antimicrobial constituents of *Terminalia chebula* Retz against methicillin-resistant *Staphylococcus aureus*. *Biol Pharm Bull* **20:** 401–404.

24. Funatogawa K., Hayashi S., Shimomura H., Yoshida T., Hatano T., Ito H., Hirai Y. (2004) Antibacterial activity of hydrolysable tannins derived from medicinal plants against *Helicobacter pylori*. *Microbiol Immunol* **48:** 251–61.
25. Peters D., Clissold S. (1992) Clarithromycin. A review of its antimicrobial activity, pharmacokinetic properties and therapeutic potential. *Drugs* **44:** 117–64.
26. Cellini L., Grande R., Di Campli E., Traini T., Giulio M.D., Lannutti S.N., Lattanzio R., Lannutti S.N. (2008) Dynamic colonization of *Helicobacter pylori* in human gastric mucosa. *Scand J Gastroenterol* **43:** 178–85. Apr;20(4):401–4.
27. Hayashi S., Hirai Y., Isogai E., Yokota K., Oguma K. (2000) Treatment of *Helicobacter pylori* infection. *Res Adv Antimicrob Agents Chemother* **1:** 7–12.
28. Spoering A.L., Lewis K. (2001) Biofilms and planktonic cells of *Pseudomonas aeruginosa* have similar resistance to killing by antimicrobials. *J Bacteriol* **183:** 6746–51.

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ORIGINAL ARTICLE

Assessment of *in vitro* biofilm formation by *Helicobacter pylori*

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Key words
auto-aggregation, biofilm, *Helicobacter pylori*, hydrophobicity, motility, TK1402.

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Conflict of interest
No conflict of interest has been declared by the authors.

Abstract

Background and Aims: Biofilms are surface-bound communities of bacterial cells that are implicated in their survival. As with various bacteria studied to date, *Helicobacter pylori* can have an alternate lifestyle as a biofilm. We previously reported that strain TK1402 showed a strong biofilm-forming ability *in vitro*. However, the mechanisms of its biofilm development remain unclear. We analyzed the basic characteristics of the biofilm-forming ability in strain TK1402.

Methods: In order to characterize the biofilm-forming ability of the *H. pylori* strains, auto-aggregation, motility and hydrophobicity, which are important factors in biofilm formation by other bacteria, were analyzed. Further, we tested whether cell growth participated in biofilm formation in strain TK1402.

Results: There were no significant differences in the auto-aggregation, motility and hydrophobicity of strain TK1402 compared with the other strains. On the other hand, pre-culture of this strain for 24–48 h resulted in decreased biofilm formation.

Conclusion: TK1402 is a strong biofilm-forming strain of *H. pylori* in Brucella broth supplemented with 7% fetal calf serum. It is possible that biofilm-forming cell growth is a principal factor in biofilm development.

Introduction

Biofilms are surface-bound communities of microbial cells found in oligotrophic environments and are strongly implicated in bacterial virulence.[1] Many bacteria in aquatic ecosystems exist in biofilms on a wide variety of surfaces. Biofilm formation is critical not only for their environmental survival but also for successful infection by numerous pathogenic bacteria.

Helicobacter pylori is a spiral, microaerophilic, non-invasive, Gram-negative bacterium that colonizes the human gastrointestinal tract, primarily the stomach.[2] Previous studies have alluded to the ability of *H. pylori* to form biofilms.[3–6] A polysaccharide-containing biofilm was observed at the air–liquid interface when *H. pylori* was grown in a glass fermenter.[3] *H. pylori* was also capable of binding to a heterotrophic mixed species biofilm grown on stainless steel coupons.[4] We have previously reported that the strain TK1402 has strong biofilm-forming ability compared to other strains.[7] In addition, this property is dependent upon direct cell–cell binding patterns mediated by the outer membrane vesicles (OMV) produced from strain TK1402. However, it is recognized that an understanding of *H. pylori* biofilm formation is still in its infancy. The purpose of the present study was to investigate what factors are involved in biofilm formation in strain TK1402.

Methods

Bacterial strains and culture conditions

The following *H. pylori* strains were used: SS1, ATCC 49503, ATCC 43579, NCTC 11638, TK1029, TK1402, KR2003 and KR2005. The last four are clinical isolates from Japanese patients. Strains TK1029 and TK1402 were used as described previously.[8] Strains KR2003 and KR2005 were isolated in our laboratory from a gastritis patient. All clinical isolates contained *vacA* and *cagA* genes as demonstrated by polymerase chain reaction. All strains were maintained at –80°C in Brucella broth (Difco, Detroit, MI, USA) with 20% (v/v) glycerol. These strains were cultured under microaerobic conditions at 37°C on a Brucella agar plate containing 7% fetal calf serum (FCS).

Biofilm formation and its quantification

Biofilm formation was carried out as previously described.[7] Briefly, sterilized glass coverslips (~22 mm × 22 mm, 0.12–0.17 mm thick; Matsunami Glass, Tokyo, Japan) were placed into 12-well microtiter plates. Each well was filled with 2 mL of Brucella broth supplemented with 7% FCS (Brucella-serum) to allow adherence of *H. pylori* at the air–liquid interface. The formation of

biofilms was initiated by inoculating 10 μL of pre-cultured cell suspension (~5×10^5 cells in Brucella broth) into each well. The cultures were incubated under microaerobic conditions at 37°C for 3 days with shaking (80–100 rpm). After incubation, the coverslips were removed and washed with phosphate-buffered saline (PBS). The samples were then air-dried and stained with crystal violet (CV) for 30 s. After staining, the coverslips were rinsed with distilled H_2O to remove excess dye and then air-dried at room temperature for 30 min. For biofilm quantification, all dye associated with the biofilms was dissolved with 1 mL of ethanol and 200 μL of the solutions were used to measure the absorbance at 594 nm with a microplate reader to determine the intensity of biofilm formation.

Cell surface hydrophobicity

Cell surface hydrophobicity of the different *H. pylori* strains was measured as described previously.[9] Briefly, the strains were grown overnight at 37°C in Brucella-serum broth and subsequently diluted 1:10 in 10 mL of fresh medium. The subculture was further incubated at 37°C for 20 h. Stationary-phase bacteria were harvested by centrifugation, washed three times with PUM buffer (0.15 mol/L potassium phosphate buffer [pH 7.1] containing 0.3 mol/L urea and 6.7 mmol/L $MgSO_4$), and resuspended in PUM buffer. To 1.2 mL of bacterial suspension in a glass tube normalized to an optical density (OD_{400}) of 1.0, 600 μL of *n*-hexadecane (Sigma, St Louis, MO, USA) was added, and the tubes were vigorously mixed for 60 s. Next, the mixtures were incubated for 15 min at room temperature to ensure complete separation of the organic and aqueous phases. The absorbance of the aqueous layer was measured at 400 nm. The percent cell surface hydrophobicity was calculated as: (OD_{400} before mixing – OD_{400} after mixing) / OD_{400} before mixing × 100. Each determination was performed in triplicate.

Motility assay

Motility of the *H. pylori* strains was assessed by the method of Osaki *et al.*[10] Briefly, the bacteria grown in Brucella-serum broth for 24 h were collected and centrifuged. Pellets were inoculated into medium using a sterile picker. At 3–5 days after incubation under microaerobic condition at 37°C, the diameter of the halo was measured. At least three plates were used for each experiment.

Auto-aggregation reaction

Auto-aggregation in the eight strains of *H. pylori* was determined with cells washed and suspended in PBS. The suspension of each strain was adjusted to an OD of 1.0 at 600 nm. The suspensions were incubated standing at room temperature. The optical density at 600 nm was determined over time. The percent auto-aggregation was calculated as: auto-aggregation = ([pre-incubation value [OD_{600}] – sample value [OD600]) / (pre-incubation value [OD_{600}]) × 100.

Statistical analysis

Statistical analysis was performed using the Mann–Whitney *U*-test. *P*-values of 0.05 or less were considered to indicate statistical significance.

Results

Hydrophobicity assay

All of the eight strains of *H. pylori* used in the present study formed a biofilm at the abiotic glass surface. The CV-stained biofilms after 3 days cultivation on the coverslip surface are shown in Figure 1. Many cells in the TK1402 biofilm remained firmly attached to the glass surface compared with the other strains. In studies of other biofilm-forming bacteria, cell-surface hydrophobicity was reported as an important factor in bacterial adherence to a glass surface.[11] We examined the hydrophobicity levels of eight *H. pylori* strains and there were no significant differences among them; however, the value for TK1402 was comparatively high (Table 1), which suggest that cell surface hydrophobicity might be involved in the strong biofilm-forming ability of the TK1402 strain.

Motility assay

The eight strains of *H. pylori* were cultured on Brucella-serum medium containing 0.3% agar and after 3–6 days of incubation, there were variations in the halo diameter. Strain TK1402 was motile, but its halo diameter after 6 days of culture was not the broadest, being the third widest (Fig. 2).

Auto-aggregation assay

The auto-aggregation of each strain was divided into high for strains ATCC 49503, TK1029 and TK1402, and low for strains SS1, ATCC 43579, NCTC11638 and KR2003 (Fig. 3). Strain TK1402 with its strong biofilm-forming ability belonged to the high aggregative group, but did not exhibit a significantly high level of auto-aggregation.

Influence of cell growth on biofilm formation

When strain TK1402 was grown under conditions similar to those used for the biofilm-formation assay, but without a glass coverslip, its growth kinetic peaked within 2 days. After 3 days of incubation, its growth rate was slightly decreased, and then reached a plateau. In order to characterize the strong biofilm formation by strain TK1402, cells in the stationary phase were used in a biofilm-formation assay (Fig. 4). Strain TK1402 was cultured in a 12-well plate for 24–48 h without glass coverslips, and then sterilized glass coverslips were placed into the culture for formation of a biofilm. After an additional 72-h incubation, biofilm formation was measured. In the previous 24-h sample, biofilm formation tended to decrease and there was a significant decrease in biofilm formation in the previous 48-h culture. These results indicated that cell growth was required for biofilm development by strain TK1402.

Discussion

Biofilm formation is critical, not only for environmental survival but also for successful infection by numerous pathogenic bacteria. Furthermore, biofilm cells growing on a surface exhibit properties distinct from those of planktonic cells, such as increased resistance to biocides and antimicrobial agents. Several reports indicate that

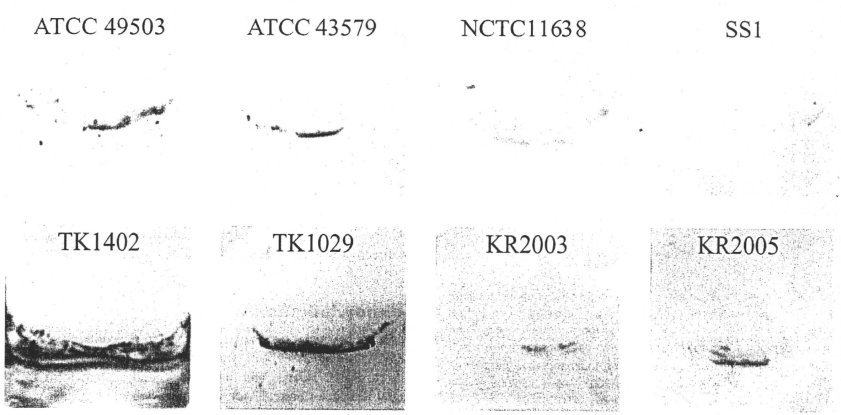

Figure 1 Biofilm formation by eight strains of *Helicobacter pylori* on glass coverslips after 3 days of culture in Brucella-serum broth.

Table 1 Hydrophobicity of eight *Helicobacter pylori* strains

Strain	Hydrophobicity (%)
SS1	66.10 ± 9.89
ATCC 49503	78.45 ± 4.28
ATCC 43579	66.50 ± 5.23
NCTC11638	58.65 ± 14.36
TK1029	66.25 ± 6.42
TK1402	73.95 ± 2.17
KR2003	69.35 ± 6.21
KR2005	72.70 ± 8.79

H. pylori has the ability to form biofilms on abiotic surfaces *in vitro* as well as on the human gastric mucosa.[4–6,12,13] In our previous study, the strain TK1402, which was isolated from a Japanese patient with duodenal and gastric ulcers, had a strong biofilm-forming ability compared with the other seven strains. We also demonstrated that the OMV produced from this strain play an important role in biofilm formation. However, little is known regarding the *H. pylori* biofilm formation. In the present study, we investigated the other factor for the strong biofilm-forming ability in strain TK1402.

Cell surface hydrophobicity is an important factor in bacterial attachment to a glass surface,[11] aggregation and hence biofilm formation. In the present study, the hydrophobicity value for strain TK1402 was of a similar level for all of the strains examined. Further, similar results were observed for the values of auto-aggregation and cell motility between strain TK1402 and the other strains. A high hydrophobic value following increasing auto-aggregation is consistent with enhanced interactions during biofilm formation. Similarly, motility mediated by pili or fimbriae is required for biofilm formation.[14] Our results indicated that these

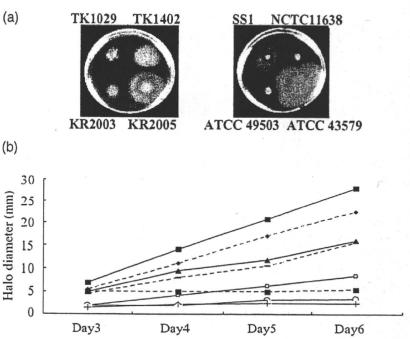

Figure 2 Motility and its kinetics of *Helicobacter pylori* strains. (a) Motility of eight *H. pylori* strains incubated on Brucella-serum semi-solid agar after 6 days culture. (b) Kinetics of motility. The eight strains were cultured on Brucella-serum semi-solid agar at 37°C for 3–6 days and the diameter of each halo was measured. The result is expressed as the mean of at least three independent experiments. —□—, TK1029; —▲—, TK1402; --■--, KR2003; —◆—, KR2005; ——, SS1; —○—, NCTC; —⊣—, ATCC49503; —■—, ATCC43579.

properties might not be responsible for the strong biofilm-forming activity of strain TK1402. However, the most motile strain, ATCC 43579, was not an auto-aggregative strain. Further, the high hydrophobic and aggregative strain, ATCC 49503, was not motile under our experimental conditions. However, all of the values for strain

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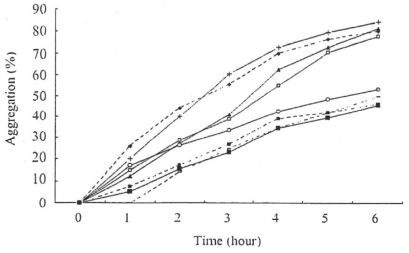

Figure 3 Auto-aggregation assay for the eight strains of *Helicobacter pylori*. The suspensions of each strain were adjusted to an optical density of 1.0 at 600 nm and incubated at room temperature without shaking. The percent auto-aggregation was calculated and the result expressed as the mean of at least three independent experiments. —□—, TK1029; —▲—, TK1402; --■--, KR2003; —◆—, KR2005; ——, SS1; —○—, NCTC; —+—, ATCC49503; —■—, ATCC43579.

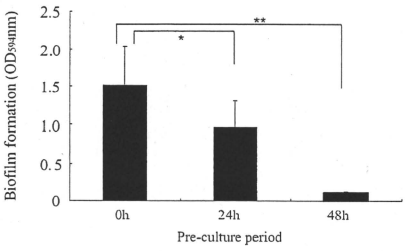

Figure 4 Effect of pre-cultivation on biofilm formation by *Helicobacter pylori* strain TK1402. Strain TK1402 was cultured in a 12-well plate for 0, 24 or 48 h without glass coverslips, and then sterilized glass coverslips were placed into the culture. After an additional 72 h incubation, the biofilm on the glass surface was measured. All results expressed as the mean ± standard deviation from at least three independent experiments. $*P < 0.05$; $**P < 0.005$. OD, optical density.

TK1402 were comparatively high among the strains used. Our results suggest that these factors are not related to the biofilm-forming ability of this strain. The factors in the development of TK1402 biofilm remain to be determined.

In the present study, we found that biofilm development by strain TK1402 required cell growth. When TK1402 cells were grown fully and then formed a biofilm, biofilm formation was significantly decreased. Some other bacterial species form a biofilm in two distinct stages: (i) primary adhesion to the surface by means of adhesins; and (ii) accumulation of multilayered clusters of cells through cell growth and self-production of the extracellular matrix.[15–17] Our present results imply that biofilm formation by *H. pylori* proceeds by the same mechanism.

Strain TK1402 reportedly exhibits virulence in gnotobiotic mice.[18] C57BL mice[8] and Mongolian gerbils.[19] Those reports also indicated that the TK1402 strain had the ability to efficiently colonize the stomach of these animals. Their results and our present findings suggest that colonizing ability might be correlated with strong biofilm-forming ability in the case of strain TK1402. Therefore, we speculate that strong biofilm-forming ability is related to gastric colonization by *H. pylori* in various animals. We suggest that to elucidate the meaning of the biofilm formation in this microorganism may provide important clues for understanding the mechanism involved in the diseases concerning *H. pylori*. Additional research is now in progress to determine what factors are directly involved in biofilm formation by strain TK1402.

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References

1 Hall-Stoodley L, Costerton JW, Stoodley P. Bacterial biofilm: from the nature environment to infectious diseases. *Nat. Rev. Microbiol.* **2**: 95–108.
2 Marshall BJ, Warren JR. Unidentified curved bacilli in the stomach of patients with gastritis and peptic ulceration. *Lancet.* **16**: 1311–15.
3 Mackay WG, Gribbon LT, Barer MR, Reid DC. Biofilms in drinking water systems-a possible reservoir for *Helicobacter pylori*. *Water Sci. Technol.* **38**: 181–5.
4 Stark RM, Gerwig GJ, Pitman RS *et al.* Biofilm formation by *Helicobacter pylori*. *Lett. Appl. Microbiol.* **28**: 121–6.
5 Cole SP, Harwood J, Lee R, She R, Guiney DG. Characterization of monospecies biofilm formation by *Helicobacter pylori*. *J. Bacteriol.* **186**: 3124–32.
6 Cellini L, Grande R, Di Campli E *et al.* Characterization of an *Helicobacter pylori* environmental strain. *J. Appl. Microbiol.* **105**: 761–9.
7 Yonezawa H, Osaki T, Kurata S *et al.* Outer membrane vesicles of *Helicobacter pylori* TK1402 are involved in biofilm formation. *BMC Microbiol.* **9**: 197.
8 Yamaguchi H, Osaki T, Taguchi H, Hanawa T, Yamamoto T, Kamiya S. Flow cytometric analysis of the heat shock protein 60 expressed on the cell surface of *Helicobacter pylori*. *J. Med. Microbiol.* **45**: 270–7.
9 Rosenberg M, Perry A, Bayer EA, Gutnick DL, Rosenberg E, Ofek L. Adherence of *Acinetobacter calcoaceticus* RAG-1 to human epithelial cells and to hexadecane. *Infect. Immun.* **33**: 29–33.
10 Osaki T, Hanawa T, Manzoku T *et al.* Mutation of luxS affects and infectivity of *Helicobacter pylori* in gastric mucosa of a Mongolian gerbil model. *J. Med. Microbiol.* **55**: 1477–85.
11 Peng M, Ping Gong J, Osada Y. Substrate effect on the formation of hydrogels with heterogeneous network structure. *Chem. Rec.* **3**: 40–50.
12 Carron MA, Tran VR, Sugawa C, Coticchia JM. Identification of *Helicobacter pylori* biofilms in human gastric mucosa. *J. Gastrointest. Surg.* **10**: 712–17.
13 Park SR, Mackay WG, Reid DC. *Helicobacter* sp. recovered from drinking water biofilm sampled from a water distribution system. *Water Res.* **35**: 1624–6.

14 Mattick JS. Type IV pili and twitching motility. *Annu. Rev. Microbiol.* **56**: 289–314.
15 Costerton JW, Lewandowski Z, Caldwell DE, Korber DR, Lappin-Scott HM. Microbial biofilms. *Annu. Rev. Microbiol.* **49**: 711–45.
16 Sambanthamoorthy K, Schwartz A, Nagarajan V, Elasri MO. The role of MSA in *Staphylococcus aureus* biofilm formation. *BMC Microbiol.* **168**: 221.
17 Ma L, Conover M, Lu H, Parsek MR, Bayles K, Wozniak DJ. Assembly and development of the *Pseudomonas aeruginosa* biofilm matrix. *PLoS. Path.* **5**: e1000354.
18 Osaki T, Taguchi H, Yamaguchi H, Kamiya S. Detection of *Helicobacter pylori* in fecal samples of gnotobiotic mice infected with *H. pylori* by an immunomagnetic-bead separation technique. *J. Clin. Microbiol.* **36**: 321–3.
19 Nakagawa S, Osaki T, Fujioka Y, Yamaguchi H, Kamiya S. Long-term infection of Mongolian gerbils with *Helicobacter pylori*: microbiological, histopathological, and serological analyses. *Clin. Diagn. Lab. Immunol.* **12**: 347–53.

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ORIGINAL ARTICLE

Analysis of the microflora in the stomach of Mongolian gerbils infected with *Helicobacter pylori*

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Key words
gastric flora, *Helicobacter pylori*, Mongolian gerbil.

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Conflict of interest
No conflict of interest has been declared by the authors.

Abstract

Background and Aims: Mongolian gerbils are frequently used to study *Helicobacter pylori*-induced gastritis and its consequences. The presence of some gastric flora with a suppressive effect on *H. pylori* suggests inhibitory microflora against *H. pylori* infection. The aim of the present study was to analyze the microflora in the stomach of Mongolian gerbils with *H. pylori* infection.

Methods: *H. pylori ureA* was detected by polymerase chain reaction (PCR) in the fecal samples of infected Mongolian gerbils. *H. pylori* was isolated from the gastric mucosa of the gerbils by microaerophilic cultivation. Gastric microflora were isolated by aerobic and anaerobic culture, and the identification of gastric bacterial species was performed by API20E and API20A.

Results: Oral administration of *H. pylori* TK1402 induced colonization and gastric inflammation of the stomach of the Mongolian gerbils. According to the frequency of detection of *H. pylori ureA* in fecal samples, the gerbils were divided into three groups (frequently detected, moderately detected and infrequently detected). According to the analysis of the gastric microflora in the frequently and infrequently detected groups, *Lactobacillus* spp. and *Eubacterium limosum* were isolated from the former and latter group, respectively.

Conclusion: Some gastric flora, such as *Lactobacillus* spp., may inhibit colonization by *H. pylori*.

Introduction

Helicobacter pylori is a Gram-negative microaerobic bacterium that was originally isolated by Marshall and Warren.[1] It colonizes the gastric mucosa of approximately 50% of the world's population and is a major cause of chronic gastritis and peptic ulcers, as well as being a risk factor for gastric adenocarcinoma and mucosa-associated lymphoid tissue lymphoma.[2,3] It has been estimated that *H. pylori* colonization increases the risk of gastric cancer approximately 10-fold.[4] In 1994, a working group of the World Health Organization International Agency for Research on Cancer classified *H. pylori* as a group 1 carcinogen in humans.[5]

There have been many challenges to establishing experimental infection with *H. pylori* in animals, but recent studies have reported an infection model using the Mongolian gerbil.[6,7] Naturally acquired gastritis among gerbils is rare, and natural infection of gerbils with *Helicobacter* species apparently does not occur. Based on these characteristics, Mongolian gerbils are used for analysis of the pathogenesis and carcinogenesis of *H. pylori* infection.

Probiotic bacteria such as *Lactobacillus, Bifidobacterium, Saccharomyces* and *Clostridium butyricum* reportedly inhibit the adhesion and growth of *H. pylori* in *in vitro* and *in vivo* studies.[8–10] In addition, these probiotic bacteria can be used to supplement eradication therapy for patients with *H. pylori* infection, either to increase the eradication rate or to prevent the occurrence of the side-effects of the antimicrobial drugs.[11,12] Based on the results of those studies, it is possible that gastric bacteria might affect the colonization of *H. pylori* in the gastric mucosa.

In the present study, gerbils were divided into groups according to the frequency of detecting of *H. pylori ureA* in fecal specimens and their gastric microflora were investigated.

Methods

Bacterial strain

The *H. pylori* TK1402 strain isolated from gastric biopsy specimens of patients with gastric and duodenal ulcers was used.[13] The TK1402 strain was cultured for 2 days in *Brucella* broth containing 1.5% agar (Difco, Detroit, MI, USA) and 7% horse serum (Sigma, St Louis, MO, USA) (BHS-agar) under microaerobic conditions at 37°C using GasPak jars (Mitsubishi Gas Chemical, Tokyo, Japan) containing 85% N_2, 10% CO_2 and 5% O_2.

Animals

We purchased 5-week-old female Mongolian gerbils (MGS/Sea; specific-pathogen-free; bodyweight 25–35 g) from Kyudou

(Fukuoka, Japan) and they were bred under specific-pathogen-free conditions (room temperature, 23 ± 2°C; relative humidity, 40–60%; 12:12-h light : dark cycle) in the animal facility of Kyorin University. They had free access to standard rodent food pellets (CE-2, 30 Gr irradiated; Clea Japan, Tokyo, Japan), and sterilized tap water was provided ad libitum in micro-isolator units as described before.[14] The experiments were approved by the Experimental Animal Ethics Committee of Kyorin University School of Medicine on 1 April 2008 (approval no. 75).

Infection of Mongolian gerbil with *H. pylori*

Eleven Mongolian gerbils were orally inoculated with *H. pylori* TK1402 strain. After incubation, *H. pylori* TK1402 were harvested in Hanks' balanced salt solution (HBSS) (Sigma) and 1 mL of bacterial suspension containing 1×10^7 colony-forming units (CFU) was inoculated once p.o. in 11 Mongolian gerbils.

Detection of *H. pylori ureA* by polymerase chain reaction (PCR) in fecal specimens

After inoculation of *H. pylori* TK1402, fecal samples were collected at 1-week intervals and preserved at −80°C until use in PCR. To extract DNA, we used the bead-phenol method as described by Matsuki *et al.*[15] Fecal samples were suspended in 500 μL of HBSS, then 200 μL of the suspension was added to a solution containing 250 μL of extraction buffer (200 mmol/L Tris-HCl, 80 mmol/L ethylene diamine tetra acetate [EDTA]; pH 9.0) and 50 μL of 10% sodium dodecylsulfate. Next, 300 mg of the glass beads (diameter, 0.1 mm) and 500 μL of buffer-saturated phenol were added to the suspension, and the mixture was vortexed vigorously at 4200 rpm for 30 s using a Mini Bead Beader (Wakenyaku , Kyoto, Japan). After centrifugation at 14 000*g* for 5 min, 400 μL of the supernatant were collected for phenol–chloroform extractions, and 250 μL of the supernatant were subjected to isopropanol precipitation. Finally, the DNA was suspended in 1 mL of Tris-EDTA buffer. We used the primer set of 2F2 (5′-ATATTATGGAAGAAGCGAGAGC-3′), 2R (5′-ATGG AAGTGTGAGCCGATTTG-3′) and 2F3 (5′-CATGAAGTG GGTATTGAAGC-3′) to detect the *ureA* gene. The primers of 2F2 and 2R were used for the first round of PCR, and those of 2R and 2F3 for the second PCR (nested PCR). Genomic DNA (1 μL) extracted using a MagExtractor (Toyobo, Osaka Japan) was mixed with each primer (5 pmol) and 0.5 U *Taq* polymerase in a final volume of 20 ± 1 μL. The nested PCR proceeded for 30 cycles at 94°C for 1 min, 94°C for 1 min, 52°C for 1 min and 72°C for 1 min using a thermal cycler (GeneAmp PCR system 9600-R; Perkin-Elmer Japan, Yokohama, Japan).

Isolation of *H. pylori* from gastric mucosa

After killing the gerbils 8 weeks after the inoculation of *H. pylori* TK 1402, their stomachs were dissected along the greater curvature and the contents emptied so the organ could be divided into two parts. One half of the stomach, excluding the forestomach was scraped off with a spatula, collected in 500 μL HBSS and homogenized for determination of the number of the microorganisms in the mucus layer. *H. pylori*-selective medium (Nissui Pharmaceutical, Tokyo, Japan) was inoculated with 50 μL of the gastric sample and incubated at 37°C for 5 days. Purple colonies were counted, and the number of viable *H. pylori* cells was expressed as the number of CFU/g of gastric mucus. Brucella agar medium supplemented with 7% horse serum was inoculated with a single colony for identification of the bacteria. The isolated strain was shown to be positive for urease, catalase and oxidase with a Gram-negative helical form and thus identified as *H. pylori*.

Differentiation of *H. pylori*-positive and -negative gerbil according to frequency of its detection by PCR

On the basis of the frequency of the PCR, two gerbils were selected as the *H. pylori*-positive one (gerbil no. 5) from the frequently detected group and as the *H. pylori*-negative one (gerbil no. 4) from the infrequently detected group. It was necessary to compare the gastric flora of the two gerbils differentiated as the highly infected one (1.3×10^4 CFU/g mucus) and as the non-infected one ($<2.0 \times 10^2$ CFU/g mucus) according to the high density of *H. pylori* and the low density of *H. pylori*, respectively.

Analysis of gastric flora

To isolate the dominant bacterial species from each gerbil, the gastric specimen and 10-fold dilutions of the suspension were inoculated on Gifu anaerobic medium (GAM) agar (Nissui Pharmaceutical), phenyl ethyl alcohol (PEA)-blood agar (Nissui Pharmaceutical) supplemented with 5% horse blood and DHL agar (Nissui Pharmaceutical). GAM agar and PEA-blood agar were incubated under anaerobic conditions at 37°C in an Anaero incubator (Hirasawa, Tokyo, Japan) for 48 h. DHL and PEA agar were aerobically incubated at 37°C for 24 h. After incubation, each different type of colony was inoculated for single colony isolation and incubated under the same conditions. These isolates were identified using API 20E (a commercially available kit used for the identification of *Enterobacteriacae* and some other Gram-negative bacteria, and incubation period is 18–24 h) and API20A (a commercially available kit used for the identification of anaerobes, and incubation time is 24 h) (SYSMEX bioMerieux, Tokyo, Japan).

Results

Detection of *H. pylori ureA* in fecal samples and isolation of *H. pylori* from gastric mucosa

In three gerbils (nos. 2, 5 and 11), *H. pylori ureA* was detected in four or five of six fecal specimens (i.e. frequently detected group) (Table 1). In contrast, *H. pylori ureA* was infrequently detected in four gerbils (nos. 1, 3, 4 and 6) in zero to one of six fecal specimens (i.e. infrequently detected group). In four gerbils (nos. 7–10), the detection rate of *H. pylori ureA* was moderate (moderately detected group). Figure 1 shows a representative result of the detection of *H. pylori ureA* by PCR in fecal specimens obtained at 1 week after infection. Amplified DNA bands were detected in the fecal samples of gerbil numbers 4 and 7–11. There was no significant difference among the three groups in the number of *H. pylori* isolated from the gastric mucosa. In gerbil numbers 4 and 9, *H. pylori* was not isolated.

Table 1 Detection of *Helicobacter pylori ureA* by polymerase chain reaction in fecal samples and isolation of *H. pylori* from gastric mucosa by cultivation

Group	Gerbil no.	PCR* (no. of positive/total)	No. of *H. pylori* (CFU/g mucus)
Frequently detected	2	4/6	8.8×10^5
	5	5/6	1.3×10^4
	11	5/6	5.4×10^5
Moderately detected	7	3/6	1.2×10^3
	8	3/6	2.0×10^3
	9	3/6	$<2.0 \times 10^2$
	10	3/6	4.0×10^2
Infrequently detected	1	1/6	2.0×10^3
	3	1/6	9.6×10^2
	4	1/6	$<2.0 \times 10^2$
	6	0/6	8.6×10^2

*Fecal samples were used for polymerase chain reaction (PCR).
CFU, colony-forming units.

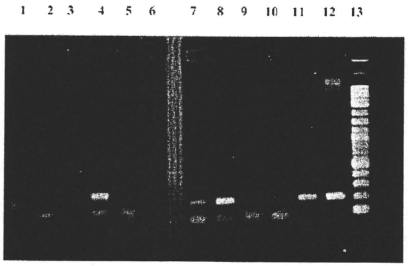

Figure 1 Detection of *Helicobacter pylori ureA* by polymerase chain reaction (PCR) in fecal samples. At 1 week after *H. pylori* infection, fecal samples obtained from gerbil numbers 1–11 were used for DNA extraction followed by PCR. Lanes 1-11, fecal samples of gerbil numbers 1–11; lane 12, positive control; lane 13, molecular weight marker.

Isolation and identification of the gastric flora of Mongolian gerbils

Gastric bacteria were isolated from the gastric mucosa of gerbil number 4 (infrequently detected group) and 5 (frequently detected group), and identification of the isolated gastric bacteria was performed using API 20E and API 20A (Table 2). The same facultative anaerobic bacterial species (*Escherichia coli* and *Kluyvera* spp.) were identified in both gerbils. However, different obligate anaerobic bacterial species were identified: *Lactobacillus* spp. was isolated from the gastric mucosa of gerbil number 4 and *Eubacterium limosum* was isolated from gerbil number 5. One other different anaerobic bacterial species was isolated from both gerbils and it was difficult to identify the species: the API 20A system indicated the possibility of *Actinomyces* spp. or *Bifidobacterium* spp. with a percentage identification of 51.1% or 48.7%, respectively.

Table 2 Isolation and identification of the gastric bacterial species in two Mongolian gerbils

Group	Gerbil no.	Facultative anaerobes*	Obligate anaerobes*
Infrequently detected	4	*Escherichia coli* *Kluyvera* spp.	*Lactobacillus* spp. *Actinomyces* spp. *Bifidobacterium* spp.†
Frequently detected	5	*Escherichia coli* *Kluyvera* spp.	*Eubacterium limosum* *Actinomyces* spp. *Bifidobacterium* spp.†

*Bacterial species were identified by API 20E and API20A.
†Identification of *Actinomyces* and *Bifidobacterium* was 51.1% and 48.7%, respectively.

Discussion

Bik *et al.*[16] reported the molecular analysis of the bacterial microbiota in the human stomach with or without *H. pylori* infection. In their study, genomic analysis of the gastric flora using 23 gastric biopsy specimens obtained from 12 *H. pylori*-positive and 11 *H. pylori*-negative adults was performed by a molecular method using the 16SrRNA clone library. Interestingly, *H. pylori* was isolated by cultivation in 12 of 23 cases, but the recombinant DNA of *H. pylori* was detected in 19 of 23 cases. There was no significant difference between the *H. pylori*-positive and -negative cases in the profile of the gastric microbiota, suggesting that the gastric flora will not affect the persistence of *H. pylori* in the gastric mucosa. Sun *et al.*[17] reported the profiling and identification of Eubacteria in the stomach of Mongolian gerbils with and without *H. pylori* infection. They showed that *Lactobacillus* spp. was the dominant bacteria in the stomach of *H. pylori*-infected, as well as control, gerbils. They also demonstrated that *Lactobacillus gasseri* and *Lactobacillus reuteri*, which are present in the stomach of most Mongolian gerbils, inhibited the growth of some *H. pylori* strains. Based on their results, it is possible that indigenous bacteria such as lactobacilli may have an effect on the colonization and growth of *H. pylori* strains in the stomach of Mongolian gerbils.

In this study, the same strain of *H. pylori*, TK1402, as Nakagawa *et al.* used[18] was selected for the inoculation to gerbils, but the comparable level of serum anti-*H. pylori* immunoglobulin (Ig) titer was not detected 8 weeks after the inoculation. However, Suzuki *et al.*[19] used a different strain for the inoculation in Mongolian gerbils. In their study, they observed the comparable level of serum anti-*H. pylori* 6 or 12 weeks after the inoculation. The tissue myeloperoxidase (MPO) activity was significantly elevated within 6 weeks and additionally increased at 12 weeks and this parameter was totally paralleled with the histological score for neutrophil infiltration. It is necessary to study preventive microflora using tissue MPO activity and IgG,[20] but we checked neither IgG level nor the tissue MPO activity to determine the status of inflammation in our study. We need to check both the tissue MPO activity and the IgG level in our future studies.

In the present study, the rate of detecting *H. pylori ureA* in fecal samples of Mongolian gerbils after p.o. *H. pylori* inoculation was compared. According to the frequency of detection, the gerbils were divided into three groups (frequently, moderately and infre-

quently detected groups). We suspected that some gastric bacteria may inhibit persistent infection of *H. pylori*, and so the gastric bacterial microflora isolated from the gastric mucosa of the frequently and infrequently detected groups were analyzed and compared. *Lactobacillus* spp. were isolated from the gastric mucosa of the infrequently detected group, suggesting the possibility of an inhibitory effect by this species of gastric bacteria on colonization by *H. pylori*. Further study examining the direct effect of the isolated *Lactobacillus* strain on the colonization of *H. pylori* in the stomach of the Mongolian gerbil remains to be done.

Acknowledgments

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References

1 Marshall BJ, Warren JR. Unidentified curved bacilli in the stomach of patients with gastritis and peptic ulceration. *Lancet* 1984; **i**: 1311–15.
2 Moss SF, Blaser MJ. Mechanisms of disease: inflammation and the origins of cancer. *Nat. Clin. Proct. Oncol.* 2005; **2**: 90–7.
3 Suzuki H, Iwasaki E, Hibi T. *Helicobacter pylori* and gastric cancer. *Gastr. Cancer* 2009; **12**: 79–87.
4 Suzuki H, Hibi T, Marshall BJ. *Helicobacter pylori*: present status and future prospects in Japan. *J. Gastroenterol.* 2007; **42**: 1–15.
5 Asaka M, Kudo M, Kato M, Sugiyama T, Takeda M. Long-term *Helicobacter pylori* infection: from gastritis to gastric cancer [Review Article]. *Aliment. Pharmacol. Ther.* 1998; **12** (Suppl 1): 9–15.
6 Tatematsu M, Tsukamoto T, Toyoda T. Effects of eradication of *Helicobacter pylori* on gastric carcinogenesis in experimental models. *J. Gastroenterol.* 2007; **42** (Suppl 17): 7–9.
7 Peek RM. *Helicobacter pylori* infection and disease: from humans to animal models. *Dis. Model. Mech.* 2008; **1**: 50–5.
8 Kabir AM, Aiba Y, Takagi A, Kamiya S, Miwa T, Koga Y. Prevention of *Helicobacter pylori* by lactobacilli in a gnotobiotic murine model. *Gut* 1997; **41**: 49–55.
9 Johnson-Henry KC, Mitchell DJ, Avitzur Y, Galindo-Mata E, Jones NL, Sherman PM. Probiotics reduce bacterial colonization and gastric inflammation in *H. pylori*-infected mice. *Dig. Dis. Sci.* 2004; **49**: 1095–102.
10 Takahashi M, Taguchi H, Yamaguchi H, Osaki T, Kamiya S. Studies of the effect of *Clostridium butyricum* on *Helicobacter pylori* in several test models including gnotobiotic mice. *J. Med. Microbiol.* 2000; **49**: 635–42.
11 Wang KY, Li SN, Liu CS *et al.* Effects of ingesting *Lactobacillus*- and *Bifidobacterium*-containing yogurt. I: subjects with colonized *Helicobacter pylori*. *Am. J. Clin. Nutr.* 2004; **80**: 737–41.
12 Tursi A, Brandimarte G, Giogetti GM, Modeo ME. Effect of *Lactobacillus casei* supplementation on the effectiveness and tolerability of a new second-line 10-day quadruple therapy after failure of a first attempt to cure *Helicobacter pylori* infection. *Med. Sci. Monit.* 2004; **10**: 662–6.
13 Osaki T, Hanawa T, Manzoku T *et al.* Mutation of luxS affects motility and infectivity of *Helicobacter pylori* in gastric mucosa of a Mongolian gerbil model. *J. Med. Microbiol.* 2006; **55**: 1477–85.
14 Nakagawa S, Osaki T, Fujioka Y, Yamaguchi H, Kamiya S. Long-term infection of Mongolian gerbil with *Helicobcter pylori*: microbiological, histopathological, and serological analyses. *Clin. Diagn. Lab. Immunol.* 2005; **12**: 347–53.
15 Matsuki T, Watanabe K, Fujimoto J, Takada T, Tanaka R. Use of 16S rRNA gene-targeted group-specific primers for real-time PCR analysis of predominant bacteria in human feces. *Appl. Environ. Microbiol.* 2004; **70**: 7220–8.
16 Bik EM, Eckburg PB, Gill SR *et al.* Molecular analysis of the bacterial microbiota in the human stomach. *Proc. Natl Acad. Sci. USA* 2006; **103**: 732–7.
17 Sun Y-Q, Monstein H-J, Nilsson LE, Petersson F, Borch K. Profiling and identification of eubacteria in the stomach of Mongolian gerbils with and without *Helicobacter pylori* infection. *Helicobacter* 2003; **8**: 149–57.
18 Nakagawa S, Takako O, Fujioka Y, Yamaguchi H, Kamiya S. Long-term infection of Mongolian gerbils with *Helicobacter pylori*: Microbiological, histopathological, and serological analyses. *Clin. Diag. Lab. Immunol.* 2005; **12**: 347–35.
19 Suzuki H, Mori M, Seto K *et al. Helicobacter pylori*-associated gastric pro- and antioxidant formation in Mongolian gerbils. *Free. Radic. Biol. Med.* 1999; **26**: 679–84.
20 Nomura S, Suzuki H, Masaoka T *et al.* Effect of dietary anti-urease immunoglobulin Y on *Helicobacter pylori* infection in Mongolian gerbils. *Helicobacter* 2005; **10**: 43–52.

Mutual correlation between gastric flora and *Helicobacter pylori* in gastric mucosa of Mongolian gerbil

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Mutual correlation between gastric flora and *Helicobacter pylori* in gastric mucosa of Mongolian gerbil

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To compare the reciprocal correlation between gastric flora and *H. pylori* in gastric mucosa of Mongolian gerbil, gastric microflora of Mongolian gerbil were analyzed at first after infected with *H. pylori* T.K 1402.On the basis of the results of PCR of the extracted DNA from the fecal samples, *H. pylori* positive and *H. pylori* negative gerbils were differentiated whose gastric samples were used in the next experiment. *Actinomyces* spp., *Lactobacillus* spp. and *Bifidobacterium* spp. in *H. pylori* negative gerbil and *Actinomyces* israeli and *Eubacterium limosum* were identified. In this result, the gastric flora of the *H. pylori* (-) gerbil [No.4] were considered as the suitable microflora against the *H. pylori* colonization and so using the gastric sample of that gerbil in the next experiment, the mutual correlation between gastric flora and *H. pylori* were analyzed. *H. pylori* (-) by the real time-PCR among 5 *H. pylori* (-) gerbils which were clarified. The *Lactobacillus* spp. was also clarified by the real time-PCR among all of the gerbils.

Keywords: *H. pylori,* gastric flora, Mongolian gerbil

I Objective

To find out the mutual correlation between gastric flora and *Helicobacter pylori*, microflora were analyzed in *H. pylori* infected Mongolian gerbil's stomach and also their interaction with *H. pylori* was observed.

II Materials and Methods

Twelve Mongolian gerbils (5 wks old) were inoculated with *H. pylori* (1.04×10^7 cfu). Nested PCR using *ureA* gene primer was done using extracted DNA from collected fecal samples. Gastric mucus samples were obtained from gerbils sacrificing after eight wks of inoculation for the detection of *H. pylori* culture and PCR.On the basis of the results of PCR , gerbils were selected as high frequency, variable and low frequency groups and according to the highest and the lowest density of *H. pylori, H. pylori* positive and *H. pylori* negative gerbils were differentiated.

The gastric samples of the *H. pylori* (+) gerbil and the *H. pylori* (-) gerbil were inoculated into another twelve Mongolian gerbils (5 wks old) dividing them into two groups. One wk after this inoculation, all of the gerbils were inoculated with *H. pylori* ($1x\ 10^9$ cfu) twice. Bacterial numbers of gastric flora and *H. pylori* were compared between two groups.

III Results and discussion

After using the extracted DNA of the gastric samples, gerbil (No.5) was shown as positive in the result of nested PCR (Table 1). It was shown that in high frequency group number of *H. pylori* in gastric sample was higher than other groups. It was necessary to compare the stomach flora between gerbil No.4 (low frequency group) and No.5 (high frequency group) as *H. pylori* negative and positive. In this result, in the stomach of No. 4 and No. 5 gerbils, same aerobic bacteria (*E. coli* and *Kluyvera* spp.) were found. However, different species of anaerobes (*Lactobacillus* spp., *Actinomyces* spp. / *Bifidobacterium* spp. in gerbil No. 4 and *Actinomyces israeli* and *Eubacterium limosum* in gerbil No. 5) were found respectively.

In this result, the gastric flora of the *H. pylori* (-) gerbil [No.4] were considered as the suitable

Table 1. Isolation of *H. pylori* in fecal and gastric samples

Term	Gerbils No	PCR* (No. of positive/Total)	No. of *H. pylori* CFU / g mucus
High frequency	#2	4/6	8.8×10^2
	#5	5/6	1.3×10^4
	#11	5/6	5.4×10^3
Variable	#7	3/6	1.2×10^3
	#8	3/6	2.0×10^3
	#9	3/6	<200
	#10	3/6	4.0×10^3
Low frequency	#1	1/6	2.0×10^3
	#3	1/6	9.6×10^2
	#4	1/6	$<2.0\times10^3$
	#6	0/6	8.6×10^2

*Fecal samples were used for PCR

Table 2. Detection of *H. pylori* and *Lactobacillus* spp. by real time PCR in both *H. pylori* (+) and *H. pylori* (-) groups

	H. pylori (-) group					*H. pylori* (+) group					
Gerbil No	1	2	3	4	5	6	7	8	9	10	11
pH	2.5	3.0	3.0	3.0	3.0	3.0	3.0	4.5	4.5	4.5	3.0
No. of *H. pylori* (Real time PCR)	5.31	(-)	4.31	(-)	(-)	-/4.00	9.56	4.42	4.54	(-)	4.79
No. of *Lactobacillus* spp./g mucus	10.58	12.18	10.76	(-)	10.80	8.78	10.65	11.40	10.97	11.18	9.77

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特集

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Helicobacter 研究の年間レビュー

感染ルートはどこまで明らかになったか

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2009年に報告された *Helicobacter pylori*（*H. pylori*）の感染ルートに関する研究を中心にレビューした．ヒト-ヒト感染が主であり，家族内感染，とくに母-子感染が重要であることはこれまでも多くの報告があり確定的な感染ルートのひとつと考えられる．唾液や扁桃，嘔吐物から *H. pylori* が検出されたという報告も集積しており，口-口感染や嘔吐物を介した感染経路も重要であろう．一方，疫学研究では水からの感染を支持する報告が多いが，生活水や飲料水の解析では *H. pylori* を検出するのは困難であるとの報告も多く今後の検討課題である．新生児期に便から *H. pylori* が検出されるという報告も散見され，産道感染について大いに関心があるが，持続感染したという報告はほとんどなく，新生児ではなぜ定着しないのか“謎”のひとつである．

KEY WORDS
***Helicobacter pylori*，感染経路，新生児**

はじめに

Helicobacter pylori（*H. pylori*）感染ルートは少しずつ解明されてきたが，予防策を講じるのはいまだ困難である．本稿では2009年に報告された感染ルートに関する論文を中心に関連する過去の論文も含めて紹介する．

1．家族内感染

家族内感染ではとくに母-子がおもな感染経路であるということは多くの論文が示している．Weyermann[1]らはドイツの検討で母，父，同胞について個別に検討したところ，感染している母親が子供への主たる感染源となっていることを疫学的に示した．家族内を PCR-based random amplified polymorphic DNA fingerprinting（RAPD）法で検討したバングラデシュの報告[2]でも母子の菌のタイプが一致し，母子感染がおもな感染経路であると報告した．Konno ら[3]も日本の家族の検討で *H. pylori* 感染小児の約70%は母親の菌株と一致していることを示しており，現在の感染経路として母から子への感染が重要であることは明らかである．スナネズミで母子感染について検討した報告[4]であるが，4週齢のメスのスナネズミに *H. pylori* を感染させ，2ヵ月後に感染してい

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表❶ 生後1ヵ月以下の便中抗原陽性率の報告

報告者	検査時期	対象数	便中抗原陽性（%）	PCR法陽性（%）	備考
Baldassarre ME ら[8]	生後1ヵ月	172	5（2.9）	施行せず	陽性5名中3名の母親は *H. pylori* 抗体陰性 追跡では全例便中抗原陰性となった
Stray-Pedersen A ら[9]	生後7日未満	69	36（52.2）		陽性のうち26名にPCR法施行し，陽性9名（35%）
	生後7日～1ヵ月未満	46	7（15.2）		
Fujimura S ら[10]	生後3日	50	1（2.0）	15（30）	PCR法陽性15名のうち8名を24ヵ月で追跡したが陰転

ないオスとつがいにした．生まれた仔スナネズミは生後1週から1週間ごとに検討し，胃を矢状断で二分し培養，RT-PCR法，その他の免疫染色に供した．また血清と胃内にある母乳中の抗 *H. pylori* 抗体を測定した．仔の *H. pylori* は生後4週目から検出されるようになった．抗 *H. pylori* 抗体は生後3週で最高値となり，急激に低下した．これらの結果から，スナネズミの仔は感染している母から糞-口感染したことが示唆された．この動物実験では産道感染はしなかったということ，抗体が低下したころに感染しており，母乳中の抗体などの予防効果のため感染時期が遅くなったと推察できる．

夫婦間感染経路に関する報告はさまざまである．十二指腸潰瘍患者のパートナーの検討では44%が同じ菌株を保有していた[5]，Indexとなった子供の14組の両親をRAPD法で検討したところ，6組（42%）の両親の菌株が一致していた[3]などがある一方，夫婦とも陽性の21組で一致したのはわずか1組という報告[6]もある．Dasanu ら[7]はあいついで胃癌が発症したルーマニアから移住した夫婦を報告した．まず妻に胃癌がみつかり，3年後，55歳の夫も胃癌と診断された．ともに *H. pylori* 関連の活動性胃炎があり，胃癌患者の家族は *H. pylori* を共有するため同じ疾患の発症のリスクとなりうる．このような家族では発癌前に *H. pylori* 感染診断と除菌治療をおこなうことが適切であると述べている．

2．新生時期，乳児期早期の感染（表❶）

家族外，医療スタッフからの感染の可能性を示唆する周産期感染を検討したイタリアからの報告[8]である．180名の母親から出生した小児を対象とし，母親は産後4日までに血清抗体あるいは便中抗原を検査し，陽性のものは尿素呼気試験（UBT）で確認した．出生児は生後1，6，12，18ヵ月に検査した．同時に新生児室のスタッフの *H. pylori* 感染状況も検討した．母親の感染率は34.4%，スタッフは34.8%であった．生後1ヵ月で172名中5名（2.9%）が便中抗原陽性となったが，3名の母親は *H. pylori* が陰性であった．人工乳と中間リスクの新生児ユニットへの入院が感染のリスク因子となった．追跡では5名ともに自然消失（便中抗原陰性化）した．新生時期に便中抗原あるいはPCR法で *H. pylori* が検出された報告が散見される．ノルウェーの新生児の検討[9]では，249名の乳幼児の *H. pylori* 便中抗原を検討し，生後7日未満の児は69名中36名（52.2%）が便中抗原陽性であったが，生後1ヵ月以降は3.7%と低くなり，新生時期は一過性の感染が多いと述べられている．生後7日未満の新生児では経腟分娩で陽性率58.8%，帝王切開では10%であった．Fujimura ら[10]は生後3日目の新生児50名の便を検討したところ15名（30%）でPCR法の *H. pylori* DNAが陽性であったが，便中抗原陽性はわずか1名であった．母親の尿中 *H. pylori* 抗体が陽性であると児の *H. pylori* DNA陽性率が高くなったが母親の抗体が陰性でも陽性者がいた．陽性15名のうち生後24ヵ月時に8名の検査をおこなったが，PCR法，便中抗原ともに陰性であった．新生時期早期に *H. pylori* が検出されることは産道での曝露あるいはスタッフからの感染などが示唆されるが，いずれの報告でも一過性の検出であり持続感染にならないのは“謎”である．前出のスナネズミの実験[4]でも生後早期には定着しておらず，母親からの抗体による防御も考えられるが，イタリアからの報告[8]

では *H. pylori* 陰性の母親から出生したにもかかわらず便中抗原陽性が確認された乳児でも定着せずに後に消失していた．ただし，注意すべきは乳児における便中抗原の有用性で，UBT が施行できず，*H. pylori* 抗体も有用性が低いのでスタンダードが取れないため十分な検討がされていないことである．感染ルート研究のためにも生後早期の乳児における便中抗原の精度を検証する必要性を感じる．われわれはポリクロナール抗体を用いた便中抗原は乳児で偽陽性になりやすいという印象をもっている．

3．感染時期

イスラエルの保育所で便中 *H. pylori* 抗原を検討した[11]ものであるが，対象となったのは 316 名でこのうち 24.7%が便中抗原陽性であった．生後 3〜12 ヵ月は 98 名中陽性はわずか 7 名（7.1%）で，13〜60 ヵ月は 218 名中陽性 71 名で陽性率は 32.5%であり，多くの感染は 1 歳以降に生じると報告している．一般的に途上国は先進国より抗体陽転年齢が低い[12]と報告されているが，この検討ではドイツの便中抗原陽転時期[13]と類似していた．日本の報告[14)15)]からは 5 歳ごろまでの感染が多いと考えている．

4．感染を媒介するもの

1）耳鼻咽喉科，口腔外科領域から

耳鼻咽喉，口腔領域からの *H. pylori* 感染に関する報告が近年多く出されており，感染経路として，また病態に関与するものとして興味深い．わが国の検討[16]であるが，くり返す扁桃炎または IgA 腎症で扁桃摘出した 55 名について扁桃からの *H. pylori* 検出を試みた．55 名中 43 名（78.2%）で扁桃に *H. pylori* が存在した．15 名（27.3%）は胃にも *H. pylori* が存在し，全員扁桃でも検出された．CagA は 43扁桃中 38（88.4%）で陽性であった．扁桃からは *H. pylori* の培養は成功しなかった．IgA 腎症のあるすべての患者に扁桃で *H. pylori* が検出され，*H. pylori* が IgA 腎症の原因になっていることが示唆された．扁桃に *H. pylori* が存在することから口-口感染を支持する結果であるともいえる．口腔内に *H. pylori* が存在するかどうか，消化器症状がある患者とないもので検討した報告[17]であるが，対象は 98 名でこのうち有症状者は 43 名であった．43 名は内視鏡をおこない胃生検し，98 名全員が歯垢と唾液を採取した．胃生検組織は鏡検染色法で *H. pylori* を，歯垢と唾液は PCR 法で *H. pylori* を検出した．98 名のうち歯垢または唾液で *H. pylori* が検出されたのは 18 名（18.4%），有症状者 43 名のうち 38 名（88.4%）は胃組織で *H. pylori* 陽性であった．口腔内で *H. pylori* が検出されたのは有症状者が 15 名（34.9%）であり，有症状の口腔内では消化器症状のないものと比較して有意に *H. pylori* が多く検出され，口-口感染を支持する結果である．耳鼻咽喉科領域では中耳の浸出液中から PCR 法などで *H. pylori* を検出したという報告もある[18)19)]．

2）感染性胃腸炎（嘔吐・下痢）との関連

新たな *H. pylori* 感染は胃腸炎の流行後に多く生じるという報告[20]であるが，カリフォルニアの 1,752 名の非感染者を 1 年間追跡し，30 名が *H. pylori* 感染であった．全体の新規感染率は 7%/年であったが，新たに感染した多くは 2 歳未満の乳幼児で感染率は 21%/年であった．感染している家族が嘔吐を伴う胃腸炎であるとオッズ比は 6.3 で，下痢だけの症状では 3.0 であった．バングラデシュ，ダッカで 28 名の急性胃腸炎患者の嘔吐物，下痢便から real-time PCR 法と便中 *H. pylori* 抗原測定法を用いて *H. pylori* の分離を試みた[21]．嘔吐物では 26 名のうち 23 名（88%）で *H. pylori* が PCR 法で陽性となり，便では 23 名のうち 17 名（74%）で陽性であり，嘔吐物や下痢便は感染源になる可能性が示唆された．いずれの検体でも培養は成功しなかった．

よく引用される報告であるが，Parsonnet ら[22]は 16 名の無症状の *H. pylori* 感染者を対象とし，下剤，催吐剤投与前後の便，唾液，嘔吐物の *H. pylori* 培養をおこなった．感染者の前の唾液からは 18.8%（3/16）で少量の *H. pylori* が培養されたが，催吐剤で嘔吐後の唾液からは 56.3%と高率に培養でき，下痢便からも培養が可能であった．*H. pylori* 感染者が嘔吐・下痢を伴う病態では感染源となりうることが示唆される．嘔吐物から培養が成功した報告はほかにもあり，感染源として重要であると考える．

5．感染率・感染に関与する因子

社会経済状況や教育レベル，所得が低いと感染リスクになるという報告が多かった．マレーシアの健康な血液ドナー（10～70 歳）の血清抗 *H. pylori* 抗体陽性率は 14.2%で，民族，性別，血液型には関連がなかった[23]．バングラデシュの検討[24]では出生児 238 名を 2 歳まで定期的に測定した便中抗原の累積陽性率は 49%であり，血液型では A 型における *H. pylori* 感染率が有意に高い結果であった．また，初感染は春と秋に多かった．ギリシアにおける検討[25]であるが，上部消化管症状があり，除菌治療歴のない連続した 100 名の小児で内視鏡検査と UBT をおこなった．同様に，除菌治療の有無に関係なくすべての家族についても UBT をおこなった．子どもと親の年齢，社会経済状況，親の教育歴，住居環境の要因についても調査した．44 名（44%）の有症状小児で *H. pylori* 感染があったが，調査した要因は年齢を除いて（*H. pylori* 感染児で有意に年齢が高い）関連するものはなかった．すべての *H. pylori* 感染児，非感染児の 71.4%で家族の少なくとも 1 名は *H. pylori* 感染者であった．中国北京にある田舎（Pinggu）と都会（Haldian）の *H. pylori* 感染率を検討した報告[26]であるが，1,232 名が参加し，感染診断は UBT を用いた．同時に性別，年齢，居住地，教育レベル，喫煙，アルコール消費を評価した．田舎での感染率は 54.7%，都会では 41.3%であった．居住地域と低い教育レベル，低所得が *H. pylori* 感染のリスク因子であった．

Lanyu 島に居住している Yami 部族（台湾でもっとも小さい先住民部族）に関する報告であるが，原始的な台湾の最も小さな先住民部族である．Lanyu 島は閉鎖した環境であり，その地域の *H. pylori* 感染状況に関する情報はほとんどない．Chi ら[27]は Lanyu 島における高校生の感染率を調査し，リスクファクターと成長への影響を検討した．横断的，population-based 研究は UBT を用いて実施された．社会経済状況などの要因を感染のリスクファクターとして収集し，身長・体重の情報も収集した．106 名の高校生（平均年齢 14.3 歳，男児 55 名）が登録され，全体の感染率は 54.7%であった．Dongcing 村の居住者の感染率が最も高く，73.3%であった．性別，人種，社会経済状況，親の教育レベルと感染には関連がなかった．*H. pylori* 感染と体重，身長，BMI には関連がなかった．*H. pylori* 感染と成長に関してはもう 1 編メキシコからの報告がある[28]が，社会経済レベルの低い地域の全寮制学校における UBT を用いた横断的検討である．対象は 5～13 歳の小児 685 名で *H. pylori* 感染のある小児では感染のない小児より平均身長が低く，成長率も悪く，報告者は *H. pylori* 感染が小児の成長抑制に影響している可能性が示唆されたと述べている．

6．水からの感染

水からの感染はとくに途上国では *H. pylori* のおもな感染経路のひとつであると報告されている．バングラデシュの感染率が高い地域の水について *H. pylori* 特異的 real-time PCR 法を用いて水中の *H. pylori* DNA を定量化する検討をおこなった報告[29]で，最小検出感度は 1 検体あたり *H. pylori* 遺伝子およそ 250 ゲノムである．対象は *H. pylori* 感染率が高いバングラデシュの地域の飲用・生活水は 75 検体，天然水バイオフィルム 21 検体であるが，どのサンプルからも *H. pylori* は検出できなかった．同じ方法で以前に他の細菌が検出できた検体もあったが，*H. pylori* は検出できなかった．コントロール実験も施行しており，実験結果が偽陰性であるとは考えにくく，この結果は少なくともバングラデシュの主たる *H. pylori* 感染経路が水であるという可能性は低いことを示唆している．下水に曝露される職業に従事する者の *H. pylori* と E 型肝炎感染のリスクを検討したスイスからの報告[30]もある．下水に曝露される労働者 332 名，コントロールは市中の労働者 446 名で *H. pylori* 抗体，E 型肝炎抗体を前向き調査した．陽転は *H. pylori* 抗体，E 型肝炎抗体ともに両群で差はなく，下水に曝露される職業がこれらの感染のリスクになることはなく，良い衛生環境で個人防御を訓練されていれば感染のリスクとはならないと述べている．

おわりに

感染ルートは少しずつ明らかになっているが，蓄積し

た成果から実際の予防に応用していく検討が今後の課題と考える.

文 献

1) Weyermann M, Rothenbacher D, Brenner H : Acquisition of *Helicobacter pylori* infection in early childhood : independent contributions of infected mothers, fathers, and siblings. *Am J Gastroenterol* **104** : 182-189, 2009
2) Nahar S, Kibria KM, Sultana J *et al* : Evidence of intra-familial transmission of *Helicobacter pylori* by PCR-based RAPD fingerprinting in Bangladesh. *Eur J Clin Microbiol Infect Dis* **28** : 767-763, 2009
3) Konno M, Yokota S, Suga T *et al* : Predominance of Mother-to-child transmission of *Helicobacter pylori* infection detected by random amplified polymorphic DNA fingerprinting analysis in Japanese families. *Pediatr Infect Dis J* **27** : 999-1003, 2008
4) Oshio I, Osaki T, Yonezawa H *et al* : Vertical *Helicobacter pylori* transmission from Mongolian gerbil mothers to pups. *J Med Microbiol* **58** : 656-662, 2009
5) Georgopoulos SD, Mentis AF, Spiliadis CA *et al* : *Helicobacter pylori* infection in spouses of patients with duodenal ulcers and comparison of ribosomal RNA gene patterns. *Gut* **39** : 634-638, 1996
6) Suzuki J, Muraoka H, Kobayashi I *et al* : Rare incidence of interspousal transmission of *Helicobacter pylori* in asymptomatic individuals in Japan. *J Clin Microbiol* **37** : 4174-4176, 1999
7) Dasanu CA, Rathmann J, Alexandredscu DT : *H. pylori*-associated gastric cancer in a husband-wife pair : a veritable family affair. *South Med J* **102** : 1158-1160, 2009
8) Baldassarre ME, Monno R, Laforgia N *et al* : The source of *Helicobacter pylori* infection in the neonatal period. *J Perinat Med* **37** : 288-292, 2009
9) Stray-Pedersen A, Gaustad P, Stray-Pedersen B *et al* : Detection rate of *Helicobacter pylori* stool antigen in newborn infants and small children. *J Perinat Med* **35** : 155-158, 2007
10) Fujimura S, Kato S, Nagai K *et al* : Detection of *Helicobacter pylori* in the stools of newborn infants. *Pediatr Infect Dis J* **23** : 1055-1056, 2004
11) Kori M, Goldstein E, Granot E : *Helicobacter pylori* infection in young children detected by a monoclonal stool antigen immunoassay. *Pediatr Infect Dis J* **28** : 157-159, 2009
12) Lindkvist P, Asrat P, Nilsson I *et al* : Age at acquisition of *Helicobacter pylori* infection : comparison of a high and a low prevalence country. *Scand J Infect Dis* **28** : 181-184, 1996
13) Rothenbacher D, Inceoglu J, Bode G *et al* : Acquisition of *Helicobacter pylori* infection in a high-risk population occurs within the first 2 years of life. *J Pediatrics* **136** : 744-748, 2000
14) Konno M, Fujii N, Yokota S *et al* : Five-year follow-up study of mother-to-child transmission of *Helicobacter pylori* infection detected by a random ampligied polymorphic DNA fingerprinting method. *J Clin Microbiol* **43** : 2246-2250, 2005
15) Okuda M, Miyashiro E, Booka M *et al* : *Helicobacter pylori* colonization in the first 3 years of life in Japanese children. *Helicobacter* **12** : 324-327, 2007
16) Kusano K, Inokuchi A, Fujimoto K *et al* : Coccoid *Helicobacter pylori* exists in the palatine tonsils of patients with IgA nephropathy. *J Gastroenterol* **45** : 406-412, 2010
17) Medina ML, Medina MG, Martin GT *et al* : Molecular detection of *Helicobacter pylori* in oral samples from patients suffering digestive pathologies. *Med Oral Pathol Oral Bucal* **15** : e38-e42, 2009
18) Yilmaz MD, Aktepe O, Cetinkol Y *et al* : Does *Helicobacter pylori* have role in otitis media with effusion? *Int J Pediatr Otorhinolaryngol* **69** : 745-749, 2005
19) Morinaka S, Tominaga M, Nakamura H : Detection of *Helicobacter pylori* in the middle ear fluid of patients with otitis media with effusion. *Otolaryngol Head Neck Surg* **133** : 791-794, 2005
20) Perry S, Sanchez ML, Yang S *et al* : Gastroenteritis and transmission of *Helicobacter pylori* infection in households. *Emerg Infect Dis* **12** : 1701-1708, 2006
21) Janzon A, Bhuiyan T, Lundgren A *et al* : Presence of high numbers of transcriptionally active *Helicobacter pylori* in vomitus from Bangladeshi patients suffering from acute gastroenteritis. *Helicobacter* **14** : 237-247, 2009
22) Parsonnet J, Shmuely H, Haggerty T : Fecal and oral shedding of *Helicobacter pylori* from healthy infected adults. *JAMA* **282** : 2240-2245, 1999
23) Sasidharan S, Uyub AM : Pervalence of *Helicobacter pylori* infection among asymptomatic healthy blood donors in Northern Peninsular Malaysia. *Trans R Soc Trop Med Hyg* **103** : 395-398, 2009
24) Bhuiyan TR, Qadri F, Saha A *et al* : Infection by *Helicobacter pylori* in Bangladeshi children from birth to two years : relation to blood group, nutritional status, and seasonality. *Pediatr Infect Dis J* **28** : 79-85, 2009

25) Roma E, Panayiotou J, Pachoula J *et al* : Intrafamilial spread of *Helicobacter pylori* infection in Greece. *J Clin Gastroenterol* **43** : 711-715, 2009
26) Chang H, Hu F, Zhang L *et al* : Prevalence of *Helicobacter pylori* infection and identification of risk factors in rural and urban Beijing, China. *Helicobacter* **14** : 128-133, 2009
27) Chi H, Bair MJ, Wu MS *et al* : Prevalence of *Helicobacter pylori* infection in high-school students on Lanyu Island, Taiwn : risk factor analysis and effect on growth. *J Formos Med Assoc* **108** : 929-936, 2009
28) Vilchis J, Duque X, Mera R *et al* : Association of *Helicobacter pylori* infection and height of Mexican children of low socioeconomic level attending boarding schools. *Am J Trop Med Hyg* **81** : 1091-1096, 2009
29) Janzon A, Sjolling A, Lothigius A *et al* : Failure to detect *Helicobacter pylori* DNA in drinking and environmental water in Dhaka, Bangladesh, using high sensitive real-time PCR assays. *Appl Environ Microbiol* **75** : 3039-3044, 2009
30) Tschopp A, Joller H, Jeggli S *et al* : Hepatitis E, *Helicobacter pylori* and peptic ulcers in workers exposed to sewage : a prospective cohort study. *Occup Environ Med* **66** : 45-50, 2009