Experimental *Helicobacter pylori* Infection in Association with Other Bacteria

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Abstract: We performed surgical treatment on normal ddY mice before *Helicobacter pylori* inoculation. The treatment was expected to obstruct bacterial flow out of the stomach and increase the chance of bacterial attachment to the gastric epithelium in mice. The bacterial challenge induced inflammation in the stomach. *H. pylori* was recovered from the stomach throughout the observation period. Lactobacilli and streptococci tended to relate to the increase in number of *H. pylori* recovered. Pretreatment with atropine was considered to confuse the gastric flora and affect the number of *H. pylori* recovered. These results suggested that a certain amount of time is necessary for *H. pylori* to contact with the gastric epithelium and that the composition of flora is important for the establishment of *H. pylori* infection.

Key words: *Helicobacter pylori*, Mouse model, Gastritis, Lactobacilli, Streptococci, Atropine

*Helicobacter pylori*, a Gram-negative spiral bacterium, was first isolated in 1982 from a patient with chronic active gastritis (21). This organism has also been recently shown to be directly linked to the development of peptic ulcers in humans (2, 14, 17) and is associated with an increased risk of gastric cancer (5, 16).

Because there is increasing evidence that *H. pylori* is a significant gastroduodenal pathogen, experimental animal models are deemed appropriate to study the pathophysiology of the disease. To date, studies of the pathogenesis have been limited by the absence of adequate animal models to reproduce the various aspects of *H. pylori* disease. Laboratory strains of *H. pylori* do not infect normal laboratory rodents (3). Experimental models have been currently reported in animals such as gnotobiotic piglets (12), euthymic germ-free mice (10), athymic nude mice (11, 20) and monkeys (1), but these animals are difficult to handle in large numbers and cannot be used to study immune responses. *Helicobacter* species other than *H. pylori*, such as *H. felis* and *H. mustelae*, have been used with gnotobiotic rats (6) and ferrets (7), respectively. However, these animal models do not mimic human *H. pylori* infection. Recently, it has been reported that a mouse model of *H. pylori* infection mimics the human disease (13). In the report, an established laboratory strain (NCTC 11637) was unable to establish infection, whereas fresh clinical isolates colonized in the stomachs of normal mice.

Clinical isolates of *H. pylori* can be divided into at least two major types (22). Type I bacteria express a vacuolating cytotoxin (VacA) and an immunodominant cytotoxin-associated antigen (CagA). Type II bacteria express neither VacA nor CagA. Both types colonized in the stomachs of normal mice and the infection caused gastric injury. A critical factor in their adaptation for life in such a gastric environment is the development of a mechanism for anchorage to the mucosal cells whose surfaces are continually washed by mucus and other secretions. A laboratory strain of *H. pylori* ATCC 43504 classified as type I possesses surface components which mediate bacterial adhesion similar to those of fresh clinical isolates (unpublished data).

It has been indicated that *H. pylori* can infect mucosal surfaces under aseptic conditions (4, 10). Furthermore, we demonstrated that *H. pylori* ATCC 43504 infects the urinary tract of normal mice even if the organ is not a target for the organisms (9). Therefore, we believe that *H. pylori* ATCC 43504 can infect the stomach of mice. We speculate that the bacterial flora is important for the establishment of *H. pylori* infection in mice. In other words, *H. pylori* competes with the attachment site of flora bacteria in the stomach. If *H. pylori* is given enough time to adhere to the site, the organism can...
infect mouse stomach. In this study, we showed a labora-
tory strain of *H. pylori* can infect normal ddY mice and
that the flora of the stomach may modify the infection.

*H. pylori* ATCC 43504 was mainly used for the exper-
iments. Freshly isolated *H. pylori* KK-4 (3 passages)
from a gastric biopsy specimen of a patient with a duo-
denal ulcer was used in the part of the study in which
infectivity was compared between the laboratory strain
and freshly isolated clinical strain. These organisms
were grown for 72 hr at 37°C under microaerophilic
conditions on brain-heart infusion agar (Nissui Co., Ltd.,
Japan) supplemented with 5% horse blood in 10% CO₂
in air. They were collected, suspended in BHI broth medi-
um supplemented with 10% fetal calf serum and enu-
merated with a counting chamber under dark-field
microscopy. The inoculum was adjusted to a concen-
tration of 6.8 × 10⁶ cells/ml. The inoculation volume was
decided as 0.3 ml/mouse (2.0 × 10⁹ cells/mouse) because
it was enough to induce a gastric lesion in almost all mice
in the preliminary experiment. BHI broth without *H.
pylori* was used for the control group.

A total of 108 male ddY mice, 6 weeks old, (Nippon
SLC, Hamamatsu, Shizuoka, Japan) were used. Sev-
enty-five mice were surgically treated prior to oral inocu-
lation with samples. Briefly, the duodenum of each
mouse was clamped by forceps at a point about 5 mm
from the pyloric ring under anesthetization with pento-
barbital sodium (25 mg/kg). Thereafter, the mice were
orally inoculated with samples with or without *H. pylori*
by catheter. After 30 min, clamping was finished. At 1,
3 and 8 days postinfection, mice were examined for
bacterial culture, gastric pH, histological and serological
findings. Twenty-eight mice, including 18 mice with sur-
gical treatment and 10 mice without the treatment, were
examined for the control group. Seventy-five mice were surgically treated prior to oral inoculation with samples. Briefly, the duodenum of each mouse was clamped by forceps at a point about 5 mm from the pyloric ring under anesthetization with pentobarbital sodium (25 mg/kg). Thereafter, the mice were orally inoculated with samples with or without *H. pylori* by catheter. After 30 min, clamping was finished. At 1, 3 and 8 days postinfection, mice were examined for bacterial culture, gastric pH, histological and serological findings. Twenty-eight mice, including 18 mice with surgical treatment and 10 mice without the treatment, were used for the control group in experiments in which the effects of surgical treatment on gastric bacterial flora were examined. Forty-four mice with the treatment were used for an experiment to compare the infectivity between laboratory and clinically isolated strains of *H. pylori*. Thirty-six surgically treated mice were used for an experiment in which the effect of atropine treatment on experimental *H. pylori* infection was examined. Eighteen of the 36 mice were given atropine (3 mg/kg) 30 min before surgical treatment for the inhibition of ga-
stric acid secretion.

All animals were kept in wire-topped polyorphin
cages (CL-0104-2, Japan CLEA Co., Ltd., Japan) at
25±2°C and relative humidity of 50±5% on a 12-hr-
light-dark cycle. Each cage was maintained in a nega-
tive-pressure isolator box (BBH Micro-b-control breeding
apparatus, CL-5603, Japan CLEA Co., Ltd).

Sampling was performed on each examination day.
The stomach of each mouse was resected and divided
longitudinally into two halves. A half of the sample was
weighed, homogenized with a sterial glass homogenizer
with 10-fold BHI medium, and inoculated onto blood
agar plates supplemented with trimethoprim, vancomycin
and polymixin B for *H. pylori* detection. The plates
were incubated at 37°C in microaerophilic conditions for
3 to 7 days. Bacteria were identified as *H. pylori* by
Gram's stain and immunodiffusion by a method described
previously (9).

Lactobacilli, streptococci and other anaerobic bacteria
were also examined in the stomachs of experimental
mice to monitor the flora using homogenized samples of
half-stomach for the culture of *H. pylori*. The diluted
suspension of each sample was plated on BHI agar plate
with 5% horse blood for anaerobes, Rogosa selective
Lactobacillus agar (Difco) for *Lactobacillus* and Mitis
Salivarius agar (MS agar, Difco) for streptococci, as
previously described (8). Both lactobacilli and strepto-
cocci inhabited the stomach of the mice as major mem-
bers of the flora. Other various bacteria also inhabited
the stomach, but their isolation was not constant.

Gastric pH was measured in the mice by using a
Micro-combination pH Probe (Hitachi, Co., Ltd.).

At the necropsy, damage to the stomach and duode-
num was scored. The total scores of each mouse were
estimated by adding the following scores. For gastric
damage: edema, 1; hemorrhage of limited area, 1; he-
morrhage of large area, 2; congestion and swelling, 1;
and increase of gastric juice, 1. For duodenum damage:
edema, 1; hemorrhage of limited area, 1; hemorrhage of
large area, 2, and congestion and swelling, 1. The max-
imum scores for stomach and duodenum were 5 and 4,
respectively.

Samples for pathological examinations were taken
from the mice. Tissues were fixed in 10% formalin,
processed by standard methods and embedded in paraf-
fin: sections were cut 4 µm thick and stained with hema-
toxylin and eosin (H & E).

The qui square test and unpaired two-sample t-test were
used. The selected level of significance was *P < 0.05*.

The effects of surgical treatment on gastric bacterial
flora and tissue damage were examined. An increase in
the number of bacteria including lactobacilli and strep-
tococci in the gastric mucosa was observed immediately
after treatment (data not shown). It gradually decreased
thereafter and recovered to a normal level at 3 days after
experiment. Damage scores indicated that this treat-
ment did not induce pathological effects on the gastric or
duodenum epithelium.

Gastric pH was elevated in mice inoculated with *H.
pylori* ATCC 43504 throughout the experimental period.
The pH of gastric juice at days 1, 3 and 8 after inocula-
tion were 2.7±0.6, 3.7±1.2 and 3.6±0.6, respectively.
They were significantly higher than that of the controls (pH 1.9 ± 0.1). Gastric acidic condition is a major obstacle for colonization of H. pylori on the surface of epithelium. H. pylori is believed to escape from the acidic condition by a pH gradient effect on the gastric mucous layer and the activity of bacterial urease, which produces ammonium from urea. Therefore, the inhibition of gastric acid formation was not so effective in our model. We believe that the acidic condition was largely effective on other gastric flora.

There were no significant differences in H. pylori recovery and gastric damage between the laboratory strain and fresh isolate. A laboratory strain of H. pylori was detected in the stomachs of all mice at 8 days after inoculation and 5 of 6 mice at 14 days after inoculation (Table 1). Similar results were obtained from mice inoculated with the fresh isolate KK-4 strain, which was recovered from the stomachs of 7 of 9 mice at 14 days after bacterial inoculation. Both strains induced gastric damage in all or almost all of the mice with surgical treatment at 14 days after bacterial inoculation. The pathological findings induced by both strains were similar to each other, causing gastric erosions, ulceration of the gastric epithelium and infiltration of inflammatory cells such as polymorphonuclear cells. These facts suggest that infectivities were not different among the strains of H. pylori, if the bacteria could contact the host gastric cell surface.

The inoculation of H. pylori in mice with surgical treatment affected the microbial flora in the stomach (Table 2). The microorganisms, mainly lactobacilli and streptococci, were significantly decreased at 1 day postinoculation, but increased at 3 days. They returned to their original levels at 8 days after inoculation. H. pylori were detected at 1, 3 and 8 days after inoculation. The volumes were highest at day 1 of the experiment. The damage score of the stomach was the highest at day 1 and decreased thereafter (Table 2). The damage score of the duodenum was recognized at 3 and 8 days after H. pylori inoculation.

Table 1. Comparison of infectivity between a laboratory strain and a fresh isolate

<table>
<thead>
<tr>
<th>H. pylori strain</th>
<th>Clamping of forceps</th>
<th>No. of mice/total after</th>
<th>8 days</th>
<th>14 days</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Recovery of H. pylori</td>
<td>Recovery of H. pylori</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Gastric damage</td>
<td>Gastric damage</td>
</tr>
<tr>
<td>A laboratory strain ATCC</td>
<td>+</td>
<td>6/6*</td>
<td>6/6*</td>
<td>5/6*</td>
</tr>
<tr>
<td>43504</td>
<td></td>
<td></td>
<td>5/6***</td>
<td>5/6***</td>
</tr>
</tbody>
</table>

A fresh isolate + NT a) NT NT 7/9* 9/9*

a) Significantly higher than the group without clamping.

**, No significant difference between the laboratory strain and fresh isolate.

**a) NT, not tested.

Table 2. Number of H. pylori and other bacteria in the stomachs of experimentally infected mice with or without atropine treatment

<table>
<thead>
<tr>
<th>Atropine treatment</th>
<th>Days after inoculation</th>
<th>Number of bacteria per stomach</th>
<th>(log₁₀/g)</th>
<th>Damage score</th>
<th>Stomach</th>
<th>Duodenum</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>H. pylori</td>
<td>Lactobacilli</td>
<td>Streptococci</td>
<td>Others</td>
<td></td>
</tr>
<tr>
<td>−</td>
<td>1</td>
<td>7.2±1.6</td>
<td>3.4±1.2**</td>
<td>4.0±1.2**</td>
<td>2.7±1.2**</td>
<td>4.7±1.5*</td>
</tr>
<tr>
<td>−</td>
<td>3</td>
<td>6.0±0.5</td>
<td>7.6±0.2</td>
<td>7.4±0.2</td>
<td>6.7±0.4</td>
<td>2.7±1.5*</td>
</tr>
<tr>
<td>−</td>
<td>8</td>
<td>3.3±0.5</td>
<td>6.8±0.5</td>
<td>6.1±0.7</td>
<td>5.1±0.3**</td>
<td>2.3±0.6*</td>
</tr>
<tr>
<td>+</td>
<td>1</td>
<td>4.1±0.4</td>
<td>6.0±2.0**</td>
<td>7.8±2.0</td>
<td>4.2±1.5**</td>
<td>4.3±2.5*</td>
</tr>
<tr>
<td>+</td>
<td>3</td>
<td>6.0±0.2</td>
<td>7.5±0.3</td>
<td>7.2±0.3</td>
<td>5.0±0.1**</td>
<td>2.7±1.5*</td>
</tr>
<tr>
<td>+</td>
<td>8</td>
<td>3.5±0.4</td>
<td>7.0±0.4</td>
<td>6.9±0.7</td>
<td>4.7±0.7**</td>
<td>2.0±0.2*</td>
</tr>
<tr>
<td>Control A a)</td>
<td>−</td>
<td>−</td>
<td>6.1±0.1</td>
<td>5.1±0.3</td>
<td>4.7±0.2</td>
<td>0</td>
</tr>
<tr>
<td>Control B a)</td>
<td>0</td>
<td>−</td>
<td>7.5±0.2</td>
<td>7.4±0.3</td>
<td>6.8±0.1</td>
<td>0</td>
</tr>
</tbody>
</table>

a) Uninfected mouse without surgical operation.

b) Uninfected mouse with surgical operation.

*, Significantly higher than control A and B.

**, Significantly lower than control B.
The gastric microflora in mice with atropine treatment was different from that of non-treated mice. *H. pylori* increased in the stomach at 3 days after *H. pylori* inoculation. The number of lactobacilli decreased at day 1 although the degree was lighter than that in atropine non-treated mice. In contrast, the number of streptococci was constant during the experimental period. A peak in the number of *H. pylori* was found at day 3 in the atropine-treated group. It was found in non-treated mice at day 1. The damage score of the stomach at day 1 was the highest score of all. The damage was not associated only with *H. pylori* but also with streptococci. For this reason, numerous streptococci-like organisms were recognized on the epithelium of severe damage sites. These results suggested that streptococci was associated with the aggravation of gastric lesions primarily induced by *H. pylori*.

*H. pylori* ATCC 43504 was related to gastritis and the colonization of gastric mucosa with other microorganisms. The administration of a large number of *H. pylori* and increase in contact chance to the gastric epithelium was possible in the animal model. In such a case, *H. pylori* can obtain the attachment sites and streptococci and lactobacilli of oral origin may fail to attach and colonize.

The ecological approaches would require the estimation of the quantitative composition of the gastric mucosal microbial community. Microbial colonization has been found in most patients with gastric disease, and streptococci and *H. pylori* were the most common findings (19). Their numbers, however, varied among the stomachs even in normal humans. In non-ulcerate dysplasia patients with superficial gastritis, the high degree of colonization was due to *H. pylori*, but in the cases of gastric ulcer and duodenal ulcer with the same state of mucosa, high numbers of *H. pylori* occurred in association with other microbes (15). These studies suggest that gastric mucosa is colonized by various microorganisms, which may modify the disease. In our approach, streptococci and lactobacilli were the most common microorganisms in the stomach of mice and were frequently accompanied by *H. pylori*. When the inflammation was severe, the number of streptococci increased. In such cases, the number of *H. pylori* decreased in the stomach. These observations were similar to those in human patients.

Shobna et al reported that *Lactobacillus acidophilus* inhibited the growth of *H. pylori* in vitro (18). Karita et al suggested that the growth of *H. pylori* was eradicated by lactobacilli previously inhabiting the stomach (10). These findings were in agreement with our findings, because the number of *H. pylori* decreased when the number of lactobacilli recovered to the original level. Furthermore, the number of lactobacilli in the atropine-treated group was 100 to 1,000 times more than that of the non-treated group, whereas the number of *H. pylori* in the atropine-treated group was about 1/1,000 of that in the non-treated group at 1 day after inoculation. Thus, the balance of the gastric flora can modify *H. pylori* infection.

**References**


