Detection and location of *Helicobacter pylori* in human gastric carcinomas

Yun-Lian Tang, Run-Liang Gan, Bi-Hua Dong, Ri-Chen Jiang, Rong-Jun Tang

**Abstract**

**AIM:** To define the infection status of *Helicobacter pylori* in 109 patients with gastric cancers and *H. pylori* localization in gastric carcinoma tissues in South China.

**METHODS:** The incidence of *H. pylori* infection in gastric carcinomas was estimated by polymerase chain reaction (PCR), simultaneously; both morphological features and the localization of *H. pylori* in gastric carcinomas were demonstrated by Warthin-Starry (WS) staining. The relationships between *H. pylori* infection and the clinical-pathologic factors of gastric carcinomas were analyzed by software SPSS10.0.

**RESULTS:** *H. pylori* was found in 42 (39.03%) and 58 (53.21%) cases of 109 patients with gastric carcinomas by PCR and WS, respectively. *H. pylori* infection rate detected in gastric carcinomas by WS was higher than that by PCR ($\chi^2 = 9.735, P < 0.005 < 0.01$). WS stain showed that *H. pylori* existed in the gastric antrum mucus, mucosal gland of normal tissues adjacent to gastric carcinomas and the gland, mucus pool of cancer tissues. The positive rate of *H. pylori* in normal tissues adjacent to carcinomas was higher than that in cancer tissues ($\chi^2 = 15.750, P < 0.005 < 0.01$). No significant differences in age, sex, site, histological types and lymph node metastasis were found between *H. pylori*-positive gastric carcinomas and *H. pylori*-negative cases by both methods, but there were statistically significant differences of *H. pylori* positive rate between early and advanced stage of gastric carcinomas ($\chi^2 = 4.548$ or 5.922, $P = 0.033$ or 0.015$ < 0.05$).

**CONCLUSION:** These results suggested that *H. pylori* infection might play a certain role in the early stage of carcinogenesis of human gastric mucosa epithelia.

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**Key words:** Gastric carcinoma; *H. pylori*, Polymerase chain reaction; Warthin-Starry staining
overnight (12-24 h). 

in a swing bed.

protease K, incubated for 120 h at 55

3 min. The last precipitations were re-suspended and

extraction step. An equal volume of phenol 

DNA of H pylori was extracted from control cells, resection 

tissue and serial section of the paraffin wax embedded 

material as described previously.[8] PCR amplification of 

411-bp fragment from the ureA gene of H pylori was carried 

out as described by Monstein et al.[9]

With 1 mL saline, 400 mg frozen fresh tissue blocks 

for each case of 32 human gastric carcinomas were washed 

thrice, sheared to microparticles in an Eppendorf tube and 

then suspended in 400 µL TNE buffer containing 1 mg/mL 

protease K, incubated at 55 °C overnight (12-24 h).

Twenty 6-µm-thick sections of gastric carcinoma tissues 

were cut from each case of 77 paraffin wax embedded 

block and placed in an Eppendorf tube and then thoroughly 

dewaxed in warm xylene four times and re-hydrated by 

passage through graded alcohols (100, 95, 75, 50% for 

30 min, respectively). The supernatant was decanted with 

each tube vortexed again and centrifugated at 14 000 

for 3 min. The last precipitations were re-suspended and 

vortexed in 700 µL 1×SSC buffer. The supernatant was 

decanted again after another 3-min centrifugation, and the 

remaining ethanol was removed with a micro capillary 

pipette. The precipitations were dried, and then resuspended 

in 400 µL paraffin wax digestion buffer containing 1 mg/mL 

protease K, incubated for 120 h at 55 °C in a swing bed. 

On the fourth d, 10 µL×20 µg/mL protease K was added 

to each tube.

DNA of each specimen was purified by an organic 

extraction step. An equal volume of phenol/chloroform: 
isoamyl alcohol (25:24:1) was added to each sample after 

digestion. After 3-min centrifugation at 14 000 g, it was 
mixed thoroughly and centrifuged to separate the aqueous 

and organic layers. The upper aqueous layer containing DNA 

was collected and the extraction was repeated. DNA samples 

were precipitated with two volumes of ethanol precooled 

(-20 °C) and one-tenth volume of 3 mol/L sodium acetate 

(pH 5.2) at -20 °C for 1 h, centrifuged for 10 min of 

14 000 g, dried and redissolved in 50 µL Tris-EDTA buffer 

containing 20 µg/µL RNase. DNA samples extracted were 

assayed quantitatively with ultraviolet spectrophotometer 

with the value of A_{260}/A_{280} from 1.7 to 2.0, finally stored 

at -20 °C.

PCR amplification

Two primers for the detection of H pylori were designed 

according to DNA sequences described by Monstein et al.[8], 

and synthesized in Shanghai Sangon Biological Engineering 

Company. The ureA-H pylori primers used were the 

upstream: 5’GCCAATGGTAAATTAGTT; and the 

downstream: 5’CTCCATATGTTTTTAC, to amplify a 

411 bp fragment of ureA gene region of H pylori DNA. 

The PCR reaction mixture contained 2.5 µL 10×Taq 

polymerase buffer (500 mmol/L KCl, 100 mmol/L Tris-

HCl, 1.5 mmol/L MgCl2 and 0.1% gelatin), 200 µmol/L 
dNTPs, 0.2 µmol/L primers, 1.25 u Taq polymerase and 

0.25 µg DNA samples purified in a final reaction volume 
of 25 µL. After an initial 5-min incubation at 94 °C in the 

Biometra Thermal Cycler to fully denature the template DNA, 
the reaction mixtures were processed through 35 PCR cycles 
of 1-min denaturation at 94 °C, 1-min annealing at 47 °C, 
and 1-min extension at 72 °C, followed by 10 min at 72 °C 
to ensure that all the products were fully extended. Each 
PCR experiment included a positive control of DNA from H pylori NCTC 11 637 and a negative control of TE in 
place of DNA. Ten microliters of reaction mixtures were 
loaded to 1.5% agarose gel for electrophoresis. Being stained 
with ethidium bromide in a DNA subcell at 100 V for 30 min, 
the gel was then observed under ultraviolet ray. The amplified 
products of ureA gene were at the band of 411 bp. If an 
orange band appeared on the band of 411 bp, which was 
identical to the product from positive control well, the result 
was thought to be positive or the sample was thought to 
have been infected with H pylori.

Warthin-Starry staining

Five-micrometers thick paraffin sections from each case 
of 109 gastric carcinoma tissues were mounted on slides 
and backed for 1 h at 60 °C. After routine dewaxing and 
re-hydration with deionized water, tissue sections were 
washed twice with 0.2 mol/L acetic acid buffer, incubated 
at 56 °C for 1 h in 1% silver nitrate buffer in the dark box, 
then dipped in developer solution and stained for 3-8 min. 
After dipping into distilled water at 56 °C and washed for 
2 min, sections were washed once more with distilled water, 
then dehydrated with 100% alcohol, cleared with xylene, 
and mounted with neutral gum. Under microscope 
observation, H pylori was stained into buffy or black color, 
and the background was light yellow.

Statistical analysis

Results of H pylori detection and the clinical-pathologic 
parameters of 109 cases of gastric carcinomas were 
statistically analyzed with software SPSS10.0.

RESULTS

PCR detection of H pylori in gastric carcinomas

PCR amplification products of H pylori DNA were presented 
in 42 (39.03%) cases of gastric carcinomas. Only one 
positive orange band was presented on the band of 411 bp 
from positive control and positive samples, but no 
amplification band was found from negative control 
and negative samples. PCR products were evaluated by 1.5% 
agarose gel electrophoresis, as shown in Figure 1. Positive 
bands were presented in 17 (53.12%) from frozen tissues 
with gastric carcinomas and in 25 (32.47%) from paraffin-
section tissues with gastric carcinomas. It is quite obvious 
that there was a statistically significant difference between 
frozen tissues and paraffin sections (0.01<P<0.05).

Detection of H pylori by WS stain

WS stain showed 58 out of 109 cases (53.21%) of tissue 
sections carrying bacterial bodies of H pylori from 109 gastric
carcinomas, and the positive rate was higher than that detected by PCR method. Simultaneously, shape and location of H pylori could be clearly seen (Figure 2), H pylori of different sizes showed different forms such as curve, S-shape, arc or stem. In non-tumor tissues adjacent to gastric cancers, some bacteria gathered together just like shoals of fish. However, in tumor tissues, they diffused in every direction. In 25 of 58 cases with H pylori-positive gastric carcinomas, bacterial bodies of H pylori were observed in the mucus, mucosal gland cavum, epithelial cell plasmas of normal tissues adjacent to cancers. Bacterial bodies of H pylori in 3 of 58 cases were observed in the gland, mucous pool and plasma of cancer cells. H pylori was observed in both tumor and non-tumor sites in 30 of 109 cases with gastric carcinomas (Table 1). By χ² test (χ² = 15.750, P<0.005 <0.01), H pylori positive rate in non-tumor sites was higher than that in tumor sites.

The results of H pylori in 109 cases with gastric carcinomas were statistically analyzed by Pearson Chi-Square of SPSS10.0, which were χ² = 9.735, P<0.005<0.01 (Table 2). There was significant difference in H pylori positive rates detected by PCR and WS methods, respectively.

**Relationships between H pylori infection and the clinical-pathologic characteristics of human gastric carcinomas**

On the basis of data in Table 3, the results were statistically analyzed by Pearson Chi-Square of SPSS10.0. No statistically significant differences in age, sex, site and lymph node metastasis were found between H pylori-positive cases and H pylori-negative cases of gastric carcinomas by both methods of PCR and WS.

On the basis of data in Table 4, the results were statistically analyzed by Pearson Chi-Square of SPSS10.0, and no significant differences of H pylori detection were shown among four types of gastric carcinoma according to historical morphology by both PCR and WS stain.

The positive rates of H pylori DNA were 62.5 and 34.4% in 16 cases of early gastric carcinomas and 93 cases of advanced gastric carcinomas by PCR detecting H pylori, respectively. Whereas, the positive rates of H pylori were 75.0 and 49.5% in early gastric carcinomas and advanced gastric carcinomas by WS, respectively. The results showed that statistically significant differences between H pylori-positive gastric carcinomas and H pylori-negative cases were found in clinical stages of gastric carcinomas (χ² = 4.548, 5.922, P = 0.033 or P = 0.015<0.05), and H pylori positive rate of early gastric carcinomas was higher than that of the advanced gastric carcinomas (Table 5).

![Figure 1](image1.png) 1.5% agarose gel electrophoresis of PCR products, showing amplification of 411-bp fragment of ureA gene from DNA extracted.

**Table 1 Numbers of H pylori positive cases in different histological sites**

<table>
<thead>
<tr>
<th>Tumor site</th>
<th>No tumor site</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>30</td>
<td>33</td>
</tr>
<tr>
<td>-</td>
<td>25</td>
<td>51</td>
</tr>
<tr>
<td>Total</td>
<td>55</td>
<td>109</td>
</tr>
</tbody>
</table>

**Table 2 Detection of H pylori by WS stain and PCR**

<table>
<thead>
<tr>
<th>PCR</th>
<th>WS stain</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>38</td>
<td>42</td>
</tr>
<tr>
<td>-</td>
<td>20</td>
<td>47</td>
</tr>
<tr>
<td>Total</td>
<td>58</td>
<td>109</td>
</tr>
</tbody>
</table>

**Table 3 Relationships between H pylori infection and gastric carcinomas in age, sex, site and lymph node metastasis**

<table>
<thead>
<tr>
<th>Factors</th>
<th>Total cases</th>
<th>H pylori positive</th>
<th>χ²</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yr)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥60</td>
<td>29</td>
<td>7</td>
<td>1.999</td>
<td>0.159</td>
</tr>
<tr>
<td>&lt;60</td>
<td>80</td>
<td>35</td>
<td>3.705</td>
<td>0.054</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>64</td>
<td>23</td>
<td>1.131</td>
<td>0.288</td>
</tr>
<tr>
<td>Female</td>
<td>45</td>
<td>19</td>
<td>2.500</td>
<td>0.114</td>
</tr>
<tr>
<td>Locus</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gastric antrum</td>
<td>55</td>
<td>22</td>
<td>1.221</td>
<td>0.269</td>
</tr>
<tr>
<td>Gastric fundus and gastric corpus</td>
<td>54</td>
<td>20</td>
<td>0.079</td>
<td>0.776</td>
</tr>
<tr>
<td>Lymph node metastasis</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LN+</td>
<td>66</td>
<td>26</td>
<td>0.052</td>
<td>0.819</td>
</tr>
<tr>
<td>LN-</td>
<td>43</td>
<td>16</td>
<td>0.120</td>
<td>0.440</td>
</tr>
</tbody>
</table>

**Table 4 H pylori infection comparison with histological types of gastric carcinoma**

<table>
<thead>
<tr>
<th>Histological types</th>
<th>Total cases</th>
<th>PCR</th>
<th>WS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Well-differentiated adenocarcinoma</td>
<td>23</td>
<td>9</td>
<td>14</td>
</tr>
<tr>
<td>Poorly-differentiated adenocarcinoma</td>
<td>49</td>
<td>16</td>
<td>33</td>
</tr>
<tr>
<td>Signet-ring cell carcinoma</td>
<td>17</td>
<td>7</td>
<td>10</td>
</tr>
<tr>
<td>Mucous adenocarcinoma</td>
<td>20</td>
<td>10</td>
<td>10</td>
</tr>
</tbody>
</table>

**Table 5 Relationships between H pylori infection and clinical stages of gastric carcinomas**

<table>
<thead>
<tr>
<th>Clinical stage</th>
<th>Total cases</th>
<th>Positive</th>
<th>Negative</th>
<th>χ²</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Early</td>
<td>16</td>
<td>10</td>
<td>6</td>
<td>4.548</td>
<td>0.033</td>
</tr>
<tr>
<td>Advanced</td>
<td>93</td>
<td>32</td>
<td>61</td>
<td>5.922</td>
<td>0.015</td>
</tr>
</tbody>
</table>

P: PCR results, W: WS results.
DISCUSSION

*UreA* gene ordinarily exists in the genome of *H pylori*, so amplification of 411-bp fragment nucleotides is used to detect the presence of *H pylori*. However, its sensibility is determined by the primers selected, the source of DNA and quantity of bacterial presented in tissues. The fragment of 411 bp of *ureA* gene was amplified by PCR to detect the presence of *H pylori* genome in this study. The positive rate detected by PCR method was lower than that by WS stain. The possible explanation: (1) The order of magnitude of bacterial detected by PCR was over one hundred, whereas *H pylori* existing in the section could be easily detected by WS stain when it was carefully observed under light microscope by an experienced observer; (2) When the presence of *H pylori* genome was detected by PCR, its sensibility was closely related to the size of fragment amplified by PCR. Because the fragment amplified by PCR was longer, DNA degradation or fragmentation might take place in treatment process of tissue samples, consequently, the amplification efficiency was lower. Since major samples of gastric carcinomas in the present study were paraffin wax blocked (77/109 cases), DNA degradation or fragmentation might take place in fixation and section of tissue samples and amplification efficiency was lower. The positive rate of *H pylori* was 53.12% from fresh tissues, whereas 32.47% from paraffin wax embedded blocks. The difference might be avoided by small fragment primers to amplify DNA fragments, Fabre et al. detected the presence of *H pylori* genome by using fresh tissues and primers to amplify 210 bp fragments, and their result showed that the positive rate by PCR was identical to that by Giemsa stain.

By WS stain technology *H pylori* were observed under light microscope, which lied in the mucus, mucosal gland cavum, epithelial cell plasma of normal tissues adjacent to cancer and the gland, mucous pool, cancer cell plasmas of cancer tissues. But the *H pylori* positive rate of normal tissues adjacent to cancer was higher than that of cancer tissues. In non-tumor tissues, bacteria gathered together just like shoals of fish. However, *H pylori* diffused in every direction in tumor tissues. The reason was regarded that acidity environments in stomach were suitable for *H pylori* breeding, but with the formation of stomach cancer, constitution structure and microenvironments of gastric mucosa were changed correspondently, which were not suitable for *H pylori* surviving and led to loss of *H pylori*.

In addition, *H pylori* positive rate at early gastric cancer was higher than that at advanced gastric cancer, which suggested *H pylori* might be partly involved in the occurrence and development process of early gastric cancer. Accumulative infection and movement of *H pylori* might play an important role in the development of gastric cancer. In *in vivo* experiments showed that *H pylori* at low inocula stimulated cell proliferation. But at higher inocula (bacteria to cell ratio >100), it caused a time- and concentration-dependent reduction of cell cycle, which would be arrested at G1 phase, inhibit gastric cancer cell proliferation and induce apoptosis. *H pylori* infection can lead to excretion of gastric acid and decrease in ascorbic acid in the gastric gland, cause gastric epithelial cell proliferation by long-term urgent and chronic damage to gastric mucosa and induce long-term tolerant inflammation response, so as to increase carcinogenesis of carcinogen. In a human model of gastric carcinogenesis, Correa thought that *H pylori* might play a role of precursor in gastric cancer, colonic metaplasia was a high-risk -factor of gastric cancer and *H pylori* positive colonic metaplasia along, but should also be considered as a high risk factor of gastric cancer. Cahill divided 151 patients into normal mucosa and *H pylori* negative group, chronic active gastritis and *H pylori* positive group, chronic atrophic gastritis group, intestinal metaplasia group and gastric carcinoma group. Gastric antral epithelial cell proliferation was assessed as the labeling index percent. The results showed that from chronic active gastritis group to gastric carcinoma one, epithelial cell proliferation increased when compared with that of normal mucosa, which was associated with *H pylori* infection. The increase in gastric epithelial cell proliferation associated with *H pylori* infection was not significantly different from that associated with the gastric precancerous lesions. *H pylori* infection, however, did not seem to influence the changed gastric epithelial cell proliferation in subjects with precancerous lesions or gastric cancer, which suggested that *H pylori* played a certain role in early gastric carcinogenesis, although it might not have so strong an influence in the later stages of the disease as that in early ones.

![Figure 2](image)

Figure 2 *H pylori* in different sites. A: *H pylori* in cancer cell plasmas and cancer tissues; B: *H pylori* in mucous pool of mucous adenocarcinoma; C: *H pylori* in the mucus of gastric cavum; D: *H pylori* in mucosal gland cavum, epithelial cell plasmas of normal tissues adjacent to cancer. WS stain ×1 000.
the pyloric region. After the 26th wk, severe active chronic gastritis, ulcers, and intestinal metaplasia could be observed in the infected animals. After the 62nd wk, adenocarcinoma had developed in the pyloric region of 37% (10/27) of the infected animals. It was found that adenocarcinoma development seemed to be closely related to intestinal metaplasia. After this, 5-wk-old Mongolian gerbils were infected with \textit{H. pylori} ATCC-43504 strain by Honda \textit{et al}.\textsuperscript{[24]}.

It was reported that atrophic gastritis and intestinal metaplasia also appeared in the lesser curvature of the ventral mucosa 6 mo after inoculation. Eighteen months after \textit{H. pylori} inoculation, 40% (2/5) infected Mongolian gerbils showed three well-differentiated gastric cancer. Because of the use of 5-wk-old Mongolian gerbils for study, it suggested \textit{H. pylori} infection of early stage might be one of the risk-factors increasing carcinogenesis of gastric cancer. Both of the two groups showed the pathway of \textquoteleft H. pylori\textrightharpoonup atrophic gastritis\textrightharpoonup intestinal metaplasia\textrightharpoonup atypical hyperplasia\textrightharpoonup intestinal-type gastric cancer\textright.

These results suggested that \textit{H. pylori} infection might play a certain role in the early stage of carcinogenesis of gastric mucosa epithelia.

\textbf{REFERENCES}


Assistant Editor Li WZ. Edited by Gabbe M