Vertical *Helicobacter pylori* transmission from Mongolian gerbil mothers to pups

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To identify the time frame and route of mother-to-child *Helicobacter pylori* infection, a Mongolian gerbil model was used. Four-week-old female Mongolian gerbils were infected with *H. pylori*, and then mated with uninfected males 2 months after infection. The offspring were sacrificed weekly after birth, and then serum, mother's milk from the stomach and gastric tissues were obtained from pups. Anti-*H. pylori* antibody titres were measured in sera and maternal milk using an ELISA. The stomach was cut in two in the sagittal plane, and then *H. pylori* colonization in mucosa was confirmed by culture and real-time RT-PCR in one specimen and by immunochemical staining in the other. Faeces and oral swabs were obtained from infected mothers, and *H. pylori* 16S rRNA was measured using real-time RT-PCR. *H. pylori* was not identified in cultures from the gastric mucosa of pups delivered by infected mothers, but *H. pylori* 16S rRNA was detected from 4 weeks after birth, suggesting that Mongolian gerbil pups become infected via maternal *H. pylori* transmission from 4 weeks of age. The anti-*H. pylori* antibody titre in sera of pups from infected mothers was maximum at 3 weeks of age and then rapidly decreased from 4 weeks of age. High antibody titres in mother's milk were detected during the suckling period, and GlcNAc α was detectable at 2–4 weeks of age, but disappeared as the offspring aged. Thus *H. pylori* seems to infect Mongolian gerbil pups from 4 weeks of age, in parallel with decreasing GlcNAc α expression in the gastric mucosa. These results suggested that *H. pylori* infection of Mongolian gerbil pups occurs via faecal–oral transmission from an infected mother.

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INTRODUCTION

Helicobacter pylori, a spiral-shaped pathogenic bacterium found in human gastric mucosa, was originally isolated by Warren and Marshall (Warren & Marshall, 1983) in 1982 and it was soon linked with chronic antral gastritis and peptic ulceration (Marshall & Warren, 1984).

Infection with *H. pylori* can persist for years or for a lifetime (Rowland, 2000; Taylor *et al.*, 1995), although spontaneous clearance often occurs in childhood (Granstrom *et al.*, 1997; Malaty *et al.*, 1999). Infection with *H. pylori* is closely associated with gastritis and peptic ulcers (Goodwin, 1997; NIH Consensus Conference, 1994), and it is a risk factor for gastric cancer (IARC Working Group, 1994) and mucosa-associated lymphoid tissue lymphoma (Blecker *et al.*, 1995). The mode(s) of *H. pylori* transmission is not fully understood despite considerable research. Epidemiological studies have demonstrated the importance of close person-to-person contact and intrafamilial spread (Drumm *et al.*, 1990), but whether this bacterium is primarily transmitted through the faecal–oral or oral–gastric route remains uncertain (Parsonnet *et al.*,

1999). Others (Kivi *et al.*, 2003; Konno *et al.*, 2005; Malaty *et al.*, 1991; Oderda *et al.*, 1991) have suggested intrafamilial clustering of *H. pylori* infections, and molecular DNA analyses of familial *H. pylori* strains have indeed revealed intrafamilial infections with a single *H. pylori* strain (or a common source of infection within the family) (Bamford *et al.*, 1993; Nwokolo *et al.*, 1992). Transmission among family members is considered to constitute the main route of *H. pylori* infection (Covacci *et al.*, 1999). Infected parents, particularly mothers, have been considered likely to play a key role in the intrafamilial transmission of *H. pylori* (Rothenbacher *et al.*, 1999; Brenner *et al.*, 2000; Weyermann *et al.*, 2006), whereas environmental factors might be more important than intrafamilial transmission in developing countries (Rothenbacher *et al.*, 2000; Sarker *et al.*, 1997). However, these epidemiological studies were not based on molecular biological analyses of *H. pylori* strains.

Here, we used molecular analyses to examine *H. pylori* transmission in the stomachs of Mongolian gerbil pups, as well as in the faeces and oral cavity of infected or

non-infected mothers. We also examined how and when Mongolian gerbil pups become infected with *H. pylori* derived from their mothers.

METHODS

Animals. Four-week-old Mongolian gerbils (MGS/Sea; specific pathogen free; body weight 20–30 g) purchased from Kyudo were maintained in plastic cages under standard laboratory conditions (room temperature 23 ± 2 °C; relative humidity 40–60%; 12 h light–dark cycle) and fed with a standard diet (CE-2; Clea Japan) and sterilized tap water *ad libitum*.

Bacterial strain. We inoculated Mongolian gerbils with *H. pylori* TK1402, which was isolated from gastric biopsy specimens of a patient with gastric and duodenal ulcers. Analysis of the strain using PCR indicated the presence of the vacuolating cytotoxin gene (*vacA*) and the *cagA* gene. We used the primer set F1 and B1 to detect *cagA*, and the primer sets VA3-F and VA3-R and VA4-F and VA4-R to detect *vacA* (Atherton *et al.*, 1995). Genomic DNA (1 µl) extracted using MagExtractor (Toyobo) was mixed with each primer (5 pmol) and 0.5 U *Taq* polymerase in a final volume of 20 ± 1 µl. The PCRs 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). The bacterial strain expressed Lewis^y antigen (Le^y) on the cell surface. The bacteria were cultured for 2 days in Brucella broth containing 1.5% agar (Difco) and 7% horse serum (Shiguma) (BHS-agar) under microaerobic conditions at 37 °C using GasPak jars (Mitsubishi Gas Chemical) containing 85% N₂, 10% CO₂ and 5% O₂.

Preparation of *H. pylori* and inoculation. *H. pylori* was incubated on BHS-agar at 37 °C for 48 h under a microaerophilic atmosphere, suspended to a final density of 1.0×10^9 c.f.u. per ml Hanks' balanced salt solution (HBSS) and then orally administered to Mongolian gerbils twice at a 1 day interval. The sera of challenged animals were confirmed as being *H. pylori*-antibody positive by ELISA as described by Osaki *et al.* (2006). Anti-*H. pylori* antibody was detected in all of the gerbils inoculated.

Experimental design. Two months after infection with *H. pylori*, infected female Mongolian gerbils and uninfected males were transferred to separate cages for mating. As soon as pregnancy was confirmed, infected females were separated from the group and cared for until delivery, when mother and pups were housed in one cage per family. Two to four litters were sacrificed under anaesthesia with diethyl ether at 0, 1, 2, 3, 4, 5, 6, 7, 10 and 22 weeks post-partum. Numbers of micro-organisms in the stomach, and antibody titres against *H. pylori* in serum and ingested milk, were then determined as described below. One or two control uninfected gerbils were sacrificed at the same time as the infected animals.

Fresh faeces and throat swabs obtained from infected and uninfected mothers at 2 and 4 months after *H. pylori* inoculation were examined by real-time RT-PCR as described below.

The experiments were approved by the Experimental Animal Ethics Committee at Kyorin University School of Medicine. All experimental animals were housed in the specific-pathogen-free unit of the animal facility and provided with sterile bedding, food and water.

Quantification of gastric *H. pylori* in vitro. Stomachs extirpated from sacrificed gerbils at various times were dissected along the greater curvature and the contents were removed. The stomachs were then divided longitudinally into two halves, including the forestomach-to-pylorus region. The mucous layer collected from the

stomach using Spatel was homogenized in 1 ml HBSS and then the homogenate was inoculated onto *H. pylori* selective medium (Nissui Pharmaceutical) and incubated at 37 °C for 5 days. Thereafter, purple colonies were counted and the numbers of viable *H. pylori* in the gastric mucosa were calculated.

Real-time RT-PCR assay. Real-time RT-PCR proceeded as described by Osaki *et al.* (2006). Tissue samples were lysed by vortex mixing with lysozyme ($400 \mu\text{g ml}^{-1}$) in 100 µl TE buffer (10 mM Tris/HCl, 1 mM EDTA; pH 8) and then mixed with lysis buffer containing guanidine isothiocyanate. Total RNA isolated from the lysate according to the instructions provided with the RNeasy Mini kit (Qiagen) was quantified by measuring the A_{260}/A_{280} ratio. Contaminating chromosomal DNA was digested with DNA-free DNase I [$1 \text{ U } (\mu\text{g RNA})^{-1}$; Ambion] at 37 °C for 20 min. DNase I-treated total RNA (1 µg) was incubated with avian myeloblastoma virus reverse transcriptase with random primers in 20 µl of a reaction mixture (AMV reverse transcription system; Promega) and then first-strand cDNA was synthesized. The cDNA was amplified using PCR primers for *H. pylori* 16S rRNA, 16SB-F (5'-GCTAAGAGATCAGCCTATGTCC-3') and 16SB-R (5'-TGGCAATCAGCGTCAGGTAATG-3') (Engstrand *et al.*, 1992). The *G3PDH* gene on cDNA derived from Mongolian gerbils was amplified using the primers G3PDH-F (5'-ACCACAGTCCATGCCATCAC-3') and G3PDH-R (5'-TCCACCACCCTGTTGCTGTA-3') as a non-infected control for total RNA extraction and for standardization of target gene transcriptional activity. Quantification using SYBR Green staining was based on the PCR kinetics of samples expressing various levels of target genes and from comparisons with standard curves that were generated by amplifying serial dilutions of a known quantity of amplicons. With each primer set, PCR was performed in parallel reactions using different amounts of *H. pylori* strain TK1402 chromosomal DNA. Data were analysed using 7500 quantification software (Applied Biosystems) in which background fluorescence was removed by manually setting a noise band.

ELISA. Whole antigen of *H. pylori* for ELISA was prepared based on our previous report (Nakagawa *et al.*, 2005). We cultured *H. pylori* TK1402 on BHS-agar at 37 °C for 3 days and then whole organisms were suspended in 0.01 M PBS (pH 7.4) and disrupted using an ultrasonic Sonifier 250 (Branson Ultrasonics) for 5 min at 20 kHz. The supernatant was separated from the insoluble cells by centrifugation. Microtitre plates (Greiner Labortechnik Japan) were coated at 4 °C for 18 h with whole sonicate *H. pylori* antigens (3 µg per well) and then washed three times with PBS. Antigens were blocked with PBS containing 1% skim milk (PBS-S; Yukijirushi Nyugyo) at 37 °C for 1 h. Serum samples from infected or uninfected gerbils were added to each well, washed with PBS and then diluted 300-fold with PBS-S. Milk samples were diluted fivefold in PBS. Diluted serum and milk samples (100 µl) were added to the plates and incubated at 37 °C for 2 h, washed three times with PBS, and then 100 µl horseradish peroxidase–protein G (Sigma) at $25 \mu\text{g ml}^{-1}$ in PBS-S was added to the plates and reacted with antigen–antibody (not specific subtype of immunoglobulin) complexes at 37 °C for 1 h. The plates were incubated with 0.1% *o*-phenylenediamine in developing buffer (0.1 M citric acid, 0.07 M sodium phosphate dibasic, 0.035% H₂O₂) at room temperature for 5 min and then the reaction was stopped by adding 50 µl 1 M H₂SO₄. The A_{490} was measured using a model 550 microplate reader (Bio-Rad).

Immunohistochemical analysis. The stomach and gastric gland including the gastric mucosa obtained from pups delivered by infected female Mongolian gerbils were fixed in Carnoy's solution (ethanol/chloroform/acetic acid, 6:3:1) at 4 °C for 48 h, cleared in xylene and embedded in paraffin. Mouse mAb HIK1083 specific for *N*-acetylglucosamine- α (GlcNAc α) was purchased from Serotec. Paraffin sections (5 µm thick) were immunostained using mAb

HIK1083 and the EnVision System (Dako). Briefly, endogenous peroxidase activity was blocked with 0.3% H₂O₂ and then the tissue was sequentially incubated with the mAb HIK1083 and horseradish peroxidase-labelled polymer bound goat anti-mouse antibody. Positive staining was visualized using 3,3'-diaminobenzidine tetrahydrochloride in imidazole buffer containing H₂O₂ and all sections were counterstained with haematoxylin and eosin.

RESULTS AND DISCUSSION

Detection of *H. pylori* in gastric mucosa by culture method and real-time RT-PCR

We cultured the gastric mucosa of gerbil pups aged from 0 to 22 weeks to determine the timing of infection from *H. pylori*-infected mothers. No *H. pylori* colonies were isolated from the gastric mucosa of these animals.

To investigate the timing of vertical *H. pylori* infection, we collected total RNA from the gastric mucosa of pups from infected and uninfected mothers and assembled a complementary DNA library using the reverse transcriptase. Mucosal expression of *H. pylori* 16S rRNA was quantified using real-time RT-PCR with *H. pylori*-specific sequences (Table 1). A product of the 16S rRNA was amplified in mucosa samples from 4–22-week-old pups delivered by infected mothers, but not from uninfected pups. The numbers of *H. pylori* in the gastric mucosa estimated by real-time PCR were 1.3×10^2 , 3.4×10^4 , 3.1×10^4 and 8.5×10^5 per stomach at 4, 5, 6 and 22 weeks of age, respectively. Direct sequencing of the 16S rRNA gene showed that the products of real-time PCR were *Helicobacter*-specific sequence (data not shown).

We tried to quantify the number of colonized organisms in the stomach by the routine culture method in addition to the real-time RT-PCR method. With the culture method, many investigators have reported that they could detect 10^4 – 10^5 c.f.u. of *H. pylori* in the gastric mucosa when 10^7 – 10^9 c.f.u. of *H. pylori* were inoculated (Matsumoto *et al.*, 1997; Sawada *et al.*, 1999; Watanabe *et al.*, 1998). In contrast, the real-time RT-PCR method detected only low numbers of *H. pylori*. In our case, we could not detect *H. pylori* in the stomach by the routine culture method. However, we could detect *H. pylori*, even

4 weeks after birth, and obtained better sensitivity with the real-time RT-PCR method. It was suggested that the number of *H. pylori* organisms infecting from mother to pups was not enough for detection by the culture method, but was detectable by the real-time RT-PCR method.

Antibody titres in maternal gerbil milk and in sera from pups

Mother's milk is considered to suppress mother-to-child transmission. We therefore used an ELISA to measure titres of antibodies against *H. pylori* in milk collected from the stomachs of 0–3-week-old pups immediately after suckling. Fig. 1(a) shows that the A_{490} of anti-*H. pylori* antibodies ranged from 2.5 to 3.5 in pups from infected mothers within 3 weeks post-partum, whereas these antibodies were undetectable in milk from uninfected mothers at any time.

To determine whether anti-*H. pylori* antibody from maternal gerbils protects pups from *H. pylori* infection, we measured titres of serum antibody against *H. pylori* in pups at various times until 22 weeks post-partum (Fig. 1b). Serum antibody against *H. pylori* was detected in 0–5-week-old pups from infected mothers, but not at any time in pups from uninfected mothers. The maximum antibody titre ($A_{490} = 2.867 \pm 0.424$) detected at 3 weeks of age rapidly decreased from 4 weeks of age. These results indicated that anti-*H. pylori* antibodies that developed in offspring were maternally derived.

Although the Ig subclass was not determined for the anti-*H. pylori* antibody in the present study, the results obtained suggest that antibodies against *H. pylori* in gerbil pup serum and mother's milk prevent colonization of *H. pylori* in gastric mucosa during the suckling period.

Determination of *H. pylori* 16S rRNA in the oral scrub and fresh faeces of infected mothers

To understand the route of maternal transmission, we tested oral swabs and fresh faeces from infected mothers for *H. pylori* 16S rRNA at 2 and 4 months after infection using real-time RT-PCR. *H. pylori* 16S rRNA was undetectable at 2 months and detectable in a very low number at 4 months

Table 1. Detection of *H. pylori* in the gastric mucosa of pups of infected mothers estimated by real-time RT-PCR

	Age (weeks)							
	0	1	2	3	4	5	6	22
Mean no. of <i>H. pylori</i> (relative no. per stomach)	0	0	0	0	1.3×10^2	3.4×10^4	3.1×10^4	8.5×10^5
No. of pups tested	3	3	3	2	6	9	2	2
No. of <i>H. pylori</i> 16S rRNA-positive gerbils	0	0	0	0	2	5	2	2
Positive ratio for <i>H. pylori</i> infection (%)	0	0	0	0	33.3	55.6	100.0	100.0
Accumulated positive ratio for <i>H. pylori</i> (%)	0	0	0	0	11.8	26.9	32.1	36.7

Fig. 1. Antibody titres measured using ELISA in milk from maternal Mongolian gerbils and in sera from pups. (a) Anti-*H. pylori* antibody titres in ingested milk from the stomachs of pups after suckling from infected (□) and uninfected (■) mothers. (b) Anti-*H. pylori* antibody titres in sera from 0-, 1-, 2-, 3-, 4-, 5-, 10- and 22-week-old Mongolian gerbil pups delivered by infected (□) or uninfected (■) mothers. Blood was removed from the hearts of 1–10 sacrificed pups per time point and from the hearts of pups from uninfected mothers at 1 and 3 weeks post-partum.

in oral specimens (Table 2), whereas relatively high numbers of *H. pylori* (10^2 – 10^3) were detected in the faeces at both time points after infection.

The presence of *H. pylori* 16S rRNA in fresh faeces from infected mothers indicated that live *H. pylori* migrates from the stomach to the intestine. Mongolian gerbil pups have a habit of eating faeces, indicating that faeces-to-mouth transmission was the infection route. If *H. pylori* is transferred through the faeces of an infected person to water for public use and consumption, then the spread of infection could be a concern.

The present results showed that *H. pylori* survives in the faeces of infected mothers, indicating transmission via nappy changes, dish-washing, careless personal hygiene or from public swimming pools. However, detection of the presence of *H. pylori* by real-time RT-PCR in the faeces does not necessarily mean proliferation of *H. pylori*.

Several epidemiological reports have detected *H. pylori* in the oral cavity of infected persons. However, we did not detect *H. pylori* 16S rRNA in the oral cavity of infected Mongolian gerbils. Rodents lack a vomiting reflex (Kuss *et al.*, 2003; Horn *et al.*, 2007), indicating that gastric juice

Table 2. Detection of *H. pylori* by real-time RT-PCR in the oral scrub and fresh faeces from infected mothers

Sample	Months after infection	<i>H. pylori</i> relative bacterial no. (g specimen) ⁻¹ (mean $\pm$ SD)
Oral scrub	2	0 ± 0
	4	$1.4 \times 10^0 \pm 2.8 \times 10^0$
Faeces	2	$1.5 \times 10^3 \pm 8.6 \times 10^2$
	4	$7.6 \times 10^2 \pm 6.8 \times 10^2$

Fig. 2. Expression of GlcNAc α in gastric mucosa of Mongolian gerbil pups delivered by *H. pylori*-infected mothers. GlcNAc α (arrows) is expressed in gastric mucosa of gerbil pups at 2 (a) and 4 (b) weeks, but not at 10 weeks (c) post-partum.

in Mongolian gerbils cannot flow back from the stomach, and thus *H. pylori* probably does not migrate from the stomach to the oral cavity of these animals.

Immunohistochemical analysis of gastric mucosa of pups from infected mothers

GlcNAc α in gastric mucus constitutes an important defence against *H. pylori* infection. We thus immunohistochemically analysed the gastric mucosa of 2–10-week-old gerbil pups using HIK1083, an anti-GlcNAc α mAb. HIK1083 reacted weakly with the immature gastric mucosa of 2-week-old suckling pups (Fig. 2). In contrast, the gastric mucosa of 4-week-old weaned pups was intensively stained with HIK1083. However, the gastric mucosa of 10-week-old pups did not react with HIK1083.

The sugar chain of the gastric mucin GlcNAc α has an inhibitory effect on growth of *H. pylori* (Kawakubo *et al.*, 2004). We found that the anti-*H. pylori* titre in sera of pups from infected mothers increased at 2–4 weeks of age, and then decreased after 4 weeks of age. These results suggest that the development of GlcNAc α from 2 to 4 weeks post-partum suppresses the growth of *H. pylori*. Real-time RT-PCR showed that the estimated numbers of *H. pylori* increased from 4 to 22 weeks of age. This is considered to be partly due to decreased GlcNAc α expression, which

confers an environment conducive to *H. pylori* proliferation. Levels of GlcNAc α increased at 4 weeks of age, thus providing defence against *H. pylori* infection, but after GlcNAc α expression decreased, the bacterial cells started to proliferate. Therefore, whether or not *H. pylori* can persist in the gastric mucosa for 4 weeks after birth is critical to its future colonization.

Minoura *et al.* (2005) have speculated that the components of maternal milk such as lactoferrin, glycoconjugates and secretory IgA inhibit bacterial growth, and that the numbers of colonizing bacteria in the stomach are reduced while breast-feeding. We found in this study that suckling pups were not infected, due to a high anti-*H. pylori* antibody titre in the mother's milk during this period. Therefore, anti-*H. pylori* antibody in mother's milk might also contribute to defending gerbil pups against *H. pylori* infection.

Nude mice become infected with *H. pylori* when housed in cages that allow faeces to be consumed, but not when kept in cages that prevent this behaviour (Yoshimatsu *et al.*, 2000; Karita *et al.*, 2005). Transmission of *H. pylori* from faeces to mouth was suggested as the infection route, as *H. pylori* has been cultured from saliva. We detected *H. pylori* rRNA in faeces from infected mothers, supporting the notion of a faecal–oral route of transmission. We did not detect *H. pylori* in the oral cavity of infected maternal

gerbils, indicating that either this organism did not reach the oral cavity, or proliferation was suppressed by salivary antibodies. Further studies are required to clarify this issue.

In the present study, we examined the timing and route of mother-to-child *H. pylori* transmission in the Mongolian gerbil model. We detected *H. pylori* 16S rRNA in the gastric mucosa of pups delivered by infected mothers from 4 to 22 weeks post-partum, suggesting that mother-to-child transmission occurred later than 4 weeks after birth. The anti-*H. pylori* antibody in mother's milk seemed to protect against *H. pylori* infection. By analogy with humans, children of infected mothers are probably not infected with *H. pylori* while breast-feeding, but the likelihood of becoming infected increases after weaning.

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