventually leads to the abnormal distribution of the cytoplasm, causing damage to the H. pylori cell. GM is the mono-ester formed from glycerol and lauric acid. H. pylori is extremely sensitive to GM, however there are no reports of L or GM killing H. pylori in vivo. Since both have had a safe record in the food industry, we introduced them into the oral cavity to see whether they could eliminate H. pylori.

MATERIAL AND METHODS

This study was approved by the ethical committee of the hospital. The trial registration number is NCT01863823. Informed consent was obtained from all the participants.

Recruitment of patients

Symptomatic patients were recruited using the following criteria:

1) Inclusion: patients with gastrointestinal complaints, who had been diagnosed with the H. pylori infection using the standard method of UBT C13, regardless of the duration or severity of their illnesses, or whether those illnesses may have been combined with other diseases. After all samples were collected, these patients then received various treatments.

2) Exclusion: patients who had taken antibiotics and/or proton pump inhibitors within four weeks prior to sample collection.

There were 73 symptomatic men (average age: 43.6, range 3–77 years) and 86 symptomatic women (average age: 44.3, range 4–75 years). Of the asymptomatic groups there were 48 asymptomatic men (average age 40.9 years, range 19–72 years) and 70 asymptomatic women (average age 34.4 years, range 18–73 years).

Clinical data were collected from the Beijing University First Hospital, Beijing, China. The results of PCR were collected by the H. pylori Institute, Research Division, Ameritek USA. A double-blind, controlled trial was carried out for this study.

Groupings of patients and sub-groups

Patients were assigned randomly to various sub-groups:

Group A: These patients had been diagnosed by UBT C13, HPS, HPF and PCR (polymerase chain reaction) as all positive, which indicated that they suffered from both stomach and oral H. pylori infections. There were 159 patients divided into three sub-groups. Each sub-group received a different treatment protocol.

A1: a total of 62 patients received treatment with mouthwash (LGM) for the oral infection, plus drug eradication (DE) for the stomach infection.

A2: patients received DE treatment only. There were 75 patients in this subgroup.

A3: a total of 22 patients received teeth cleaning (TC). There was a smaller number of patients in this group because the TC required keeping three cleaning appointments within one month, and some of patients did not complete their TC treatments.

Group B: Patients were diagnosed as positive by HPS, HPF and PCR, but as negative with UBT C13, indicating that the patients had only an oral infection. There were 118 individuals, who were divided into four sub-groups. Each sub-group received a different treatment protocol:

B1: a total of 34 patients received treatments with L-GM and TC.

B2: these patients received L-GM treatment only. There were 35 patients in this sub-group.

B3: a total of 31 patients received DE treatment. There were fewer patients in this group because some of patients did not complete the UBT C13 test.

B4: these patients received the TC treatment. There were 18 individuals in this sub-group.

Tests

Detection of the H. pylori antigens: HPF, HPS and PCR in saliva were compared with UBT C13.

1. UBT C13

Each person swallowed a capsule of urea (UBT C13). Breath was collected and analyzed according to standard procedures (14).

2. HPF

H. pylori flagellin antigen was specifically detected in saliva, using a lateral flow, immuno-chromatographic test device. The device for flagellin detection in saliva was identical to the device used for stool flagellin detection. The saliva flagellin test employed a monoclonal antibody that was developed against semi-purified H. pylori protein (3).

Test procedure:

(a) No food or drink was allowed for one hour prior to the test.

(b) A swab was inserted under the tongue for at least 1 minute.

(c) The swab was removed and swirled vigorously for 15 seconds in a buffer solution.

(d) The wet swab had as much liquid as possible expunged from the swab by pressing and rotating the fiber portion against the wall of the tube.

(e) Two to 3 drops of saliva buffer mixture were added to the sample well of the test device.

As the test kit we developed begins to work, a purple color moves across the result window in the center of the test disk. The presence of two color bands ("T" band and "C" band) within the result window indicates a positive result. The presence of only one purple color band indicates a negative result.

Specificity

An in-house study was conducted with 3 separate lots of the H. pylori flagellin antigen test, to determine specificity. The following common oral bacteria were applied: Actinomyces naeslundii, Actinomyces odontolyticus, Bifidobacterium dentium, Corynebacterium matruchotii, Gemella haemolysans,
Granulicatella adiacens, Streptococcus gordonii, Streptococcus salivarius, Streptococcus sanguinis, and Veillonella parvula as well as E. coli having flagella. All of these were analyzed and did not show interference or cross reactivity with the test.

Sensitivity: 10 ng/ml of H. pylori flagellin antigen.

3. HPS

H. pylori urease antigen was specifically detected in saliva using a lateral flow, immuno-chromatographic test (3). The HPS test is similar to UBT C13 in detecting urease released by H. pylori. The test employed monoclonal antibodies which were developed against semi-purified urease protein.

Specificity and sensitivity were the same, as noted above. The procedure for the HPS test was similar to the HPF: no food and water were allowed for one hour prior to the test.

4. PCR

PCR with primers was targeted to the glycerol monolaurate (GlmM) sequence of H. pylori on the specimens of saliva and dental plaque (15). The scientists of the H. pylori Institute, Research Division, Ameritek USA ran the PCR tests, which were blinded after assignment to interventions.

5. Saliva H. pylori culture

- (a) Add 500 µl of 0.4 M urea (filter sterile) into 13 mm tube;
- (b) Add 1 ml of 0.15 M HCl (diluted with filter sterile H2O) into the tube;
- (c) Add 1 ml of saliva sample into the tube. (final urea conc.; 0.08 M, final HCl conc. 0.06 N);
- (d) Mix gently using pipetting;
- (e) Incubate at 37°C for 5 min;
- (f) Dilute 1:10 with filter sterile PBS (0.5 ml sample + 4.5 ml PBS);
- (g) Inoculate 500 µl of diluted solution on TSA+ 5% blood plate;
- (h) Incubate at 37°C in a CampyJar with a Campypack and water for 5–7 days.

We ran all the following tests to confirm the colonization of H. pylori:

- (i) Oxidase test: the oxidase test determines the presence or absence of cytochrome c oxidase activity in bacteria. Organisms containing cytochrome c as part of their respiratory chain are oxidase positive and turn the reagent purple.
- (j) Catalase test: most cytochrome containing organisms produce a catalase enzyme which breaks down hydrogen peroxide into oxygen and water. When a small amount of a catalase producing organism is introduced into hydrogen peroxide, bubbles of oxygen form as a result of the enzyme’s activity.
- (k) H. pylori antigen and antibody test: the red color indicated a positive.
- (l) Microscopy: see Gram negative staining that show pink and spiral bacteria.

Treatments

LGM: the mouthwash solution contained lysine (0.4%) and glycerol monolaurate (0.2%). Oral rinsing with 20 ml of solution was done twice a day (in the morning and at night before bed) for one month.

DE: standard dose proton-pump inhibitors (PPIs) were used twice a day for 14 days. (PPIs include: lansoprazole 30 mg, omeprazole 20 mg, pantoprazole 40 mg, rabeprazole 20 mg, and esomperazone 40 mg). Also used were: 500 mg of tetracycline, twice a day, and 1000 mg of amoxicillin, twice a day for a total 14 days; 525 mg of bismuth subsalicylate, once daily, and 250 mg of metronidazole, four times a day (taken with meals and at bedtime), both for 14 days.

TC: teeth cleaning. Professional dental prophylaxis/periodontal scaling was required with three appointments during a one-month period.

Statistical analysis

The data for each diagnostic test method were analyzed by symptomatic grouping and also by UBT C13 subgrouping. The symptomatic group was analyzed in three ways: as a group, as a UBT + subgroup, and as a UBT – subgroup. Positive rates (P) for each diagnostic test within all groupings were calculated using the number of individuals with positive results, divided by the total number of individuals (N) in the group or subgroup. Normal approximation was used to calculate the 95% of the CI ranges for each diagnostic method, (P±1.96 sqrt [P(1–P)/N]), where P is the positive rate for a diagnostic test and N is the total number of individuals. For comparison within each UBT subgroup, the sample sizes were relatively small. An exact method based on binomial distributions was used to calculate the 95% of CI ranges. Since test results from any single individual may not have been independent, the paired chi-square test (McNemar test) was used to compare the differences in positive rates among the diagnostic methods. For comparison of test results within the overall symptomatic and asymptomatic groups, a generalized estimating equation method was used. A p-value of <0.05 indicated that the paired test results differed significantly. A statistical analysis software (SAS) package was used for data analysis (16).

RESULTS

The types of testing assigned to individuals were based on whether or not they presented with chronic symptoms of indigestion/stomach complaints. Both symptomatic and asymptomatic groups were assigned HPS, UBT C13, and PCR testing. There were 73 symptomatic men (average age 43.6 years, range 3–77 years), and 86 women (average age 44.3 years, range 4–75 years). Of the asymptomatic groups, there were 48 men (average age 40.9 years, range 19–72 years), and 70 women (average age 34.4 years, range 18–73 years).

Effectiveness of treatment in group A

In Group A, patients were diagnosed using UBT C13, HPS, HPF and PCR, which showed all as positive, and which indicated that patients suffered from both stomach and oral infections.

There were three sub-groups:

A1: patients received treatment with LGM for oral infections, and DE for stomach infections. The results indicated a 72.58% effectiveness rate on oral infections in the 95% of the CI ranges, as determined by changing HPS, HPF and PCR tests to negative (see Fig. 1).

A2: Patients received DE, which was only 10.67% effective in the 95% of the CI ranges on oral infection.

A3: Patients received teeth cleaning (TC). No patients showed changes in HPS, HPF or PCR.

There was a significant difference of p<0.05 between A1 and A2, and between A1 and A3.

The effect of stomach infection in treatment Group A varied, as determined by UBT C13 (see Fig. 2).

Sub-group A1 showed an effectiveness rate of 82.26% in the 95% CI.
Sub-group A2 showed an effectiveness rate of 61.54% in the 95% CI. Sub-group A3 showed an effectiveness of 0%. There was a significant difference of p<0.005 between both A1 and A2, and A1 and A3.

In Group B, a total of 118 asymptomatic individuals were diagnosed with positive HPS, HPF and PCR, but negative for UBT C13. There were four sub-groups;
B1: a total of 34 patients received treatments of LGM and TC and showed a treatment effectiveness rate 73.53% in the 95% of the CI ranges on oral infection.

B2: these patients received LGM treatment treatment without TC. They showed an effectiveness rate of 77.14% in the 95% of the CI ranges on oral infection. There were 35 people in this sub-group.

B3: a total of 31 patients received DE treatment and showed an effectiveness rate of only 9.68% in the 95% of the CI ranges on oral infection.

B4: a total of 18 patients received TC treatment. They showed only an effectiveness rate of 5.56%.

Performance and reliability of oral H. pylori antigen tests

In our previous multi-center screening trial of two rapid HPS and HPF tests (urease and flagellin), we studied the existence of oral H. pylori using the saliva of 201 men and women (3). They were also tested by the gold standard of H. pylori diagnoses: endoscopy, UBT C13, Campylobacter-like organism (CLO), silver stain, and culture, serology, and stool tests. The analysis (Table 1) showed that overall, a high percentage of UBT C13 + individuals were also positive for serum tests, oral tests, or both (>82% in the 95% of the CI ranges). Very low percentages of UBT C13 + people were negative for serum or oral tests (57.8%). These observations indicated that both the serum and oral antigen tests performed reliably, by detecting evidence of H. pylori in infected individuals.

In contrast, all Table 1 percentages for UBT C13 – individuals were quite similar. (Mean = 45±2% in the 95% of the CI ranges).

Examination of UBT C13 – symptomatic and asymptomatic sub-groups, however, revealed that this was because the percentages were the sum of high numbers of both test-positive symptomatic people and high numbers of test-negative asymptomatic people. Normalized data indicated that both antigen tests were strong indicators of the presence of H. pylori antigen in the mouth. (Fig. 4).

Normal oral test results for individual people. Each person was either +/+ (both oral tests were positive), +/− (only one oral test was positive), or −/− (both oral tests were negative). Each subgroup has been normalized to represent the same group size as the UBT C13 + symptomatic group (81 people).

Table 1 presents individual-level presentation of oral and serum test results. Both Table 1 and Fig. 4 show that 44.2% of all 201 people were negative for both oral antigen tests, and that these were distributed primarily in UBT C13 – asymptomatic individuals. The incidence of both tests being negative was high in UBT C13 – people and declined stepwise to zero, as the evidence of disease increased (Fig. 4). Just 3.5% of UBT C13 + people were negative for both oral tests, and these were only in the asymptomatic subgroup. (Well defined negative results are essential for meaningful testing.)

This study of 201 people demonstrated that oral H. pylori antigens could identify individuals who have no risk for H.
Table 1. Individual-level presentation of oral and serum test results. (A) The number of individuals who were positive or negative for the indicated tests is recorded, and the ratios of UBT status within the indicated test(s) results are calculated. (B) Data were also summarized as the percent of UBT group total. For example, the number of people in the UBT group was 57 + 29 = 86 people. Serum positive people in this group were 17 + 22 = 39. So, 39/86 = 45.3% of individuals in the UBT group were serum positive.

<table>
<thead>
<tr>
<th>Subgroup</th>
<th>Serum positive</th>
<th>Serum and 1 or 2 oral tests positive</th>
<th>2 oral tests positive</th>
<th>Serum negative</th>
<th>1 or 2 oral tests negative</th>
<th>Serum and 2 oral tests negative</th>
<th>2 oral tests negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>UBT– Asymptomatic (n=57)</td>
<td>17</td>
<td>11</td>
<td>20</td>
<td>40</td>
<td>37</td>
<td>27</td>
<td>31</td>
</tr>
<tr>
<td>UBT– Symptomatic (n=29)</td>
<td>22</td>
<td>21</td>
<td>21</td>
<td>7</td>
<td>8</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>UBT+ Asymptomatic (n=34)</td>
<td>28</td>
<td>23</td>
<td>25</td>
<td>6</td>
<td>9</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>UBT+ Symptomatic (n=81)</td>
<td>78</td>
<td>72</td>
<td>75</td>
<td>3</td>
<td>6</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>UBT– total (n=86)</td>
<td>39</td>
<td>32</td>
<td>41</td>
<td>47</td>
<td>45</td>
<td>34</td>
<td>38</td>
</tr>
<tr>
<td>UBT+ total (n=115)</td>
<td>106</td>
<td>95</td>
<td>100</td>
<td>9</td>
<td>15</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Ratio of UBT+ : UBT– totals</td>
<td>2.7</td>
<td>3.0</td>
<td>2.4</td>
<td>5.2</td>
<td>3.0</td>
<td>8.5</td>
<td>9.5</td>
</tr>
</tbody>
</table>

**Table 2.** The test results of HPS and HPF confirmed by S-Hp-C.

<table>
<thead>
<tr>
<th>Subgroup</th>
<th>HPS &amp; HPF +</th>
<th>HPS &amp; HPF –</th>
<th>HPS &amp; HPF +</th>
<th>HPS &amp; HPF –</th>
</tr>
</thead>
<tbody>
<tr>
<td>S-Hp-C+</td>
<td>159</td>
<td>34</td>
<td>118</td>
<td>52</td>
</tr>
<tr>
<td>S-Hp-C-</td>
<td>138</td>
<td>1</td>
<td>101</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>21</td>
<td>33</td>
<td>17</td>
<td>50</td>
</tr>
</tbody>
</table>

**DISCUSSION**

The detection of *H. pylori* within dental plaque or saliva would provide a foundation for a role of the oral cavity in *H. pylori* transmission, since there has been disagreement as to whether or not oral infection of *H. pylori* exists. UBT C13 is the gold standard for diagnosis of stomach *H. pylori*, but is not so for detection in the mouth. Difficulties in culturing oral samples are caused by the presence of a viable coccoid form of *H. pylori*, which is unculturable by conventional technology (11). Although PCR allows for the rapid detection of even small numbers of *H. pylori*, it is not a convenient diagnostic method for use in medical clinics (17). There have been disputes as well regarding high positive rates, which may be caused by fragments of *H. pylori* (10). It has been proposed that salivary *H. pylori* originates from gastrointestinal reflux (10, 18). Our study shows that saliva *H. pylori* exists when patients present with a negative UBT C13. In the absence of stomach infection, patients may still have the *H. pylori* antigen in the mouth, even in the absence of disease. Both oral antigen tests were negative for 38 of the UBT C13 – people, indicating they likely carried no *H. pylori*.

**Confirmation of S-HP-C**

We ran S-HP-C on saliva sample individuals with all negative and positive test results of HPS and HPF. Based on the S-HP-C data, we calculated the sensitivity, specificity, accuracy, and positive and negative predictive values of HPS and HPF. For the results of the comparison study see Table 2 and Fig. 5.

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conditions promote a viable habitat for micro-aerophilic and anaerobic microorganisms. Because dental biofilms can provide urea, urease-producing bacteria such as *H. pylori* may have improved viability in this periodontal environment. Because antibiotics have difficulty penetrating bacterial biofilm structures (19). The microbial ecology of the oral cavity is highly complex, richly diverse, and not yet well understood. Poor periodontal health may be associated with *H. pylori* infections in the oral cavity and with poverty status. This is why the efficacy of drug treatment on gastric *H. pylori* is lower (60%) than has been reported when good patient compliance is achieved to 80–90%.

Meta-analysis indicated that *H. pylori* gastric infection doubles the likelihood of finding *H. pylori* in the mouth (19). The search of Scopus detected 221 articles that have ‘*Helicobacter pylori*’ and ‘oral’ in the title. Their conclusions showed a close relationship between infection of *H. pylori* in the oral cavity and in the stomach. *H. pylori* in the oral cavity is more difficult to eradicate and thus may be a source of recurrence.

We observed that *H. pylori* directly infected the oral mucosa in a manner similar to that seen in the gastric mucosa and it does infect the periodontium as well. *H. pylori* colonize the gastric mucosa causing inflammation and being of potential risk for gastric cancer development that may due to the contribution of fibroblasts (20). The proteinase-activated receptors may involve inflammatory responses of gastric mucosa (21). We note that the presence and severity of periodontal disease in patients were frequently associated with oral *H. pylori* carriage rate (22). The treatment of LGM on oral *H. pylori* infection improves the severity of periodontium diseases.

The Centers for Disease Control and Prevention estimate that approximately two-thirds (2/3) of the world’s population harbors *H. pylori* bacterium, with infection rates much higher in developing nations than in Europe and North America. There are 800,000 new cases of stomach cancer and 640,000 deaths per year worldwide. *H. pylori* is the only proven oncogenic bacterial species (23), and detecting and/or curing the infection in its early stages is essential if gastric disease is to be prevented. More than half of all newly diagnosed human stomach cancers occur in Eastern Asia (24). The discovery of oral *H. pylori* is particularly significant and meaningful because it is possible that approximately 75% of the population of Asia suffers from gastric diseases (3). In China alone, more than 900 million people (25%) carry oral *H. pylori* (25–27).

In summary, both saliva antigen tests and PCR were positive in UBT – test subjects. This study indicates that these individuals have had an oral infection from *H. pylori* but have had no stomach *H. pylori* infection or complaints. LGM proved effective in eradicating oral *H. pylori* infection. Drug eradication and teeth cleaning did not have any effect. Treatment of oral infection significantly increased the success rate of eradication of stomach *H. pylori* infection.

**Authors’ contributions:** X.M. participated in part of the PCR studies, the clinical trial and also drafted the manuscript. K.C. made contributions to the conception, design, analysis and interpretation of data. J.L., H.Y., M.L. and G.Y. performed various tests and collected data in the clinical trial. Noriko participated in the PCR studies. All authors read and approved the final manuscript.

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