

Experimental *Helicobacter pylori* gastric infection in miniature pigs

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An experimental *Helicobacter pylori* infection in miniature pigs was developed and investigated. Eighteen miniature pigs were inoculated with an *H. pylori* strain that has high virulence in mice at $c. 5 \times 10^{10}$ cfu. *H. pylori* infection in miniature pigs was achieved by the administration of agar 1% in brucella broth with fetal bovine serum 10% just before inoculation. The bacterial colonisation and distribution were analysed by mapping of viable cell counts in the stomach in pigs of three different ages. The mapping assay was achieved on post-infection day 3 for the 5-day-old and 2-week-old pigs, and between days 41 and 43 for 3-month-old pigs. The highest cell counts were observed in 5-day-old pigs, which averaged 4.9×10^6 cfu/g of mucosa ($n = 4$). The bacteria were colonised mainly in the cardiac and fundus gland region in the 5-day-old and 2-week-old pigs, whereas the colonisation sites did not depend on the region in the 3-month-old pigs. Biopsy assay of the antral mucosa of a 3-month-old pig after *H. pylori* infection showed that this infection persisted for >22 months. Serum antibody against *H. pylori* was detected in the infected pigs but not in the uninfected animal. Immunostaining demonstrated the presence of bacteria on the epithelial surface of the infected pigs. A microscopic finding common to all the infected pigs, focal gastritis with infiltration of lymphocytes detected on the lesser curvature of the stomach, resembled the microscopic appearance in *H. pylori*-infected human patients. These results suggest that miniature pigs might be a suitable model for studying *H. pylori* infection.

Introduction

Helicobacter pylori infection is strongly associated with gastroduodenal diseases [1, 2] and has also been linked to gastric carcinoma and lymphoma [3, 4]. An appropriate animal model is needed for understanding *H. pylori* infection and its relationship with these diseases. The animals now considered available for this infection model include pigs [5, 6], monkeys [7], dogs [8], mice [9], gerbils [10], rats [11] and guinea-pigs [12]. As pigs are similar to man in gastric physiology and anatomy [13], the first experimental infection with *H. pylori* was conducted in gnotobiotic pigs soon after the discovery of this organism in the human stomach [5]. Understanding of the mechanisms of diseases and bacterial virulence factors in the pig infection model has made considerable progress [14, 15]. Pigs infected

with *H. pylori* had severe epithelial cell vacuolation and significant reduction of the gastric mucosal surface area [16]. Furthermore, the size of areas colonised by bacteria has been shown to vary and the distribution of bacteria has shown a patchy pattern of infection in the pig model [17]. These studies indicate that among animal models, pigs reproduce the greatest number of the features of the disease associated with *H. pylori* infection in man. However, the size of the animals, cost and handling are all disadvantages.

Miniature pigs have characteristics in common with full-size pigs [18]. Furthermore, like their full-sized relatives, miniature pigs can be endoscoped easily, and it is possible to follow *H. pylori*-induced pathological changes and the effects of therapeutic regimens in the same animal. As it is easier to maintain and handle miniature pigs than full-sized pigs, these animals are more convenient for observation of the infection. Moreover, the degree of genetic homogeneity has been improved as an experimental animal species. Haring *et al.* have described the successful production of miniature pigs at Göttingen in Germany [19]. CSK

miniature pigs were originally derived from Göttingen miniature pigs, in a closed colony for 18 years from 1977 to 1994. However, earlier attempts to colonise *H. pylori* in miniature pigs were unsuccessful [20]. In this study, experimental *H. pylori* infection was developed in CSK miniature pigs and its characteristics were studied.

Materials and methods

Animals

Male specific pathogen-free (SPF) CSK miniature pigs (CSK Research Park, Nagano, Japan) were used in this study. The pigs were confirmed to be free of the following common porcine pathogens: *Mycoplasma* spp., *Bordetella* spp., *Haemophilus* spp., *Pasteurella multocida* and *Salmonella* spp. Pigs aged 2 days (body weight 550 g), 11 days (body weight 1350 g) and 11 weeks (body weight 10 kg) were purchased and housed individually in suitably adapted cages in the animal facility in a 12-h light-dark controlled room. A constant temperature of 22–25°C and humidity of c. 55% was maintained in the facility. The 2- and 11-day-old miniature pigs were inoculated with *H. pylori* after an acclimatisation period of 3 days and the 11-week-old miniature pigs were inoculated after an acclimatisation period of 2 weeks. To feed the animals age-suitable standard diets, the following specifically formulated diets were used: Weanylobulin (Nihon Nosan Kogyo KK, Kanagawa, Japan), a milk-based diet given three times daily for the 2-day-old animals; Weanymashace (Nihon Nosan Kogyo KK), a grain-based diet given twice daily for the 11-day-old animals; and combination diets of NS and Lucerne pellet (NS:Lucerne Pellet = 4:1; Nisseiken, Tokyo, Japan) given once daily for the 11-week-old animals. Six-week-old SPF BALB/c (*nu/nu*) athymic nude mice and 4-week-old SPF outbred ddY mice (Nippon SLC, Shizuoka, Japan) were used in this study. The mice were housed in a 12-h light-dark controlled room, and fed a normal commercial pellet diet (F-2; Funabashi Farms, Chiba, Japan). Drinking water was freely available to all animals. All animal experiments were performed according to the guidelines provided by the Institutional Animal Care and Use Committee of Sankyo Co. Ltd.

Helicobacter spp. status and histological evaluation of the pig herd

As gastric colonisation with *Helicobacter* spp. has been reported in domestic pigs [21, 22], the presence or absence of gastric *Helicobacter* spp. in the miniature pigs was ascertained before admission to the study. Ten 3-month-old miniature pigs were kept for 2 months in the animal facility of this institution. The 16 tissue sections (sites 1–16) shown in Fig. 1 were taken from 10 5-month-old miniature pigs and two 2-week-old miniature pigs and tested by bacterial culture. The

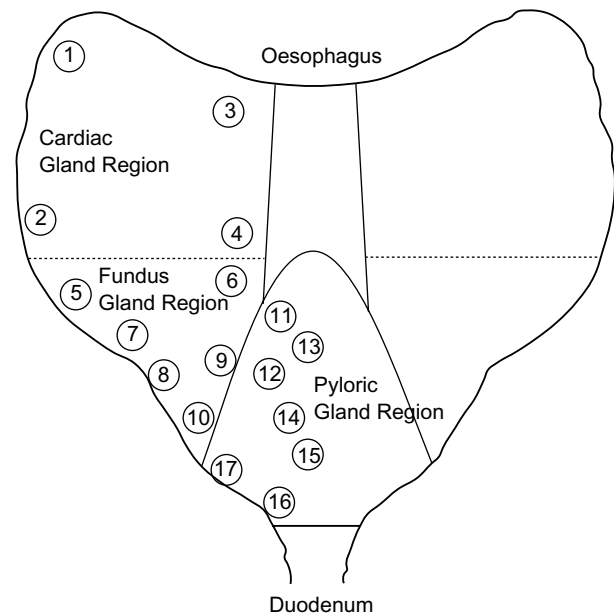


Fig. 1. Sites of biopsy for culture. The stomach was cut open along the greater curvature.

culture methods used have been described previously [23]. Sixteen tissue sections from sites opposite those sampled for culture were immunostained by the method described below. Four tissue sections were taken from the cardiac gland regions of 12 pigs for PCR analysis. DNA was extracted from the tissue sections as described previously [24]. PCR was performed with primers that amplify the urease B gene of *H. pylori* and *H. heilmannii* [25]. PCR was performed as described previously [25], and PCR products were separated on an agarose 1% gel and visualised by ethidium bromide. The same sections used for immunostaining were stained with haematoxylin and eosin and examined by light microscopy.

Bacterial inocula

H. pylori strain no. 9839 used in this study was isolated from gastric biopsy samples from a patient with gastric ulcer. *H. pylori* no. 9839 was kindly supplied by Dr T. Fujioka (Oita Medical University, Oita, Japan). This strain had the *cagA* gene and vacuolating cytotoxin, as confirmed by PCR amplification with primers specific for *cagA* and cytotoxicity assay, respectively [26, 27]. *H. pylori* 26695 is *cagA* and *VacA* positive [20], colonises neonatal gnotobiotic pigs [5], and was kindly provided by Professor S. Krakowka (Ohio State University, USA). Stock cultures were stored at –80°C in Brucella Broth (Becton Dickinson, Cockeysville, MD, USA) supplemented with fetal bovine serum (FBS Brucella Broth; Dainippon Pharmaceutical, Osaka, Japan) 2%. Bacteria were cultured in FBS brucella broth on a gyratory shaker at 110 rpm for 40 h at 37°C in micro-aerobic conditions. The culture was harvested by centrifugation and the cells were suspended in FBS brucella broth to 1×10^9 cfu/ml. The bacterial cell counts of all inocula were done with Brain Heart

Infusion Agar (Difco Laboratories, Detroit, MI, USA) supplemented with horse blood 5%. Plates were incubated at 37°C in a GasPak jar (Becton Dickinson) for 5 days with Campy Paks (Becton Dickinson).

Experimental design

All miniature pigs were rendered temporarily achlorhydric by intramuscular administration of famotidine (Yamanouchi Pharmaceutical, Tokyo, Japan) at 10 mg/kg, 0 and 3 h before inoculation. All animals except for the 5-day-old subjects were pre-treated with atropine (Tanabe Seiyaku, Osaka, Japan) 0.05 mg/kg, medetomidine (Meiji Seika Kaisha, Tokyo, Japan) 0.08 mg/kg and butorphanol (Bristol-Myers Squibb KK, Tokyo, Japan) 0.22 mg/kg, and just before inoculation they were anaesthetised with ketamine hydrochloride (Sankyo) 5 mg/kg intramuscularly. Four experiments were conducted as described below.

Experiment 1. Four pigs aged 3 months were fasted for 24 h before inoculation. During anaesthesia, animals received 50 ml of bacterial suspension of 1×10^9 cfu/ml in FBS brucella broth *via* a silicone stomach catheter (4 mm \times 120 cm; Japan Medical Supply, Hiroshima, Japan). The pigs were killed 41–43 days after infection for mapping of viable cell counts in the stomach.

Experiment 2. Five pigs aged 3 months were fasted for 24 h before inoculation, and another served as a control. During anaesthesia, 50 ml of FBS brucella agar were administered just before inoculation of 50 ml of bacterial suspension of 1×10^9 cfu/ml in FBS brucella broth, to delay gastric emptying. The FBS brucella agar was prepared by adding FBS 10% to the brucella broth, which was supplemented with Bacto-Agar (Difco Laboratories) 1%, autoclaved and cooled to *c.* 50°C. A syringe was prefilled with the FBS brucella agar. After gelatinisation at 4°C, the FBS brucella agar was injected by force through the catheter into the stomach. The control pig received FBS brucella broth and FBS brucella agar. Four of the infected pigs and the control animal were killed 41–43 days after infection. The remaining infected pig was observed for >22 months. Endoscopy was performed as described below.

Experiment 3. Five pigs aged 2 weeks were fasted for 6 h before inoculation. FBS brucella agar (10 ml) was administered before inoculation of 10 ml of bacterial suspension of 1×10^9 cfu/ml in FBS brucella broth *via* a silicone stomach catheter (2.67 mm \times 40 cm, Japan Medical Supply). The pigs were killed 3 days after infection.

Experiment 4. Four pigs aged 5 days were inoculated as described in Experiment 3 without anaesthesia. The pigs were killed 3 days after infection.

Infection of BALB/c nu/nu mice, ddY mice and miniature pigs

Bacterial inocula of *H. pylori* 9839 or *H. pylori* 26695 were prepared for mice as described previously [28]. The mice were killed 1 week after infection. Two 2-week-old miniature pigs were inoculated as described in Experiment 3. The pigs were killed 5 days after infection.

Culture of H. pylori from infected miniature pigs

Before they were killed, the pigs were fasted with free access to water for 18 h. Under deep sedation with atropine, medetomidine, butorphanol and ketamine hydrochloride, they were cut open and their stomachs were removed. The animals were killed by administration of an overdose of sodium pentobarbital (Dainippon Pharmaceutical). The stomach was cut open along the greater curvature, emptied and gently rinsed with saline to remove the stomach contents. Local *H. pylori* populations were sampled by taking one biopsy (*c.* 50 mg) from each of 16 representative sites: four sites from the cardiac mucosa (sites 1–4 in Fig. 1), six from the fundic mucosa (sites 5–10) and six from the antral mucosa (sites 11–16). The biopsy specimens were homogenised in 2 ml of FBS brucella broth, followed by dilution with the same broth and 100 μ l volumes of the dilutions were inoculated on to modified Skirrow's agar plates containing vancomycin (Sigma) 10 mg/L, bacitracin (Sigma) 8 mg/L, polymyxin B (Pfizer Pharmaceutical, Tokyo, Japan) 0.25 mg/L, trimethoprim (Shionogi Pharmaceutical, Osaka, Japan) 2.5 mg/L and amphotericin B (Sigma) 3 mg/L. Plates were incubated at 37°C in micro-aerobic conditions. Bacterial counts were expressed as cfu/g of tissue.

Culture of H. pylori from infected mice

Each stomach specimen was homogenised in 2 ml of FBS brucella broth, followed by dilution with the same broth and 100 μ l volumes of the dilutions were inoculated on to modified Skirrow's agar plates containing vancomycin 10 mg/L, polymyxin B 2.5 U/ml, trimethoprim 2.5 mg/L, nalidixic acid 15 mg/L and amphotericin B 3 mg/L [28]. Plates were incubated at 37°C under micro-aerobic conditions for 5 days.

Endoscopy and biopsy specimens

Before endoscopy the pigs were fasted for 18 h. Endoscopy was performed with anaesthesia and a GIF XQ240 Endoscope (Olympus, Tokyo, Japan). Four biopsy specimens (*c.* 2 mg) were taken from the antrum as indicated in Fig. 1 (sites 11, 12, 14 and 16). The specimens were homogenised in 2 ml of FBS brucella broth and cultured for *H. pylori* (see above). The numbers of *H. pylori* were counted as described above. The numbers of *H. pylori* in the

antral mucosa indicated the average of four antral biopsy specimens and were expressed as the mean and SEM.

Histopathological examination

Tissue samples for histopathology were taken from sites opposite those sampled for microbiology. Samples for histopathological examination was prepared by the method of Shimizu *et al.* [29]. Specimens were fixed in a cold Carnoy's solution (of ethanol:acetic acid:chloroform, 6:3:1 v:v:v) for 2 h at 4°C. After fixation, they were sliced longitudinally at 5-mm intervals and all tissue sections were then dehydrated in absolute alcohol, cleared in xylene and embedded in paraffin. Serial paraffin sections, 3 µm thick, were prepared. One slide from each block was stained with haematoxylin-eosin for morphological observation and one was immunostained for *H. pylori* by an indirect immunoperoxidase method. Histopathological findings were evaluated for the presence of lymphocyte aggregates or lymphoid follicles in each section. A lymphocyte aggregate was defined as >10 lymphocytes per ×400 field. After rehydration, sections from Carnoy-fixed materials were re-fixed with buffered formalin 20% for 30 min. Before the application of anti-*H. pylori* polyclonal antibody (DAKO Japan, Kyoto, Japan), hydrated sections were treated with a trypsin (Sigma) solution (trypsin 0.2%, CaCl₂ 0.1% in 0.05 M Tris buffer, pH 7.6) at 37°C for 10 min. This anti-*H. pylori* polyclonal antibody also reacts with *H. heilmannii* antigen [22]. After washing with 0.05 M Tris-HCl buffered saline (pH 7.6), the diluted horseradish peroxidase-conjugated anti-rabbit IgG antibody (DAKO Japan) was added. Antibody binding sites were visualised with 3',3'-diaminobenzidine tetrahydrochloride (Dojindo Laboratories, Kumamoto, Japan) and the sections were counterstained with haematoxylin.

ELISA for H. pylori antibody

Just before and after infection in 3-month-old pigs, serum samples were collected and frozen at -20°C for the subsequent determination of *H. pylori* antibodies. Serum IgG antibody to *H. pylori* was assayed by means of the enzyme-linked immunosorbent assay (ELISA) with a commercial kit (Helico G, International Reagents, Kobe, Japan) [30]. The serum samples were diluted to 1 in 100. Dilutions of sera were incubated for 1 h at 37°C. The secondary antibody, peroxidase-conjugated goat anti-pig IgG (Bethyl Laboratories, Texas, USA) diluted to a concentration of 1 in 1000 in commercial buffer, was applied to the wells for 30 min at 37°C, substrate was added for 10 min and then stopping solution was added. OD₄₅₀ was recorded with an ELISA plate reader (MR 580; Dynatech Laboratories, Alexandria, VA, USA).

PCR amplification and restriction fragment-length polymorphism (RFLP)

H. pylori were recovered from the 5-day-old, 2-week-old and 3-month-old miniature pigs at necropsy and from the pig observed for 22 months after inoculation, at biopsy. The DNA samples of the recovered *H. pylori* were used. Primer sequences chosen for amplification were specific for the *H. pylori flaA* gene [31]. PCR was performed as described previously [24], with some modifications. After PCR amplification, 15 µl of reaction mixture were removed and incubated at 37°C for 2 h, and the fragments were separated on an agarose 3% gel. The gels were stained with ethidium bromide.

Statistical analysis

Proportions were compared by Fisher's exact test; p values of <0.05 were considered statistically significant.

Results

Helicobacter spp. status and histological evaluation of the pig herd

Ten 5-month-old miniature pigs and two 2-week-old miniature pigs examined were negative for *Helicobacter* spp. by culture. All miniature pigs examined were negative for *H. heilmannii* and *H. pylori* by both immunostaining and PCR. There was no inflammatory response in all 16 tissue sections from the gastric mucosa of 12 pigs.

Effect of agar on infection of 3-month-old miniature pigs

No *H. pylori* were recovered from any biopsy specimens of four pigs challenged with a bacterial suspension alone. On the other hand, *H. pylori* were recovered from all five pigs challenged with a bacterial suspension immediately after administration of the FBS brucella agar.

Number of H. pylori in the gastric mucosa of miniature pigs

H. pylori were recovered from all sites in the 5-day-old infected pigs; they were mainly found in cardiac and fundus gland regions (Table 1). The highest number of viable bacteria was log₁₀ 6.69 cfu/g in sites 4 and 7. In all sites tested (1-16), 5-day-old pigs had more bacteria than the other pigs. In 2-week-old pigs, bacteria were mainly found in cardiac and fundus gland regions and the highest number of viable bacteria was log₁₀ 5.26 cfu/g in site 4. On the other hand, in 3-month-old pigs, the colonisation sites differed from the other pig groups. Bacteria were predominantly recovered from site 4 in the cardiac gland region, site 5 in

Table 1. Number of *H. pylori* in gastric regions of miniature pigs

Gastric region	Site	Mean (SEM) log ₁₀ cfu/g of tissue – positive cultures/total					
		5-day-old		2-week-old		3-month-old	
Cardia	1	6.47 (0.33)	4/4	4.15 (0.61)	4/5	3.48	2/4
	2	6.48 (0.50)	4/4	4.50 (0.30)	5/5	3.91 (0.54)	4/4
	3	5.76 (0.83)	4/4	3.75	2/5	2.51	1/4
	4	6.69 (0.082)	4/4	5.26 (0.14)	5/5	5.12 (0.11)	4/4
Fundus	5	6.67 (0.15)	4/4	4.84 (0.27)	5/5	4.57 (0.45)	4/4
	6	6.50 (0.26)	4/4	4.34 (0.29)	5/5	3.58 (0.39)	4/4
	7	6.69 (0.19)	4/4	4.24 (0.36)	5/5	3.31 (0.28)	3/4
	8	6.35 (0.23)	4/4	4.40 (0.26)	5/5	3.48 (0.52)	3/4
	9	5.80 (0.68)	4/4	4.38 (0.44)	5/5	4.06	2/4
	10	5.78 (0.68)	4/4	4.48 (0.44)	5/5	3.37	2/4
Antrum	11	5.66 (0.53)	4/4	3.97 (0.03)	3/5	4.64 (0.19)	4/4
	12	5.16 (0.55)	3/4	2.51	2/5	4.43	2/4
	13	4.81 (0.24)	4/4	4.60	1/5	3.81 (0.49)	3/4
	14	3.52 (0.24)	4/4	3.20	1/5	4.11	1/4
	15	4.48 (0.66)	3/4	2.60	1/5	3.46 (0.38)	3/4
	16	4.97 (0.74)	3/4	<2.45	0/5	2.46	1/4

the fundus gland region and site 11 in the pyloric gland region and the highest number of viable bacteria was log₁₀ 5.12 cfu/g in site 4. For the control pig, no *H. pylori* were isolated from any sites.

Histopathology

The presence of lymphoid follicle or lymphocyte aggregates is shown in Table 2. No inflammation was present in the antrum regions. All infected pigs had chronic gastritis at site 4. Focal to diffuse lymphocyte infiltration in 5-day-old and 2-week-old pigs was prominent in the cardiac and fundus gland regions (sites 1, 3, 4, 6, 8 and 9). This inflammation was also detected in cardiac and fundus gland regions (sites 1–4 and 9) in the 3-month-old animals. The histopathological intensity and severity were most pronounced in the 3-month-old infected pigs. Lymphoid follicle formation occurred in both the lamina propria and the submucosa at site 4 in the 3-month-old pig (Fig. 2a) and the lamina propria at site 4 in the 5-day-old pig (Fig. 2b). No gastric epithelial erosions or ulcerations were observed in any of the infected pigs. Lesions of chronic gastritis were not observed in the uninfected control pig. With immunostaining in the 5-day-old infected pig at site 10, *H. pylori* were detected on the epithelial surface. No bacteria were detected in the

tissues of the uninfected control pig by immunostaining.

Serum anti-*H. pylori* antibody

Fig. 3 shows the serum antibody response to *H. pylori* of 3-month-old pigs infected with *H. pylori* and the uninfected control. Anti-*H. pylori* IgG antibody levels from all infected pigs increased at 2 weeks after infection. The anti-*H. pylori* IgG levels continued to rise over the course of the experiment, suggesting that seropositivity correlated with active infection. The uninfected control pig had no antibody response specific for *H. pylori*.

Persistence of colonisation of *H. pylori*

Fig. 4 shows the number of *H. pylori* in the antral mucosa of a 3-month-old pig infected with *H. pylori*. Once the pig was infected, the infection persisted for at least 22 months and the bacterial counts recovered from the stomach were constant. No macroscopic gastritis or gastric ulcers were observed in this pig.

RFLP analysis of *H. pylori*

The identities of the strains of *H. pylori* isolated from the experimentally infected miniature pigs were com-

Table 2. Histopathological findings in miniature pigs infected with *H. pylori*

Age of pigs (n)	Number of pigs with lymphocyte aggregates or lymphoid follicles at stomach site									
	Cardia					Fundus				
	1	2	3	4	5	6	7	8	9	10
5 days (4)*	2	1	2	4	1	4	2	3	3	1
2 weeks (5)*	3	1	5	5	1	4	0	2	2	2
3 months (4)†	2	2	2	4	0	1	0	0	2	0

*The presence of histological change was assessed at 3 days after infection.

†The presence of histological change was assessed at 41–43 days after infection.

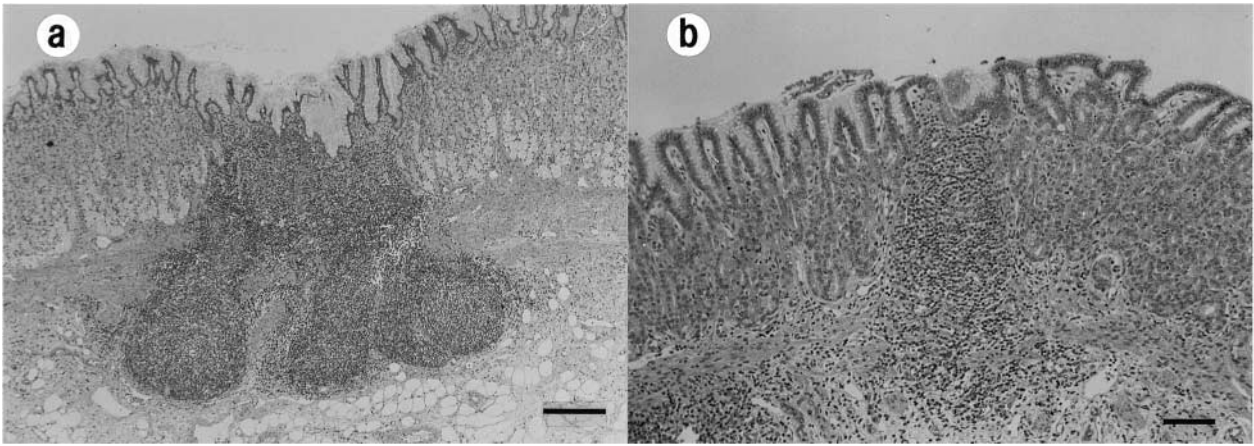


Fig. 2. Lymphoid follicle development in (a) the lamina propria and submucosa of the 3-month-old pig at site 4 (bar = 300 μ m) and (b) the lamina propria of the 5-day-old pig at site 4 (bar = 100 μ m).

pared by RFLP analysis of the 1.5-kb *flaA* gene segment with that of the infecting strain. The RFLP pattern of the *flaA* gene PCR product of the infecting strain was virtually identical to that of the strains isolated from the infected miniature pigs (Fig. 5).

Infection of BALB/c *nu/nu* mice, ddY mice and miniature pigs

To compare the virulence of *H. pylori* 9839 and *H. pylori* 26695 in animals, BALB/c *nu/nu* mice, ddY mice and miniature pigs were inoculated with each strain. *H. pylori* 9839 colonised all inoculated mice and the numbers of colonising bacteria in BALB/c *nu/nu* and ddY mice averaged \log_{10} 4.72 and 4.99 cfu/stomach, respectively (Table 3). On the other hand, *H.*

pylori 26695 was recovered from only one of 15 BALB/c *nu/nu* and ddY mice ($p = 0.0001$) and was not recovered from miniature pigs.

Discussion

Recent studies have reported that spiral shaped gastrospirillum-like organisms (GHLOs) are often found in pigs, especially in slaughterhouse pig stomachs with ulcer or pre-ulcerous lesions in the pars oesophagea [21, 22]. Cloned 16S ribosomal DNA from GHLOs appeared to be very similar to human *H. heilmannii* type 1 [32]. The pre-study results of PCR with *H. heilmannii* and *H. pylori* specific primers and immunostaining suggested that miniature pigs examined in this study were free of *H. heilmannii* and *H.*

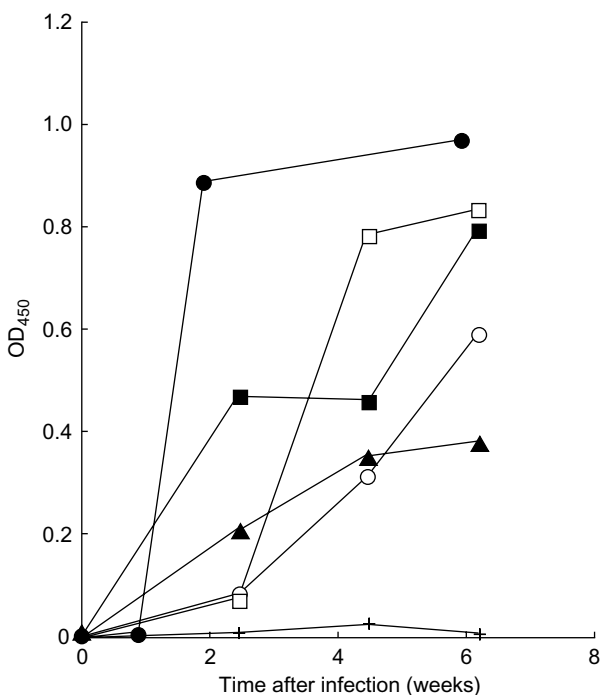


Fig. 3. Antibody response to *H. pylori* in sera of miniature pigs infected with *H. pylori* (●, ○, ■, □, ▲) and an uninfected pig (+).

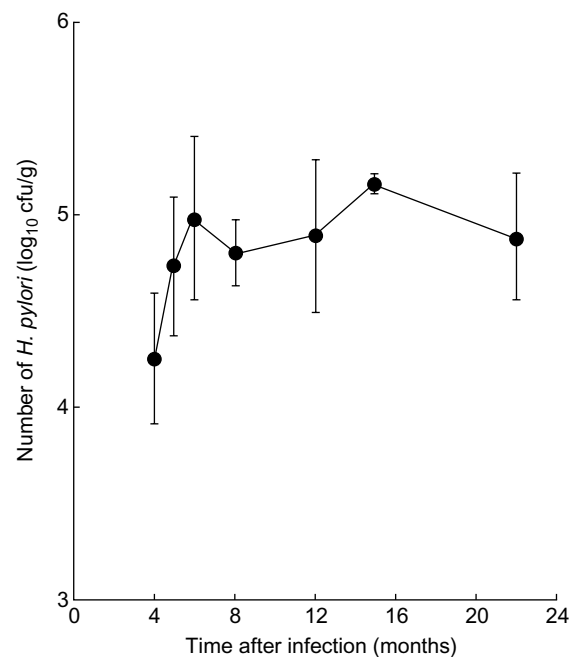


Fig. 4. The number of *H. pylori* in the antral mucosa (mean results from biopsy sites 11, 12, 14 and 17; Fig. 1) of the miniature pig infected with *H. pylori*; bar, SEM.

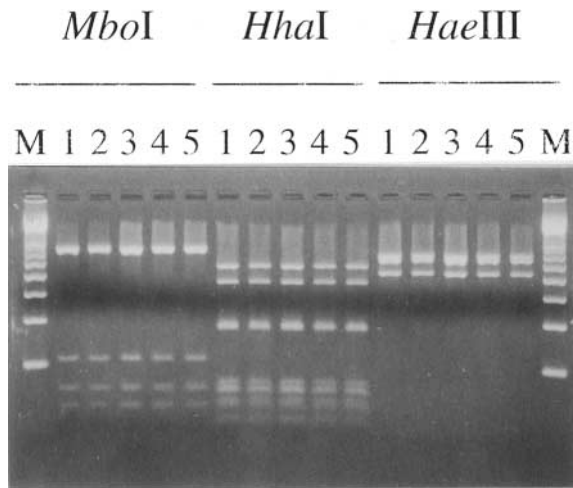


Fig. 5. RFLP analysis of the PCR-amplified 1.5-kb *flaA* segment of *H. pylori* with the indicated restriction enzymes. Lane 1, PCR product from the infecting strain *H. pylori* 9839; 2, PCR products from strains recovered from experimentally infected 5-day-old miniature pigs; 3, PCR products from strains recovered from experimentally infected 2-week-old miniature pigs; 4, PCR products from strains recovered from experimentally infected 3-month-old miniature pigs; 5, PCR products from strains recovered from experimentally infected 3-month-old miniature pigs 22 months after infection; M, 100-bp ladder markers (Amersham Pharmacia Biotech, Uppsala, Sweden).

pylori. Moreover, miniature pigs were not infected with other GHLOs, as demonstrated by culturing of gastric tissue samples. Thus, this pig herd was free of gastric *Helicobacter* organisms. These results are also supported by the observation that non-infected miniature pigs exhibited neither gastritis nor minimal focal gastritis.

These studies have demonstrated that miniature pigs are susceptible to gastric infection with *H. pylori*. Persistent *H. pylori* infection was detected by *H. pylori* culture, systemic immune response and immunostaining of gastric mucosa. The successful chronic colonisa-

tion of *H. pylori* in miniature pigs depended on the administration of FBS agar. In a study by Engstrand *et al.* [6], 8-week-old barrier-born pigs were fed gruel before and after administration of the bacterial suspension, and fat was also injected into the duodenum before bacterial suspension to delay gastric emptying. In the present study, FBS agar was administered into the stomach to delay the passage of the bacterial suspension through the duodenum, with the hope that the prolongation of exposure of the gastric mucosa to the bacteria would make the stomach more susceptible to colonisation with *H. pylori*. Another factor in the successful establishment of *H. pylori* infection was the use of strain no. 9839, a strain that can colonise the stomach of BALB/c *nu/nu* and ddY mice. The colonisation levels of *H. pylori* 9839 in BALB/c *nu/nu* mice were higher than those of *H. pylori* 2052 [28]. *H. pylori* 26695, which can colonise gnotobiotic pigs [5], could not colonise mice and miniature pigs. Strain 9839 may have the ability to colonise several hosts.

The RFLP pattern of the *flaA* gene PCR product of the infecting strain was virtually identical to those of recovered organisms. This result confirmed the lack of co-infection with other *Helicobacter* species. The patterns for all five *H. pylori* strains differed from previous reported *H. pylori* strains [24]. RFLP analysis of the 1.5-kb *flaA* gene segment with three enzymes, *HaeIII*, *MboI* and *HhaI*, would be a useful method to identify the strain.

It is now accepted that *H. pylori* infection is usually acquired early in childhood [33]. However, nothing is known about the factors of age that allow colonisation and distribution of *H. pylori* in the stomach. The present study examined the susceptibility of miniature pigs of different ages to *H. pylori* infection. This is the first evidence that the numbers and distribution of *H. pylori* in the gastric mucosa depend on the ages of the infected miniature pigs. The colonisation levels in the 5-day-old miniature pigs were higher than those in the

Table 3. Experimental infection of BALB/c *nu/nu* mice, ddY mice and miniature pigs with *H. pylori* strains 9839 and 26695

	Bacterial Strain			
	<i>H. pylori</i> 9839		<i>H. pylori</i> 26695	
Animals	Number of infected animals/number challenged	Log ₁₀ cfu/stomach* or g of tissue	Number of infected animals/number challenged	Log ₁₀ cfu/stomach† or g of tissue
BALB/c <i>nu/nu</i> mice‡	15/15	4.72 (0.10)	1/15	3.65
ddY mice ‡	15/15	4.99 (0.09)	1/15	3.50
Miniature pigs§	2/2	5.50	0/2	<2.45

*The results of mice were expressed as means (SEM) for 15 mice, and those of pigs were expressed as the highest bacterial count between two pigs.

†Only one mouse recovered from the results of both groups of mice.

‡The presence of *H. pylori* infection was assessed at 1 week after infection.

§The presence of *H. pylori* infection was assessed at 5 days after infection.

^{||}p < 0.01 mice infected with *H. pylori* 9839 versus *H. pylori* 26695.

2-week-old animals and were similar to those of human patients. The reason for the difference in colonisation levels may be due to the age differences in the pigs. However, other factors such as diet cannot be excluded and will have to be investigated by further studies. The colonisation levels in the 5-day-old miniature pigs, 3 days after infection, were also higher than those in the 3-month-old pigs at 41–43 days after infection. This difference in colonisation could be due to the amount of time elapsed.

In 5-day-old and 2-week-old pigs, bacteria were found mainly in the cardiac and fundus gland region, whereas the colonisation sites in the 3-month-old pigs did not depend on the region. However, the site-dependent colonisation observed was similar to that described for man [34]. It has been reported that differences in distribution relate to differences in local acid output in the *H. felis*-infected mouse model [35]. This difference may be related to the acid output in 5-day-old and 2-week-old miniature pigs, which is lower than that in their 3-month-old cohorts. This speculation is supported by the fact that the gastric acid secretory capacity of the pig increases slowly from birth to 4 weeks of age, and that gastric acid secretory capacity at 10 weeks of age is almost equal to that of mature pigs [36]. Five-day-old and 2-week-old pigs may be suitable as models for studying *H. pylori*-infected infants who have low acid output.

The colonisation levels of 5-day-old miniature pigs were comparable to those in gnotobiotic pigs [37]. However, the distribution of bacteria differed between miniature pigs and gnotobiotic pigs in the following respects. Bacterial distribution was predominantly in the cardiac and fundic gland region in miniature pigs but in the cardiac and antrum in gnotobiotic pigs [15]. The reasons for this difference remain unknown. The colonisation levels of 3-month-old miniature pigs were also comparable to those in barrier-born pigs [17]. In the present study, bacteria were predominantly recovered at sites 4, 5 and 11. On the other hand, in barrier-born pigs, sites 4 and 11 gave a positive urease reaction that indicated the presence of *H. pylori*. These findings suggest that this *H. pylori*-infected miniature pig model may be similar to the gnotobiotic and barrier-born pig infection models.

Histopathological examination indicated inflammatory lesions accompanied by lymphoid follicle formation localised in the cardiac and fundus gland regions along the lesser curvature of the stomach. There was a very low number of neutrophils present in the inflamed mucosa. This finding has been demonstrated in several other *Helicobacter*-infected animal models [9, 11, 12] and comprises the most striking difference between man and animal models. As the sites of inflammation did not always correlate to the sites of bacterial colonisation, the inflammatory response appears to be site-dependent. The absence of any direct correlation

between the severity of inflammation and the extent of colonisation was also reported in gnotobiotic pigs [15]. Moreover, in man, the distribution of follicles was unrelated to the intensity of *H. pylori* infection, and lymphoid follicles were more numerous on the lesser than on the greater curvature of the stomach [38]. These findings indicate that the inflammatory response produced following *H. pylori* infection may also depend on the site of stomach affected.

H. pylori were readily observed in the tissue of 5-day-old miniature pigs by immunostaining; bacteria were seen on the epithelial surface. In contrast, organisms were seen only rarely in immunostaining of tissue from 2-week-old and 3-month-old pigs. The number of bacteria at site 10 was \log_{10} 6.9 cfu/g. Histological detection of bacteria in immunostained sections requires colonisation of $>\log_{10}$ 6 cfu/g of tissue. According to Krakowka and Eaton [20], the sensitivity of the Warthin-Starry stain is limited in that *H. pylori* are identified easily in pigs with a recoverable cfu $>\log_{10}$ 6, with difficulty at \log_{10} 5 cfu and usually as falsely negative below \log_{10} 4 cfu. The sensitivity of immunostaining in the present study would be similar to that of the Warthin-Starry stain.

Most of the infected animals had a detectable specific antibody response to *H. pylori* by 2 weeks after challenge. Seroconversion may take place as early as 1 week after the exposure. A similar antibody response has been observed in several other infected animal models [11, 12] but, in man, ≥ 3 weeks may be required to produce a detectable IgG response [39]. This rapid increase in specific IgG may be related to the high oral inoculation dose of *H. pylori* or the severity of colonisation. The uninfected control had no immune response against *H. pylori*, indicating that normal miniature pigs possess no natural cross-reacting antibodies against *H. pylori*.

Once infected, bacteria were recovered from the antrum mucosa at colonisation levels ranging from \log_{10} 4 to 5 cfu/g, a range that remained constant throughout the study period (22 months). However, no macroscopic gastritis and gastric ulcer were observed in the infected pig. This observation needs further confirmation with a longer observation period and a larger number of miniature pigs.

In summary, miniature pigs were successfully colonised with *H. pylori*. The numbers and distribution of *H. pylori* in the gastric mucosa depended on the ages of the infected miniature pigs. The 5-day-old and 2-week-old miniature pigs may provide useful models for the study of the infection in human infants and the 3-month-old pigs may provide a useful model for the study of the infection in human adults. In conclusion, miniature pigs might be a suitable model for studying the relationship between age and *H. pylori* infection and *H. pylori*-associated diseases in man.

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