Short communication

The high prevalence of *Helicobacter* sp. in porcine pyloric mucosa and its histopathological and molecular characteristics

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Abstract

This study examined the prevalence of *Helicobacter* infection in the pyloric mucosa of pigs and its histopathological and molecular characteristics. Forty porcine pyloric samples were examined for *Helicobacter* infection by silver staining and PCR assay. The PCR product (376 bp) was digested with *Nde* II to differentiate between *Helicobacter heilmannii* and *Helicobacter pylori*. Another PCR assay run to produce an 1157 bp fragment was performed using a primer set designed from the 16S rRNA gene of *Candidatus H. suis*, and its product was cloned and sequenced. Infection rates were 62.5% (25/40) and 95.0% (38/40) as determined by silver staining and the PCR assay, respectively. On histopathological examination, lymphoid follicle aggregation in the pyloric mucosa and granulocytic migration into the lumen of pyloric glands were observed in 24 (60.0%) and 33 (82.5%) gastric samples, respectively. All PCR products, except that of *H. pylori*, were cut into two fragments of 147 and 229 bp by enzymatic digestion with *Nde* II. Sequencing of the 16S rRNA gene showed that the bacterium had 99.57% (1152 bp/1157 bp) homology to the 16S rRNA gene of *Candidatus H. suis*.

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1. Introduction

The *Helicobacter* sp. that colonizes pig stomachs has been named *Gastrospirillum suis* (Mendes et al., 1990), *Helicobacter heilmannii* (Queiroz et al., 1995), and *Candidatus H. suis* (De Groote et al., 1999). However, all three are considered as identical. Since the bacterium was first described by Queiroz et al. (1990), several studies about its prevalence have been performed. The results showed that this prevalence is very different according to the implemented diagnostic methods and the local area that was examined, ranging from 8.0 to 77.0% (Mendes et al., 1991; Grasso et al., 1996; Utriainen and Hänninen, 1998; De Groote et al., 2000; Park et al., 2000; Choi et al., 2001). Interestingly, when examined histologically...
with carbol fuchsin, gram, and steiner’s silver stain, infection rates were comparatively low (8.0–10.3%) (Mendes et al., 1991; Grasso et al., 1996; Park et al., 2000), whereas the studies using a PCR assay showed infection rates over 60% (Utriainen and Hänninen, 1998; De Groote et al., 2000; Roosendaal et al., 2000; Choi et al., 2001).

In the present study, based on histology and a PCR assay, we report the high prevalence and histopathological characteristics of Helicobacter infection in the pyloric mucosa of pig stomachs. We have demonstrated that an enzymatic digestion of PCR products using NdeII could differentiate between Helicobacter pylori and the bacterium regarded as Candidatus H. suis (or H. heilmannii) from 16S rRNA sequencing.

2. Materials and methods

2.1. Sample preparation

Stomach samples were obtained twice, with two weeks interval, from 10 male and female pigs (about 6 months old) weighing approximately 100–120 kg after slaughter at a slaughterhouse (Cheonan, Korea). The stomachs were opened immediately along the greater curvature and the gastric contents was gently discarded by washing. Pyloric portions containing torus were cut and divided into two parts. One was fixed in 10% buffered formalin for histological examination and the other was put into an individual polyvinyl pack in an ice box (4°C) for DNA extraction. The samples were then transported within 1 h to the laboratory.

2.2. Histological examination

The pyloric fragments were fixed in 10% buffered formalin over 24 h and dehydrated in an alcohol–xylene series and embedded in paraffin wax. From each block, 2 μm thick sections were prepared and stained by Hematoxylin and Eosin and modified Steiner’s silver method (Park et al., 2000). The pyloric gastritis was scored on a 0–4 scale as previously described (Park et al., 2000). The bacterial density was also assessed as follows. −, none; +, <20/high power (HP) field (×400); ++, 20–50/HP field; +++, >50/HP field.

2.3. DNA extraction and PCR amplification

DNA from each gastric sample was extracted as previously described (Park et al., 2003). The genomic DNA of H. pylori ATCC 43504 (American Type Culture Collection, Manassas, VA, USA) was used as a positive control. A Helicobacter genus-specific primer set was used to determine Helicobacter infection (Riley et al., 1996). The PCR was performed using the AccuPower® PCR PreMix kit (Bioneer, Daejeon, Korea). PCR conditions were as follows: 3 min of denaturation at 95 °C followed by 35 cycles consisting of 30 s of denaturation at 95 °C, 30 s of annealing at 53 °C, and 1 min of extension at 72 °C before a final 7 min of extension at 72 °C. The PCR product was detected by electrophoresis of 9 μl of reaction solution in 1.5% agarose gel, followed by staining with ethidium bromide. The bands of the PCR product were visualized on a UV transilluminator.

2.4. Enzymatic digestion of amplified DNA

A 10 μl sample of each PCR product was digested with 10 U of NdeII (Promega, Madison, WI, USA) for 4 h at 37 °C. The digested samples were analyzed by electrophoresis using 1.5% agarose gel and examined by transillumination.

2.5. Cloning and sequencing of 16S rRNA gene

For sequencing, another primer set (F: 5’-TAC-CAAATACCTACCTAC-3’, R: 5’-GCTGATTTGCGTATTACTAGC-3’) was designed from the partial 16S rRNA gene sequence of Candidatus H. suis (GenBank accession no. AF127028). Under the same PCR conditions that described above, 1157 bp of the 16S rRNA gene were amplified from a stomach sample of a H. heilmannii infected pig. The PCR product was cloned using the pGEM®-T Easy Vector System (Promega) and JM109 competent Escherichia coli cells (Promega) as previously described by Choi et al. (2001). Plasmid DNA was isolated from E. coli by the QIAprep Spin Miniprep Kit (Qiagen, Hilden, Germany). Nucleotide sequencing of the PCR product was performed by a DNA sequencing service center (Geno Tech. Corp., Taejon, Korea) with a DNA sequencer (model; Applied Biosystems 3730xl DNA Analyzer, Applied BioSystems, Inc., Foster City, CA, USA).
USA). The sequence analysis was resolved with the ABI BigDye terminator (Perkin-Elmer, Foster City, CA, USA).

### 2.6. Statistical analysis

The significance of differences between groups was determined by Duncan’s Multiple Range Test (SAS ver. 8.1; SAS Institute Inc., Cary, NC, USA) or Student’s t-test. Values of \( P < 0.05 \) were considered significant.

### 3. Results

#### 3.1. Microbiology and histopathology

Details of the microbiological and histopathological results are shown in Table 1. Helicobacter-like organisms (HLO) in porcine pyloric mucosa could be easily detected by silver stain. The bacterium had a peculiar screw-like appearance, easily differentiated from other rod like or curved bacteria (Fig. 1). The bacteria colonized the surface mucosa and the lumen of gastric foveola, and were detected in 25 (62.5%; 12 males and 13 females) samples. Lymphoid aggregates, one of the major lesions associated with *H. heilmannii* infection, were histopathologically observed in the pyloric mucosa of 24 samples (60.0%; 13 males and 11 females) (Fig. 2A). Granulocytic migration in the lumen of gastric foveola was observed in 33 (82.5%; 17 males and 16 females) samples (Fig. 2B). The pyloric gastritis was scored with on a 0–4 scale as previously described (Park et al., 2000). The gastritis level was assessed according to the bacterial density. The mean gastritis score was highest in the group of grade 1 (+). However, there was no significant difference between each group (\( P < 0.05 \), Fig. 3). The gastritis score was not significantly different between infected male (\( n = 18; \ 2.777 \pm 0.647 \)) and female groups (\( n = 20; \ 2.550 \pm 0.707 \) (\( P = 0.3008 \)).

#### 3.2. PCR amplification of 16S rRNA gene and enzymatic digestion with NdeII

Thirty-eight gastric samples (male 18 and female 20) showed positive results by PCR assay using a *Helicobacter* genus-specific primer set (Fig. 4). On histological examination of silver stained slides, *Helicobacter*-like organism was not detected in two samples showing PCR-negative results. Through the restriction enzyme analysis using the WebCutter program of BCM Search Launcher, NdeII was expected to cleave the 376 bp PCR product of the *Candidatus H. suis* 16S rRNA gene into two fragments of 147 and 229 bp but not that of *H. pylori*. As

### Table 1

Microbiology and histopathology of the porcine pyloric mucosa obtained from a slaughterhouse in Cheonan city, Korea

<table>
<thead>
<tr>
<th>Microbiologya</th>
<th>Male (( n = 20 ))</th>
<th>Female (( n = 20 ))</th>
<th>Total (( n = 40 ))</th>
</tr>
</thead>
<tbody>
<tr>
<td>–</td>
<td>8 (40.0%)(^b)</td>
<td>7 (35.0%)</td>
<td>15 (37.5%)</td>
</tr>
<tr>
<td>+</td>
<td>4 (20.0%)</td>
<td>2 (10.0%)</td>
<td>6 (15.0%)</td>
</tr>
<tr>
<td>++</td>
<td>5 (25.0%)</td>
<td>2 (10.0%)</td>
<td>7 (17.5%)</td>
</tr>
<tr>
<td>+++</td>
<td>3 (15.0%)</td>
<td>9 (45.0%)</td>
<td>12 (30.0%)</td>
</tr>
</tbody>
</table>

Histopathology

<table>
<thead>
<tr>
<th>Formation of lymphoid aggregates(^c)</th>
<th>Male (( n = 20 ))</th>
<th>Female (( n = 20 ))</th>
<th>Total (( n = 40 ))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Granulocytic exudation in the lumen of gastric foveola</td>
<td>13 (65.0%)(^d)</td>
<td>11 (55.0%)</td>
<td>24 (60.0%)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Gastritis score</th>
<th>Male (( n = 20 ))</th>
<th>Female (( n = 20 ))</th>
<th>Total (( n = 40 ))</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \leq 2 )</td>
<td>3</td>
<td>10</td>
<td>13</td>
</tr>
<tr>
<td>3</td>
<td>5</td>
<td>10</td>
<td>15</td>
</tr>
<tr>
<td>4</td>
<td>3</td>
<td>1</td>
<td>4</td>
</tr>
</tbody>
</table>

\(^a\) Assessed by modified Steiner’s silver stain. The bacterial density was also assessed as follows. –, none; +, <20/high power (HP) field (\( \times 400 \)); ++, 20–50/HP field; ++++, >50/HP field.

\(^b\) Two were negative in the PCR assay using *Helicobacter* genus specific primers. Their gastritis scores were 3 and 4, respectively.

\(^c\) Assessed only in musoca, but not in the submucosa.

\(^d\) Two were *Helicobacter*-negative in both silver stain and PCR assay.
expected, 376 bp PCR products amplified from the gastric samples were cut into two fragments for all samples except that from \textit{H. pylori} ATCC43504 (Fig. 5).

3.3. \textit{Sequencing}

Another PCR was performed from a DNA sample of \textit{Helicobacter}-infected gastric tissue using primer sets designed from the 16S rRNA gene of \textit{Candidatus H. suis} (accession no. AF127028). From this, an 1157 bp PCR product was obtained, cloned into the pGEM T-easy vector system, and sequenced. When this sequence was compared to those present in databases using BLAST software, it showed a 99.57\% (1152 bp/1157 bp) homology to the 16S rRNA gene of \textit{Candidatus H. suis} (AF127028) and a 99.48\% (1151 bp/1157 bp) to that of \textit{H. heilmannii} (AF506791). Our sequence was

Fig. 1. \textit{Helicobacter}-like organisms were seen in the surface of pyloric mucosa of a pig. Steiner’s silver stain, Bar = 20 μm.

Fig. 2. Lymphoid follicles were seen in the pyloric mucosa of a \textit{Helicobacter} infected pig (A) and granulocytic exudation in the lumen of gastric foveola (B). H&E stain, Bar = 200 μm (A) and 20 μm (B).
deposited in the GenBank database under accession number AY517854.

4. Discussion

In pigs, the appearance of the lesions caused by Helicobacter infection is controversial. Several studies elucidated that Helicobacter infection was related to the ulcerative lesions of the pars esophagea or cardiac mucosa (Queiroz et al., 1996; Roosendaal et al., 2000; Choi et al., 2001). On the other hand, others including our previous study showed that Helicobacter infection was related to chronic gastritis accompanying lymphoid follicle formation in the pyloric mucosa of pigs (Mendes et al., 1991; Park et al., 2000). In the present study, lymphoid aggregates were detected in the pyloric mucosa of 24 (60.0%) samples. However, relationships between Helicobacter infection and the presence of lymphoid aggregates could not be directly elucidated, because the Helicobacter-negative sample size was too small (only two of 40 samples) and, even in the negative samples, the lymphoid aggregates were observed (Table 1). In the present study, the rate of the presence of lymphoid aggregates was higher than that detected in the study by Mendes (24.1%, 29 of 120 pigs showing 10.8% Helicobacter infection) (1991), which might be due to the high infection rate with Helicobacter.

Ierardi et al. (2001) showed lymphocyte exudation into the lumen of gastric crypts in patients with H. heilmannii gastritis, which is a distinctive feature that was not seen in H. pylori-infected patients. However,
in the present study, mild granulocytic, but not lymphocytic, exudation was observed in the lumen of pyloric foveola. *Helicobacter* spp. colonized both the surface mucosa and the lumen of gastric foveola, and more in surface mucosa than the lumen of gastric foveola. However, such granulocytic inflammation is rarely observed in the surface mucosa of *Helicobacter*-infected pig stomachs. This fact means that granulocytic exudation in the lumen of pyloric foveola might not be related to *Helicobacter* infection.

In the present study, the PCR results indicated a 95% infection rate of *Helicobacter* spp. in pig stomachs. To confirm the results, each PCR-amplified reaction was digested with *Nde* II. As expected, all PCR-positive reactions were cut into two fragments by the digestion with *Nde* II, with the exception of that from *H. pylori* ATCC 43504. Among the genus *Helicobacter*, *H. pylori* and *H. heilmannii* can colonize in the human gastric mucosa. Accordingly, enzymatic digestion with *Nde* II will be useful to differentiate *H. heilmannii* from *H. pylori* in human gastric tissues.

In the present study, we designed a new primer set from 16S rRNA gene sequence of *Candidatus Helicobacter suis* to obtain a longer PCR product. An 1157 bp 16S rRNA gene was amplified using this primer set, cloned, and sequenced. The sequencing result showed 99.57% (1152 bp/1157 bp) homology to the 16S rRNA gene of *Candidatus H. suis*. and 99.48% (1151bp/1157bp) to those of *H. heilmannii* originating from pig stomachs, indicating the bacterium may be *Candidatus H. suis* (or *H. heilmannii*).

In conclusion, in the present study, we showed a high rate of *Helicobacter* infection in pig stomachs, especially the pyloric mucosa, and the specific bacterium may be *Candidatus H. suis* (or *H. heilmannii*), as determined by 16S rRNA gene sequencing. In addition, we described that the bacterial infection may be related to the presence of a lymphoid follicle found in the pyloric mucosa and that an enzymatic digestion with *Nde* II of the PCR product will be useful to differentiate *H. heilmannii* from *H. pylori*. These findings can be used to study the epidemiology, diagnosis, and pathogenesis of the zoonotic agent, *H. heilmannii*.

**Acknowledgment**

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**References**


