Characterization of elongated *Helicobacter pylori* isolated from a patient with gastric-mucosa-associated lymphoid-tissue lymphoma

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To date, two *Helicobacter* species, *Helicobacter pylori* and *Helicobacter heilmannii* (formerly *Gastrospirillum hominis*), have been identified from the human stomach. In this study, we observed non-*H. pylori*-shaped bacteria in gastric tissue sections and successfully isolated them by cultivation. Elongated bacteria were isolated from a patient with gastric-mucosa-associated lymphoid-tissue lymphoma who had been diagnosed as *H. pylori*-negative by culture, rapid urease test and histopathology in another hospital. The bacteria were grown only on chocolate agar in a CO₂ incubator, appeared more than 10 μm long in histological sections, formed small colonies and showed poor growth in a brain heart infusion broth; these characteristics apparently differed from common clinical isolates of *H. pylori*. However, the bacteria were identified as *H. pylori* by PCR of the urease gene, 16S rDNA sequencing, protein profile and antigenicity examined by anti-*H. pylori* polyclonal antibody. These observations suggest that the *H. pylori* strain identified in this study may contribute to the development of gastroduodenal diseases in cases judged as *H. pylori*-negative by ordinary methods.

INTRODUCTION

Chronic *Helicobacter* infection of the stomach causes gastroduodenal diseases, and two *Helicobacter* species have been identified so far from the human stomach: *Helicobacter pylori*, first isolated by Marshall and co-workers (Warren & Marshall, 1983), and *Helicobacter heilmannii* (formerly named *Gastrospirillum hominis*), reported by Dent et al. (1987). *H. pylori* is the major stomach-colonizing bacteria of humans that causes gastritis, peptic ulcer disease, mucosa-associated lymphoid tissue (MALT) lymphoma and gastric cancer. Corkscrew-shaped organisms that resemble *H. heilmannii* and are not *H. pylori*-like have sometimes been seen in histological sections of gastric biopsies. The morphology of these "tightly spiral-shaped bacteria (corkscrew-shape)" in gastric sections is suspected to be *H. heilmannii*. In particular, they are characterized by their predominantly straight appearance and large size (about 10 μm) (Holck et al., 1997; Ierardi et al., 2001). *H. heilmannii* infection is less frequent than infection with *H. pylori*, and few cases of successful cultivation from the stomach have been reported (Holck et al., 1997). Morgner et al. (2000) reported that gastric MALT lymphoma might arise in patients with *H. heilmannii* infection and that curing of this infection might lead to complete remission of MALT lymphoma.

In the present study, to investigate whether *H. heilmannii* could be isolated from Japanese patients, we tried to isolate the bacteria from gastric biopsy samples using several media under two atmospheric conditions. We isolated elongated bacteria from a patient with MALT lymphoma who had originally been diagnosed as *H. pylori*-negative by culture, rapid urease test and histopathology. The isolated bacteria were characterized morphologically by light and electron microscopy and genetically by using PCR analysis with *H. pylori*, *Helicobacter felis* and *H. heilmannii* urease-specific primers and by 16S rDNA sequencing.

METHODS

Bacterial culture Endoscopic biopsy specimens were obtained from
each patient from the greater curvature of the body and the antrum of the stomach and were homogenized and inoculated on agar plates. Four different media were used for cultivation: Brucella agar supplemented with 7% horse blood and antibiotics (5 μg trimethoprim ml⁻¹, 2.5 U polymyxin B ml⁻¹, 10 μg vancomycin ml⁻¹), Brucella agar supplemented with 7% horse blood only, blood base agar (BBA) supplemented with heat-lysed 7% horse blood (chocolate agar) and brain heart infusion (BHI) agar supplemented with heat-lysed 7% horse blood (chocolate agar). All basal media were purchased from Difco. Plates were cultured under two different atmospheric conditions: a gas-pack jar (gas content 10–15% CO₂, 10% O₂ and 75–80% N₂) with Anaeropack Helico (Mitsubishi Gas Chemical), and 10% CO₂ with 100% humidity (CO₂ incubator; TABAI). Growth of colonies was checked after incubation for 7 days. Two or three typical colonies from each individual culture were examined morphologically by observing Gram-stained smears under the light microscope (×1000). Urease, catalase and oxidase tests were also performed.

**Histopathology.** Gastric biopsy specimens were stained by haematoxylin and eosin staining and Giemsa staining. For diagnosis of low-grade MALT lymphoma, tissue specimens were examined by immunohistochemistry using antibodies to CD3, CD79a (Dako) and cytokeratin AE1/AE3 (Boehringer Mannheim). Histological grades were determined according to the criteria of the revised European–American lymphoma (REAL) classification (Harris et al., 1994).

**Protein profiles.** Protein profiles of cultured elongated bacteria were visualized and compared with those of *H. pylori* ATCC 43504 and *H. felis* (kindly provided by A. Lee) by SDS-PAGE. Sonicated bacteria were dissolved in 1% SDS containing 5% 2-mercaptoethanol, separated by SDS-PAGE (10% gel) and stained with Coomassie brilliant blue staining.

**Western blot analysis.** Western blot analysis was performed to determine the antigenicity of the cultured elongated bacteria. Anti-*H. pylori* and anti-*H. felis* polyclonal antibodies were prepared by immunization of sonicated bacteria to rabbits. Briefly, *H. pylori* and *H. felis* were cultured on BHI agar containing 7% horse blood for 5 days under microaerophilic conditions. Bacteria were collected in PBS and washed twice. After heat treatment at 60°C under microaerophilic conditions. Bacteria were collected in PBS and H. felis visualized and compared with those of *H. felis*.

Table 1. Primers used for amplification of urease B genes from *H. heilmannii*, *H. pylori* and *H. felis*

<table>
<thead>
<tr>
<th>Species</th>
<th>Primer sequence</th>
<th>Accession no.</th>
<th>Product size (bp)</th>
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<tr>
<td><em>H. heilmannii</em></td>
<td>F', 5'-GGGCGATAAAAGTGCCGGTTG-3'</td>
<td>L25079</td>
<td>580</td>
</tr>
<tr>
<td></td>
<td>R, 5'-CTGCTCAATGGAGACCGG-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>H. pylori</em></td>
<td>F, 5'-AGTAAAAGGAAAAAGATGAGGAAAAAGG-3'</td>
<td>M60398</td>
<td>1757</td>
</tr>
<tr>
<td></td>
<td>R, 5'-AAAAAATCCTAGAAAAATGCTAAGAGGTTG-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>H. felis</em></td>
<td>F, 5'-ATGAAACTAAGGCCCTAAGAAGACTAG-3'</td>
<td>X69080</td>
<td>1150</td>
</tr>
<tr>
<td></td>
<td>R, 5'-GGGAGATAAAAGTGATGCCGT-3'</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*F, Forward; R, reverse.*

PCR analysis for urease B-encoding gene. We performed a PCR analysis for the detection of the urease B-encoding gene of the cultured elongated bacteria using a modification of the method reported by Neiger et al. (1998). Sequence primers used for urease genes are shown in Table 1. Genomic DNA was purified from cultured bacteria on a BHI agar plate with horse blood. PCR analysis was performed as follows: one cycle of 95°C for 2 min, followed by 30 cycles of 95°C for 1 min, 57°C for 1 min and 75°C for 2 min. PCR products were analysed by electrophoresis on a 2% agarose gel.

**16S rDNA sequencing.** 16S rDNA sequencing was performed by a modification of the methods described by Andersen et al. (1999). A colony from a 5 day bacterial culture was picked from a BHI chocolate agar plate and intracellular DNases were inactivated by incubation at 80°C. After centrifugation, cells were disrupted by an ultrasonic sonicator (Astron) and then centrifuged at 20,000 × g for 20 min. The supernatants were subjected to a protein assay (Bio-Rad) and then subjected to SDS-PAGE. Sonicated bacteria were dissolved in 1% SDS containing 5% 2-mercaptoethanol, separated by SDS-PAGE (10% gel) and stained with Coomassie brilliant blue staining.

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Table 2. 16S rDNA sequencing primers

<table>
<thead>
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<th>Primer</th>
<th>Sequence</th>
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<tr>
<td>172 (F1)</td>
<td>5'-TCAGAGTGAAACCTGCCCCGT-3'</td>
</tr>
<tr>
<td>620 (F2)</td>
<td>5'-AACGAAATTGCACTGCTAAC-3'</td>
</tr>
<tr>
<td>1107 (F3)</td>
<td>5'-TAATTCGAGATACCAAGAAC-3'</td>
</tr>
<tr>
<td>675 (R1)</td>
<td>5'-CGCCGAGTGATTCGAGGACTTG-3'</td>
</tr>
<tr>
<td>1191 (R2)</td>
<td>5'-CAGCCGTCGCACCCGTTTCTCA-3'</td>
</tr>
<tr>
<td>1584 (R3)</td>
<td>5'-TGACTTAAGGCAACACAACACTCC-3'</td>
</tr>
</tbody>
</table>

Primer names give the position of the primer and the direction (F, forward; R, reverse).
**Electron microscopy.** Bacteria were cultured in BHI broth supplemented with 5% horse serum with or without shaking. After incubation for 3 or 5 days, bacteria were collected with centrifugation, washed gently with 0.1 M phosphate buffer and fixed with 1:0% glutaraldehyde in 0.1 M phosphate buffer. Cells were then rinsed briefly in cacodylic buffer. For scanning electron microscopy, fixed bacteria were mounted on specimen support stubs and then dehydrated in a graded ethanol series (60–100%) for 20 min each, dried to the critical point with bone-dry-grade liquid CO2. A HITACH mild sputter coater E5400 was used to coat the specimens with 5-nm gold particles, and a HITACH model-S900 scanning electron microscope was used to view and photograph the samples at 20 kV.

For transmission electron microscopy, cultured bacteria were mounted on Formvar-coated carbon-reinforced copper grids (200 mesh), stained with 2% uranyl acetate and observed under a HITACH model H-700 transmission electron microscope at 75 kV.

**RESULTS**

**Detection of bacteria**

One strain with characteristics somewhat different from those of *H. pylori* was isolated from a patient with MALT lymphoma. The strain formed smaller colonies (pinhole sized) than typical *H. pylori* on BBA chocolate agar (Fig. 1a, b). Gram staining of bacteria taken from the smaller colonies revealed Gram-negative elongated bacteria (Fig. 1c). This strain was isolated from a patient with low-grade MALT lymphoma diagnosed by histopathological examination (Fig. 1d). The elongated bacteria were also observed in Giemsa-stained sections of a gastric biopsy from this patient (Fig. 1e, f). The elongated bacteria were grown only on BBA with CO2 incubation (Table 3). Five small colonies were detected at primary cultivation. The bacteria could grow on the same medium and under the same atmospheric conditions at the first transfer. In the next transfer, the bacteria were grown successfully on all media and atmospheric conditions and retained the elongated morphology (Table 3). This strain was positive for urease, catalase and oxidase tests.

**PCR for urease and 16S rDNA sequence**

Molecular sizes of PCR products of the urease B gene were respectively 1757 and 1150 bp for *H. pylori* and *H. felis*. The
PCR product from the elongated bacteria was 1757 bp, and amplification products corresponding to either H. felis or ‘H. heilmannii’ were not detected (Fig. 2); thus, the elongated bacteria were likely to be H. pylori based on the molecular size of PCR products. A partial 16S rDNA sequence (1331 bp) obtained from PCR products of the elongated bacteria was aligned to three gastric Helicobacter sequences found in the GenBank/EMBL database. H. pylori and H. felis stocked in our laboratory were also sequenced and aligned (Table 4). The sequence of the 16S rRNA gene from the elongated bacteria was most similar to H. pylori, differing by less than 1 %.

Protein profile and antigenicity

We compared protein profiles of the elongated bacteria with those of H. pylori and H. felis (Fig. 3a). The major proteins of the elongated bacteria were also detected in H. pylori, but the protein profiles of the three strains were similar. Anti-H. pylori antibody reacted with the main proteins of both H. pylori and the elongated bacteria. The 120 kDa protein (likely to be a CagA-associated protein) and 60 and 30 kDa proteins (likely to be urease proteins) of the elongated bacteria were recognized by the anti-H. pylori antibody (Fig. 3b). The urease proteins, but not the 120 kDa protein, were also recognized by anti-H. felis antibody (Fig. 3c). Thus, the protein and antigenic profiles of the elongated bacteria appeared to be similar to those of H. pylori.

Electron microscopy

Scanning electron microscopy (Fig. 4a) and transmission electron microscopy of negatively stained specimens (Fig. 4b) revealed elongated helical organisms that ranged from 8 to 10 μm in length and 0.3 μm in width. Although flagella were observed in H. pylori ATCC 43504T (Fig. 4c, d), the elongated bacteria did not exhibit flagella.

DISCUSSION

In this study, a strain of bacteria with some characteristics that differed from those of typical H. pylori was isolated from a patient with gastric MALT lymphoma. The bacteria were primarily cultured on only BBA in CO2 incubation, formed smaller colonies than those of H. pylori and showed a longer shape under microscopy. However, genetic and protein analyses of the bacteria indicated that this strain was likely to belong to H. pylori.

In humans, ‘H. heilmannii’ has been detected and isolated from the stomach as non-pylori helicobacter. Non-pylori helicobacters are usually distinguishable from H. pylori by morphological features; tightly spiral-shaped bacteria were suspected to be ‘H. heilmannii’ in gastric sections (Dent et al., 1987; Heilmann & Borchard, 1991; Lcrardi et al., 2001). ‘H. heilmannii’ is a Gram-negative rod, characterized by its predominantly straight appearance and large size (10 μm), with three to eight coils and a wavelength of about 1 μm. Andersen et al. (1999) described characteristics of the culture conditions of ‘H. heilmannii’; it grows as small, translucent colonies on 7 % lysed, defibrinated horse-blood agar plates within 3–7 days. Thus, we speculated that the elongated Gram-negative rods isolated in this study were ‘H. heilmannii’, but its spiral forms revealed by electron microscopic observation were different from those of ‘H. heilmannii’, and
the genetic and protein profiles of the bacteria were also different from those of 'H. heilmannii', yet rather similar to those of H. pylori. The morphology of our isolate was, however, apparently different from that of typical H. pylori, as observed histologically and electron microscopically. The elongated isolate did not exhibit flagella, but we speculated that the loss of flagella was due to its culture conditions, requiring constant shaking in liquid medium. Indeed, standard H. pylori (ATCC 43504T) exhibited flagella when cultured under conditions without shaking, but lost them after culture with shaking (data not shown). Interestingly, Fawcett et al. (1999) reported that three or four transfers of culture in liquid medium induced morphological changes of a standard H. pylori strain (ATCC 43504T) to a morphology similar to that of 'H. heilmannii'. They demonstrated that these morphological changes were associated with culture conditions and were completely reversible. The bacteria isolated in this study were elongated (10 μm) both on agar plates and in BHI broth cultivation, and this elongated morphology was seen after transfer of culture and frozen stocks. In contrast, our stock strain (ATCC 43504T) showed only a short-curved morphology after several transfers in a liquid medium. The elongated morphology of our isolate was observed histologically in gastric specimens and from colonies at primary culture before transfer, suggesting that the morphological change was not induced by the culture conditions but rather might be caused by the gastric environment after infection. We are currently studying the events that account for the morphological differences between the elongated bacteria and normal-shaped H. pylori.

The elongated bacterium observed in this study was isolated from a patient with gastric MALT lymphoma who had been diagnosed as H. pylori-negative by culture, rapid urease test and histopathology in another hospital. However, when the patient was re-examined in our hospital, five small colonies of the elongated bacteria were isolated. A close link has been established between H. pylori infection and the development of gastric MALT lymphoma (Isaacson & Spencer, 1993; Parsonnet et al., 1994). MALT lymphomas often regress after eradication of H. pylori and relapse by reinfection (Cammarota et al., 1995; Wotherspoon et al., 1993). Observations in this study suggest that careful bacterial isolation using several conditions is needed to define the correct H. pylori status in conditions such as MALT lymphoma, because the identification of H. pylori can predict the efficacy of eradication as therapy for MALT lymphoma. Whether the

### Table 4. 16S rDNA similarity matrix

Percentage DNA sequence similarity is indicated.

<table>
<thead>
<tr>
<th>Strain/isolate</th>
<th>Accession no.</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Elongated bacteria</td>
<td>This study</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>2. H. pylori*</td>
<td>This study</td>
<td>99.6</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>3. H. pylori ATCC 43504^T</td>
<td>U01330</td>
<td>99.3</td>
<td>99.2</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>4. 'H. heilmannii' isolate 3</td>
<td>AF058770</td>
<td>94.4</td>
<td>94.8</td>
<td>95.0</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>5. H. felis Dog-1</td>
<td>U51870</td>
<td>94.5</td>
<td>95.0</td>
<td>94.8</td>
<td>98.6</td>
<td>–</td>
</tr>
<tr>
<td>6. H. felis†</td>
<td>This study</td>
<td>94.6</td>
<td>94.7</td>
<td>94.7</td>
<td>98.9</td>
<td>99.1</td>
</tr>
<tr>
<td>7. Helicobacter bizzozeronii CCGU 35545^T</td>
<td>Y09404</td>
<td>94.4</td>
<td>94.8</td>
<td>91.2</td>
<td>98.7</td>
<td>92.8</td>
</tr>
<tr>
<td>8. Helicobacter salomonis Inkinen^T</td>
<td>U89351</td>
<td>94.4</td>
<td>94.9</td>
<td>91.4</td>
<td>99.1</td>
<td>91.9</td>
</tr>
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</table>

*H. pylori ATCC 43504^T* stocked in our laboratory.
†H. felis kindly provided by A. Lee and stocked in our laboratory.

### Fig. 3. Protein profiles and Western blotting.

Proteins extracted from the elongated bacteria (EL), H. pylori (Hp) and H. felis (Hf) were separated by SDS-PAGE and stained with Coomassie brilliant blue (a). The separated proteins were then transferred to PVDF membrane and reacted with anti-H. pylori (b) or anti-H. felis (c) polyclonal antibody. Production of anti-H. pylori and -H. felis antibody is described in Methods. The protein profiles and antigenicity of the elongated bacteria appeared to be similar to those of H. pylori.
Fig. 4. Electron microscopy. The elongated bacteria (a, b) and H. pylori ATCC 43504T (c, d) were observed by scanning microscopy (a, c) and transmission electron microscopy of negatively stained preparations (b, d). Bars, 1 μm.

elongated H. pylori isolated in this study is specifically associated with the development of MALT lymphoma awaits further clarification.

REFERENCES


