Virulence of water-induced coccoid Helicobacter pylori and its experimental infection in mice

Fei-Fei She, Jian-Yin Lin, Jun-Yan Liu, Cheng Huang, Dong-Hui Su

Fei-Fei She, Department of Microbiology, Medical College of Wuhan University, Wuhan 430071, Hubei Province, China. Jian-Yin Lin, Department of Molecular Medicine, Fujian Medical University, Fuzhou 350004, Fujian Province, China. Jun-Yan Liu, Department of Microbiology, Medical College of Wuhan University, Wuhan 430071, Hubei Province, China. Cheng Huang, Department of Microbiology, Fujian Medical University, Fuzhou 350004, Fujian Province, China. Dong-Hui Su, Department of Immunology, Fujian Medical University, Fuzhou 350004, Fujian Province, China. Supported by the Natural Science Foundation of Fujian Province, China, No. C95031. Correspondence to: Fei-Fei She, Department of Microbiology, Fujian Medical University, Fuzhou 350004, Fujian Province, China. cyslf@163.net. Telephone: +86-591-3569309. Received: 2002-09-14 Accepted: 2002-10-17.

Abstract

AIM: To explore the virulence and the infectivity of coccoid Helicobacter pylori (H. pylori) transformed from spiral form by exposure to sterile tap water.

METHODS: Three strains of H. pylori, isolated from gastric biopsy specimens of confirmed peptic ulcer, were converted from spiral into coccoid form by exposure to sterile tap water. Both spiral and coccoid forms of H. pylori were tested for the urease activity, and the adherence to Hep-2 cells. The presence of flagella was examined under electron microscopy. In the experimental animal infection, the spiral and coccoid forms of H. pylori originated from the same strain F49 were inoculated intragastrically into BALB/c mice respectively four times at a 3-day interval. Half of the mice from each group were sacrificed at Day 21 and Day 28 after the last inoculation. Histology and H. pylori colonization were detected by urease test of gastric mucosa, cultures of H. pylori, and electron microscopy and so on.

RESULTS: The urease activity and the ability of adherence to Hep-2 cells were found to be lower in coccoid H. pylori than that in its spiral form. For example, the transformation in strain F49 led to a significant decrease of the adherence rate and adherence index from 70.0±5.3 % to 30.2±3.5 % (P<0.01), and from 2.6±0.4 to 0.86±0.3 (P<0.01), respectively. The flagella of coccoid H. pylori were observed under electron microscope. In the experimental infection in mice, the positive rate of gastric mucosa urease test was 93.8 % (15/16) in the group infected by spiral H. pylori and 50 % (8/16) in the group infected by coccoid H. pylori, and the estimated coccoid H. pylori colony number was 1.75 vs 0.56. The positive rates of H. pylori culture were 87.5 % (14/16) in spiral H. pylori group and 68.8 % (11/16) in coccoid H. pylori group. There was no significant difference in either urease test or bacterial culture rate between the groups examined at Day 21 and Day 28 after inoculation.

Electron microscopic examination of the samples taken from both groups showed the adherence of H. pylori in spiral, bacillary and coccoid shapes to the epithelial cells of gastric wall. Histological examination showed the occurrence of gastric mucosal injury as indicated by various degrees of erosion, ulcer, and inflammatory cell infiltration. Mucosal injury was slighter in the mice infected by coccoid H. pylori. No positive result was obtained in the control group that received intragastrical administration of sterile tap water.

CONCLUSION: Although the virulence of coccoid H. pylori induced by water decrease, coccoid H. pylori still remains a considerable urease activity and the adhering ability to epithelial cells. Furthermore, the flagella, an important component responsible for bacterial movement and infection, were still observed as a cellular structure of coccoid H. pylori under electron microscope. The coccoid H. pylori induced by water is capable of colonizing in gastric mucosa and causing gastrititis in mice.

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INTRODUCTION

Helicobacter pylori has been recognized as an important pathogen that causes chronic gastritis and peptic ulcer and likely as a risk factor associated with gastric carcinoma[1-9]. H. pylori infection is endemic. In despite of more than 10 years of intensive research, the precise mode and route of H. pylori transmission remain elusive. Four routes including fecal-oral, oral-oral, gastro-oral and iatrogenic transmission have been postulated[10-13]. The association between water consumption and H. pylori infection indicates that H. pylori may be transmitted through a waterborne route[14,15]. H. pylori exists in two forms: the spiral form and the coccoid form. Coccoid H. pylori is non-culturable but alive[16-20]. Some researchers have shown that H. pylori can survive water microcosms in coccoid form[21,22]. The coccoid H. pylori in water has therefore been suspected to contribute an important part to the transmission of the bacteria. However, the virulence and infectivity of coccoid H. pylori in water has not been studied. To explore the pathogenicity of the coccoid H. pylori in water, three strains of spiral H. pylori were treated by prolonged exposure to sterile tap water and examined for the presence of flagella under electron microscopy and tested for their urease activity and their adherence to Hep-2 cells. A strain was inoculated into the BALB/C mice. The gastric mucosal samples were taken to assess the bacterial in vivo colonization and pathological effects by means of urease test, bacterial culture, electron microscopy, and light microscopy.

MATERIALS AND METHODS

Animals

Female BALB/c mice were purchased from Shanghai...
Experimental Animal Center, Chinese Academy of Sciences and raised under SPF conditions. Those of 8 weeks old, weighing 20-22 g were used for bacterial inoculation. Sterile food and tap water were given ad libitum.

**Cells**

Human epithelial cell line Hep-2 cells were maintained in 1640 medium supplemented with 10% fetal calf serum, 200 IU/ml penicillin and 50 µg/ml streptomycin at 37 °C in 5% CO₂-95% air, and re-cultivated twice a week.

**Bacterial strains**

Three strains (F44, F45 and F49) of *H. pylori* were isolated in this laboratory from gastric biopsy specimens of confirmed peptic ulcer patients. The isolates were spiral in shape, positive for catalase, oxidase, urease, and cagA and vacA gene. Stock cultures were maintained in defatted milk at -80 °C.

**H. pylori cultivation and coccoid form induction**

The stored strains of *H. pylori* were cultured on Brucella agar with 5% sheep blood at 37 °C for 2-3 d under microaerophilic conditions (5% O₂; 10% CO₂; 85% N₂). After being subcultured, the bacteria were harvested and suspended in sterile tap water and the suspensions were incubated at 4 °C for a few days (about 3-4 d) until 100% transformation to coccoid form was achieved and confirmed under light microscopy. The transformed bacteria were inoculated on the Brucella agar media supplemented with 5% sheep blood for reversion trial culture. The stock suspensions were stored at 4 °C until use.

**Electron microscopy**

*H. pylori* flagella were examined under A Hu-12A transmission electron microscope. To prepare the bacterial samples, *H. pylori* suspensions were centrifuged, and the pellets were embedded in Epoxy 618. The ultra-thin sections were cut and negatively stained by 1% phosphotungstic acid.

**Assessment of cell adherence**

Hep-2 cells were grown to confluence on glass coverslips in culture flask, and 0.5 ml of the suspension of *H. pylori* (10⁶ cfu/ml) was added to culture medium (5 ml) for an additional 3.5 h culture at 37 °C in 5% CO₂-95% air. Cultures on the coverslips were washed and stained with Wright-Giemsa. One hundred Hep-2 cells were examined under light microscope for the counts of both the cells adhered by bacteria and the bacteria adhering to each cell. The adherence rate and adherence index were then calculated by the formula (described in the Results).

**Animal infection experiment**

Forty-two BALB/c mice were randomly divided into 3 groups. By oral gavage, groups 1 and 2 (16 mice each) were given 0.4 ml (10⁹ cfu/ml) of suspensions of F49 strain spiral *H. pylori* and coccoid *H. pylori* (in water for 40 days), respectively, four times at a 3-day interval. The control group (10 mice) received 0.4 ml sterile tap water. At Day 21 and 28 after inoculation, half of the mice from each group were sacrificed, respectively. Before killing, the mice were fasted for 36 hours with free access to water. At sacrifice, stomachs were removed, opened and washed with sterile saline and longitudinally divided into 3 sections in same size, which were used respectively for fast urease test and bacterial culture, electron microscopy, and histological examination.

**Urease activity assay**

Urease activity fast assay kit was purchased from Sanqiang Company (Sanming, Fujian). The assays were made according to the manufacturer’s instructions. Diluted *H. pylori* cultures (10¹⁰ cfu/ml, 5 µ), or tissue fragments (3×3 mm) obtained from the pylorus part of one-third of the mouse gastric mucosa were added to the test wells to react with the commercial reagents. To evaluate the urease activity, the colors developed in the assay were scored into five grades (+++, ++++, ++, + and -) for bacterial cultures and four grades (+++, +++, ++, + and -) for tissue fragments.

**Bacterial examination**

After collected for urease assay, the remaining one-third gastric mucosa samples were grounded into homogenate, daubed on Brucella agar with 5% sheep blood, and incubated at 37 °C for 3-4 d under microaerophilic conditions. Colonies were taken and identified under light microscopy, urease activity test and cagA gene amplification by PCR. In addition, two samples from groups 1 and 2 respectively, witch were bacteriologic positive and trimmed to 1 mm³, were embedded in Epoxy 618, then the ultra-thin sections were cut, stained by uranyl acetate and lead citrate and examined under a Hu-12A transmission electron microscope.

**Light microscopic histological examination**

The gastric mucosal samples were embedded in paraffin, cut in 5 µm sections, stained with hematoxylin-eosin, and examined under light microscope.

**Statistical analysis**

Data was analyzed using the Student *t* test. The statistically significant difference was suggested by a value of *P*<0.05, and the very significant difference by *P*<0.01.

**RESULTS**

**In vitro virulence of water-induced coccoid *H. pylori***

Flagella Three strains (F44, F45 and F49) of *H. pylori* were seen under light microscope in a typical spiral shape before their exposure to water. After 3-4 d incubation in sterile tap water at 4 °C, no spiral but only coccoid shaped bacteria were observed. Reversion trial showed that water-induced coccoid *H. pylori* failed to grow on Brucella agar supplemented with 5% sheep blood. Electron microscopy showed that the flagella remained a part of the bacterial cell structure (Figure 1).

![Image](image-url)

**Figure 1** The flagella of coccoid *H. pylori* under transmission electron microscope ×6000.

**Urease activity** Table 1 shows the urease activity assayed for three strains (F44, F45 and F49) of *H. pylori* both in spiral form (normal culture) and the respective coccoid form (subjected to water treatment). Strong urease activity (++++) was confirmed in the spiral *H. pylori* of all the three strains tested. The urease activity of the water-induced *H. pylori*, i.e., the
coccioid form of these three strains, significantly reduced, but still existed on a detectable level (+ to ++).

**Table 1** Urease activity of *H. pylori*

| Strain | Spiral form | Coccoid form |
|--------|-------------|--------------|
| F44    | ++++        | +            |
| F45    | ++++        | ++           |
| F49    | ++++        | +            |

**Adhering ability** All the three strains of *H. pylori* in both spiral form and water-induced coccoid form were tested for their adhering ability to Hep-2 cells. According to the following formula, the rate and the index of adherence were calculated. The rate of adherence = the amount of cell adhered by bacteria/100×100 %; The index of adherence = the amount of bacteria adhering to cells/100; Five groups of each 100 Hep-2 cells were counted for the number of cells adhered by bacteria and the total number of bacteria adhering to the cells. The percentages of cells adhered by bacteria (adherence rate) and the average bacteria number (adherence index) adhered to each cell are presented in Table 2. The adherence was observed in all groups tested. Student t test showed a very significant difference between the spiral and coccoid forms of *H. pylori* in either adherence rate or adherence index.

**Table 2** Adherence of *H. pylori* to Hep-2 cells

| Groups | Adherence rate (%) | Adherence index (Bacteria numbers per cell) |
|--------|--------------------|-----------------------------------------------|
|         | F44    | F45    | F49    | F44    | F45    | F49    |
| Spiral form | 70.0±5.3 | 73.0±5.1 | 72.6±4.5 | 2.6±0.4 | 3.1±0.5 | 2.9±0.4 |
| Coccoid form | 30.2±3.5 | 35.7±4.1 | 31.4±4.0 | 0.8±0.3 | 0.9±0.3 | 0.8±0.4 |
| t value | 12.3   | 11.2   | 12.8   | 7.2    | 7.8    | 7.4    |
| p      | <0.01  | <0.01  | <0.01  | <0.01  | <0.01  | <0.01  |

*a* Adherence rate = amount of cells adhered by bacteria/100×100 %; *b* Adherence index = total amount of bacteria adhering to 100 cells/100; Five groups of 100 cells on the same coverslip were counted for the bacteria adhered. Data are presented as mean ±SD. t and P values were obtained using Student t test.

**Coccioid *H. pylori* infection in mice**

**Bacterial colonization** *H. pylori* colonization in the gastric mucosa of inoculated mice was determined by the urease assay and bacterial culture of the of tissue samples. The bacterial cultures were found to be characteristic of spiral *H. pylori* as proved by the spiral shaped structure under light microscope, the positive urease activity, and the positive amplification of cagA gene fragments (data not shown). Data shown in Table 3 were the rates of positive findings in each group of mice. The positive rates of urease test of gastric mucosa, which was infected by spiral *H. pylori* and coccioid *H. pylori*, were 93.8 % (15/16) and 50 % (8/16), respectively. The positive rates of cultures of *H. pylori* were 87.5 % (14/16) and 68.8 % (11/16) respectively. Neither urease assay nor bacterial culture was found positive in the mice of the control group. Sampling at Day 21 and Day 28 after inoculation found almost no difference in both tests. In the semi-quantitative study, the color development in fast urease assay was scored. The colors distinguished at grades - , +, ++, and ++++, which were associated with the existence of the *H. pylori* in 0, 1-10, 11-30, and >30 per microscope field, respectively, according to the guide of test kit, were accordingly assigned by 0, 1, 2, and 3 point(s). Table 4 presents the number of mice scored at the same points in this assay and the average points of each group. Again, the score in group 1 was much higher than in group 2 (1.75 vs 0.56), while the score in control group was zero. In addition, electron microscopy showed the adherence of bacteria to the gastric mucosal samples taken from both group 1 and group 2 (Figure 2A and B). These bacteria were in spiral, bacillary or coccoid shapes, and some of them had invaded into the gastric epithelial cells. No similar bacterium adherence and invasion was observed in the samples from control group.

**Table 3** Tests of bacteria in gastric mucosa samples

| Group  | Total no. | Positive total | Positive rate(%) |
|--------|-----------|----------------|------------------|
|        | Fast urease test | Culture of bacterial |
|        | D21 | D28 | D21 | D28 |
|        | +ve/total | Positive rate(%) | +ve/total | Positive rate(%) |
| 1      | 16 | 7/8 | 8/8 | 93.8 | 7/8 | 7/8 | 87.5 |
| 2      | 16 | 4/8 | 4/8 | 50.0 | 5/8 | 6/8 | 68.8 |
| Control | 10 | 0/5 | 0/5 | 0 | 0/5 | 0/5 | 0 |

Group 1 was inoculated with spiral *H. pylori*, group 2 was inoculated with water-induced coccoid *H. pylori*, and control group received sterile tap water.

**Table 4** Scores for urease tests of tissue samples

| Groups | Color development | Urease activity (mean score) |
|--------|-------------------|-----------------------------|
|        | -     | +     | ++    | ++++  |
| 1      | 1     | 2     | 5     | 7     | 3     | 1.75a |
| 2      | 2     | 8     | 7     | 1     | 0     | 0.56  |
| Control | 10    | 0     | 0     | 0     |

*a* Numbers of mice; *b* Refers to text for scoring and calculation.

![Figure 2](image_url) *H. pylori* colonization in mouse stomach under transmission electron microscope. A. infection of spiral *H. pylori* ×7 000; B. infection of coccoid *H. pylori* ×9 000.
Histopathological alteration. Inflammatory pathological features were observed in both group 1 and group 2 samples under light microscope (Table 5 and Figure 3). Fifteen mice of group 1 and ten mice of group 2 developed inflammatory cell infiltration and different degrees of erosion or ulcer. The frequency and intensity of the erosion in group 1 was higher than in group 2. Two out of sixteen mice in group 1 even developed mucosal ulcers. Mucosal injury was slighter in the mice infected by coccoid H. pylori. None of these features was found in the control group.

| Groupe | Normal | Gentle erosion | Deep erosion | Ulcer | Total |
|--------|--------|----------------|--------------|-------|-------|
| 1      | 1      | 7              | 6            | 2     | 16    |
| 2      | 6      | 9              | 1            | 0     | 16    |
| Control| 10     | 0              | 0            | 0     | 10    |

DISCUSSION

Increasing reports showed that H. pylori had been detected from water by immunomagnetic separation, bacterial culture or polymerase chain reaction (PCR) technique[22-27] and that consumption of water was closely related to H. pylori infection[14-16]. Water borne route is therefore thought to be an important route of H. pylori transmission. H. pylori has been found to be able to convert from spiral form to coccoid form under certain adverse circumstances such as increased oxygen tension, extended incubation and exposure to antibiotics or water[17-28-32]. Some researches suggested that H. pylori in coccoid form can survive the water for a long time. However, it remains unknown whether coccoid H. pylori can attack and colonize in stomach., resulting in the diseases of digestive system. Going deep inside to the behavior of coccoid H. pylori will thus be very beneficial to our understandings on the transmission of H. pylori infection and its association with many severe human diseases like gastritis, ulcer and peptic carcinomas.

The putative pathogenic determinants of H. pylori have been divided into two major groups[19]: maintenance factors, which allow the bacterium to colonize and remain within the host, and virulence factors, which contribute to the pathogenetic effects of the bacterium. Flagella, urease activity and adherence to epithelial cells of H. pylori are important maintenance factors[14-37]. If coccoid H. pylori in water remains infective, they must possess maintenance factors in order to colonize and remain in stomach. In this study, it is shown that both urease activity and adherence to Hep-2 cells of coccoid H. pylori decreased as compared with the spiral forms, suggesting a reduction of virulence related to colonization of H. pylori when the transformation to coccoid form occurs. However, as shown by the microbial assays, coccoid H. pylori induced by water still remains a considerable urease activity and the adhering ability to epithelial cells. Furthermore, the flagella, an important component responsible for bacterial movement and infection, were still observed as a cellular structure of coccoid H. pylori under electron microscope. This adds to the potential of in vivo infection of the coccoid H. pylori induced by water.

In the animal experiments described here, some mice (10/16) inoculated with water-induced coccoid H. pylori developed significant pathological changes such as mucosal erosion and inflammatory cell infiltration in gastric mucosa, as were shown by histopathological examinations. The evidences of the coccoid H. pylori being the pathogen of the mucosal injury were further provided by bacteriological examinations. In this aspect, a 50 % positive rate and a considerable intensity of urease test were detected in the mucosal samples of mice inoculated with water-induced coccoid H. pylori, and the positive H. pylori cultures of these samples reached a percentage of 68.8 % in addition, electron microscopy for these samples showed the presence of spiral bacteria in gastric mucosa. All these findings reveal the ability of water-induced coccoid H. pylori in their colonization on mouse gastric wall and their injury to the mucosal tissues.

It might be reasonably queried whether there still exists an undetectable trace amount of spiral H. pylori among the huge quantity of their coccoid variance, which could be intrinsically responsible for the virulence and infectivity of the bacteria in some studies including ours. The facts that the bacteria were kept in in-nutritious water for up to 40 days and that the water-treated bacteria were assayed for in vitro virulence in real time, eliminated the possibility of an expansion of the spiral population or in-water reversion of the coccoid variance to its spiral origins. The failure of the trial reversion in supplemented Brucella medium further supported the concept of a direct virulence of the coccoid H. pylori. Now that the spiral shaped bacteria were observed in the mucosal tissues of mice inoculated with coccoid H. pylori, it seemed that the reversion took place in vivo. However, whether the reversion is a key precondition for the infection remains unclear. In despite of our ignorance in the process and mechanisms of the inter-transformation of H. pylori, conclusions can be drawn from our current study that water-induced coccoid form of H. pylori remains virulent and infective to gastric wall in mice. Water borne route transmission of H. pylori needs more attention.

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