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Helicobacter sp. MIT 01-6451 infection during fetal and neonatal life in laboratory mice

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Abstract: *Helicobacter* sp. MIT 01-6451 has been detected in SPF mice kept in Japan. To characterize strain MIT 01-6451, its infection route during fetal and neonatal life and effects on pregnancy were investigated using immunocompetent and immunodeficient mouse strains (BALB/c, C57BL/6, and SCID). MIT 01-6451 was detected in the uterus, vagina, and mammary glands of 50% of infected SCID mice, whereas these tissues were all negative in immunocompetent mice. No fetal infections with MIT 01-6451 were detected at 16–18 days after pregnancy in any mouse strain. In newborn mice, MIT 01-6451 was detected in intestinal tissue of C57BL/6 and SCID mice at 9–11 days after birth, but not in BALB/c mice. The IgA and IgG titers to MIT 01-6451 in sera of C57BL/6 female mice were significantly lower than those of BALB/c mice. Although no significant differences in the number of newborns per litter were observed between MIT 01-6451-infected and MIT 01-6451-free dams, the birth rate was lower in infected SCID mice than in control SCID mice. The present results indicated that MIT 01-6451 infects newborn mice after birth rather than by vertical transmission to the fetus via the placenta and that MIT 01-6451 infection shows opportunistically negative effects on the birth rate. In addition, the maternal immune response may affect infection of newborn mice with MIT 01-6451 through breast milk.

Key words: *Helicobacter* sp. MIT 01-6451, laboratory mice, reproductive organ, vertical transmission

Introduction

Helicobacter species have been isolated from various species of mammals including humans, and isolation of novel *Helicobacter* species has also been reported recently [18]. *Helicobacter* colonizes the stomach and intestinal tract, and some species are also present in the hepatobiliary system. The potential of many *Helicobacter* species to infect humans and mammals has been reported; for instance, *H. pylori* can induce inflammation, ulcers, and neoplasia in gastric mucosa of humans [15], and both *H. hepaticus* and *H. bilis* have been associated with hepatitis and intestinal diseases in certain strains of mice and rats [5, 6]. Furthermore, many species including *H. cinaedi*, *H. felis*, and *H. pullorum* have the potential for pathogenicity in both humans and animals and are recognized as zoonotic pathogens [4, 8, 18]. Most commercial laboratory rodents in Japan, therefore, are kept under specific pathogen free (SPF) conditions that include monitoring for either two *Helicobacter* species (*H. bilis* and *H. hepaticus*) or all *Helicobacter* species.

The transmission route of *Helicobacter* species is

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considered to be via oral-oral and fecal-oral contact in laboratory mice [12, 33]. However, *Helicobacter* species have been detected in reproductive organs, and vertical transmission of *H. hepaticus* to the fetus via the placenta has been observed in SCID mice [21, 22]. Furthermore, *Flexispira rappini*, considered a member of the *Helicobacter* family, has been isolated from aborted lambs and observed to be transmitted vertically via the placenta and abortion in experimentally infected guinea pigs [2]. In addition to *F. rappini*, infection with *H. rodentium*, *H. typhlonius*, and *H. pylori* in mice, including immunodeficient mouse strains, has negative effects on pregnancy rate and number of pups [20, 22]. It is therefore necessary to clarify the transmission route of each *Helicobacter* species other than the oral route, in particular vertical transmission, and effects of infection on pregnancy.

The detection of unidentified *Helicobacter* species strain MIT 01-6451 was reported first by Taylor *et al.* in laboratory mice obtained from research institutions in Japan but not in mice from Europe or North America [26]. Our previous study also showed that MIT 01-6451 was detected at the highest rate among *Helicobacter* species in laboratory mice obtained from commercial and academic institutions in Japan but not in mice from Europe and the US [34]. Since others have recently reported that MIT 01-6451 is present at a high frequency in laboratory mice in Thailand [3], this strain may preferentially colonize laboratory mice in Asia. Our previous study also showed that this strain was present in the large intestines and less frequently in the gallbladder of infected mice [34]. MIT 01-6451 may disturb the immune system and induce inflammation in the intestinal and hepatobiliary systems of laboratory mice. Since little information on this strain is known and the presence of this *Helicobacter* species in experimental animals may threaten not only the health of those animals but also the health of animal care and research personnel, characterization of this *Helicobacter* species is important.

In the present study, the distribution of MIT 01-6451 in reproductive organs of pregnant mice, possible vertical transmission to the fetus and newborn mice, and the effect of infection on pregnancy were investigated using both immunodeficient and immunocompetent inbred mice. Our data provide important information for understanding the characteristics of MIT 01-6451 and management of microbiological hazards in laboratory animals.

Materials and Methods

Animals and sampling

Three strains of SPF mice (BALB/cAnNCrlCrlj, C57BL/6NCrlCrlj, and CB17/Icr-*Prkdc*^{scid}/CrlCrlj) supplied by Charles River Laboratories Japan (Kanagawa, Japan) were used in this study. Mice were housed with access to food and water *ad libitum* in the Biomedical Research Center of Nagasaki University. Animal care and experimental procedures were performed in accordance with the Regulations and Guidelines for Animal Experimentation of Nagasaki University, reviewed by the Institutional Animal Care and Use Committee of Nagasaki University and approved by the president of Nagasaki University.

Four-week-old female and male mice were infected with MIT 01-6451 by two methods. BALB/c and C57BL/6 mice were housed with soiled bedding (including feces) from cages containing infected mice until detection of *Helicobacter* infections, SCID mice were housed for 7–14 days in the same cage with a mouse infected by oral administration of a bacterial suspension. Pure cultures of MIT 01-6451 were obtained from fecal extracts of infected SPF mice kept in the Biomedical Research Center of Nagasaki University. The mouse housed with the SCID mice was administered orally once every 3–4 days a bacterial suspension in PBS (approximately 5×10^8 organisms) until detection of *Helicobacter* infection. It was confirmed by PCR detection and sequencing analysis of 16S rRNA genes that fecal extracts were not contaminated with any *Helicobacter* species other than MIT 01-6451.

Each male and female mouse (9–27 weeks old) infected with MIT 01-6451 was mated, and each female mouse was housed individually after observation of a vaginal plug or pregnancy. To determine the presence of MIT 01-6451 in various organs, pregnant female mice were euthanized by cervical dislocation at days 16–18 after observation of a vaginal plug (day 0). Blood, mammary glands, uterus, vagina, whole internal organs of fetuses, and the placentas of the fetuses were collected. Newborn mice were euthanized by decapitation, and their intestinal tracts were collected; tissues of their dams were also collected as described above. All tissue samples were stored at −80℃ until DNA extraction.

DNA extraction

DNA was extracted from 40–50 mg of tissue samples

using a LaboPass™ Tissue Mini kit (Cosmo Genetech Co., Ltd., Seoul, South Korea) according to the manufacturer's instructions. For DNA extraction from feces, fecal pellets were homogenized in 1 ml of PBS and centrifuged at 3,000 × g for 30 s. The supernatant was centrifuged at 13,000 × g for 10 min, and DNA was extracted from the pellet. DNA was extracted in 100 μl of ultrapure water (Sigma-Aldrich, St. Louis, MO, USA) and stored at −30℃ until use.

PCR amplification

Detection of the 16S rRNA gene of *Helicobacter* species was performed by nested PCR using two *Helicobacter* genus-specific primer sets, He16rF3 (sequence 5′-CCAAGGCTATGACGGGTATC-3′) and He16rR6 (sequence 5′-ACTTCACCCCAGTCGCTG-3′) and He16rF5 (sequence 5′-AGGGAATATTGCTCAATGGG-3′) and He16rR5 (sequence 5′-TCGCCTTCGCAATGAGTATT-3′), as described previously [34]. All reactions were performed in a reaction volume of 20 μl containing 1 μl of extracted DNA, 10 μl of EmeraldAmp™ PCR Master Mix (Takara Bio Inc., Otsu, Japan), and 1 μM of each primer. After the first round of PCR, 1 μl of the reaction product was diluted 1:5 in ultrapure distilled water and used as template in the nested PCR. PCR products were visualized on 1.5% agarose gels.

Sequence analysis of the 16S rRNA gene

All positive PCR products were purified using a LaboPass™ Gel kit (Cosmo Genetech Co., Ltd., Seoul, South Korea), and sequencing was performed using ABI PRISM® BigDye™ Terminator v3.1 Cycle Sequencing Kits (Applied Biosystems, Foster City, CA, USA) as described previously [34]. The identities of 16S rRNA gene sequences were verified by comparison to the sequence of MIT 01-6451 (accession number: EF373968).

Detection of IgA and IgG antibodies to MIT 01-6451

To detect antibodies to MIT 01-6451 in sera, ELISA was performed. MIT 01-6451 was cultured on modified Skirrow agar (Nissui, Tokyo, Japan) under microaerobic conditions in AnaeroPack® jars (Mitsubishi Gas Chemical, Tokyo, Japan). Grown bacterial colonies were confirmed as MIT 01-6451 by microscopy and analysis of the 16S rRNA gene sequence detected by PCR. Bacterial cells were suspended in phosphate-buffered saline (PBS) and centrifuged at 13,000 × g for 3 min. The cells were resuspended and disrupted by sonication for 15 × 30 s with a cooling interval of 30 s between each sonication. The sonicated cell suspension was centrifuged at 13,000 × g, and the supernatant was used as the antigen for the ELISA. Total protein concentration was measured using a BCA protein assay kit (Thermo Scientific, Rockford, IL, USA).

MaxiSorp Immuno Plate II microtiter plates (Nunc, Roskilde, Denmark) were coated with 50 μl/well of *Helicobacter* antigen (4 μg/ml) in PBS and incubated overnight at 4℃. Plates were blocked with Blocking One (Nacalai Tesque, Kyoto, Japan) diluted 1:2 in ultrapure distilled water by incubation for more than 1 h at 37℃. Serum was diluted serially in PBS containing 0.05% Tween 20 (Tween20-PBS), 50 μl was added to individual wells, and the plates were incubated for overnight at 4℃. After washing with Tween20-PBS, 50 μl/well of anti-mouse IgA or IgG conjugated with horseradish peroxidase (Sigma-Aldrich, St. Louis, MO, USA) was added and incubated for 1 h at 37℃. For detection, 50 μl of 2,2'-azino bis (3-ethylbenzothiazoline sulfonic acid) (ABTS) (Moss, Inc., Pasadena, CA, USA) was added to each well, and absorbance at 405 nm was measured after 1 h of incubation at room temperature using a Labsystems Multiskan MS microplate reader. Absorbance greater than negative controls plus 3 × standard deviation was used as a cutoff for determination of positive samples.

Statistical analysis

The Mann-Whitney U-test was used to evaluate differences in IgA and IgG titers and the number of newborn mice per litter. The *P*-values for statistical differences were discerned at the 95% confidence interval.

Results

Vertical transmission of MIT 01-6451 to the fetus does not occur despite the presence of bacteria in reproductive organs

The possibility of vertical transmission of MIT 01-6451 via the placenta, vagina, or breast milk in both immunocompetent and immunodeficient mouse strains was determined by PCR detection of the *Helicobacter* 16S rRNA gene in various tissues. As shown in Table 1, in both BALB/c and C57BL/6 strains, no *Helicobacter* was detected in the uterus, vagina, or mammary glands of mice, although infection was confirmed by detection

Table 1. Detection of *Helicobacter* sp. MIT 01-6451 DNA in the reproductive and fetal tissue samples of BALB/c and C57BL/6 mice by single and nested PCR

Tissue	BALB/c			C57BL/6		
	1	2	3	1	2	3
Mammary gland	–/–	–/–	–/–	–/–	–/–	–/–
Uterus	–/–	–/–	–/–	–/–	–/–	–/–
Vagina	–/–	–/–	–/–	–/–	–/–	–/–
Feces	+/nd	+/nd	+/nd	+/nd	+/nd	+/nd
Placenta [a)]	–/–	–/–	–/–	–/–	–/–	–/–
Fetus [a)]	–/–	–/–	–/–	–/–	–/–	–/–

The results are presented as single PCR/nested PCR. –, negative; +, positive; nd, not done. [a)]Three fetuses and their placentas at day 16-18 after pregnancy sampled randomly from littermates were tested.

Table 2. Detection of *Helicobacter* sp. MIT 01-6451 DNA in the reproductive and fetal tissue samples of 6 SCID female mice by single and nested PCR

Tissue	SCID					
	1	2	3	4	5	6
Mammary gland	–/–	–/–	–/–	–/–	–/–	–/+
Uterus	–/±	–/–	–/–	–/–	±/+	±/+
Vagina	–/+	–/–	–/–	–/–	±/+	±/+
Feces	+/nd	+/nd	+/nd	+/nd	+/nd	+/nd
Fetal number	5	2	3 [a)]	4	6	7
Placenta	nd [b)]	–/–	–/–	–/–	nd [b)]	± [c)]/+
Fetus	–/–	–/–	nd	–/–	–/–	–/–

The results are presented as single PCR/nested PCR. –, negative; +, positive; nd, not done. [a)]Three placentas were collected because no fetuses were found. [b)]The fetus had been delivered already within 1-2 hrs. [c)]All placentas were positive except for one placenta showing indefinite result by single PCR.

of MIT 01-6451 in feces. Fetuses at gestational days 16–18 and their respective placentas also were negative for infection in BALB/c and C57BL/6 mice.

In pregnant SCID mice, MIT 01-6451 was detected in 3 of 6 vaginas and uteruses (Table 2). Two of the three positive results were detected in the first round of PCR reactions, indicating a high level of bacteria in these samples. MIT 01-6451 was also detected in 1 of 6 mammary glands of SCID mice. Despite the presence of bacteria in these reproductive tissues, infection of fetuses with MIT 01-6451 was not detected. This included 7 fetuses with placentas that were positive for MIT01-6451 in the first round of PCR reactions.

Infection of newborn mice with MIT 01-6451 occurs after birth

As shown in Table 3, no infection with MIT 01-6451 was detected in newborn pups of BALB/c or C57BL/6 mice at day 0 or 3 after birth. However, at days 9–11 after birth, MIT 01-6451 was detected in all newborn pups of C57BL/6 dams in the first round of PCR reactions, whereas no infection of newborn pups of BALB/c dams was detected. MIT 01-6451 was not detected in the mammary glands of infected dams in both BALB/c and C57BL/6 mice. There was no significant difference in the mean numbers of newborn pups per litter in both BALB/c and C57BL/6 mice infected with MIT 01-6451 as compared with MIT 01-6451-free control groups (Fig. 1).

In SCID mice, no infection of newborn mice with MIT 01-6451 was detected until day 4 (Table 4), but many newborn mice in all litters were infected by day 10. Although a significant difference in the mean number of

Table 3. Detection of *Helicobacter* sp. MIT 01-6451 DNA in mammary gland and newborn tissue samples of BALB/c and C57BL/6 mice by single and nested PCR

Tissue	BALB/c				C57BL/6				
	1	2	3	4	1	2	3	4	5
Mammary gland	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–
Feces	+/nd	+/nd	+/nd	+/nd	+/nd	+/nd	+/nd	+/nd	+/nd
Newborn [a)]									
Day 0	–/– (n=3)	–/– (n=3)	–/– (n=3)	–/– (n=3)	nd	nd	nd	–/– (n=2)	–/– (n=3)
Day 3	nd	–/– (n=1)	nd	–/– (n=3)	nd	nd	nd	–/– (n=3)	–/– (n=2)
Day 9-11	–/– (n=3)	–/– (n=3)	–/– (n=3)	nd	±/+ (n=3)	+/+ (n=3)	+/+ (n=3)	nd	nd

The results are presented as single PCR/nested PCR. –, negative; +, positive; nd, not done. [a)] Newborn tissue samples from littermates were tested.

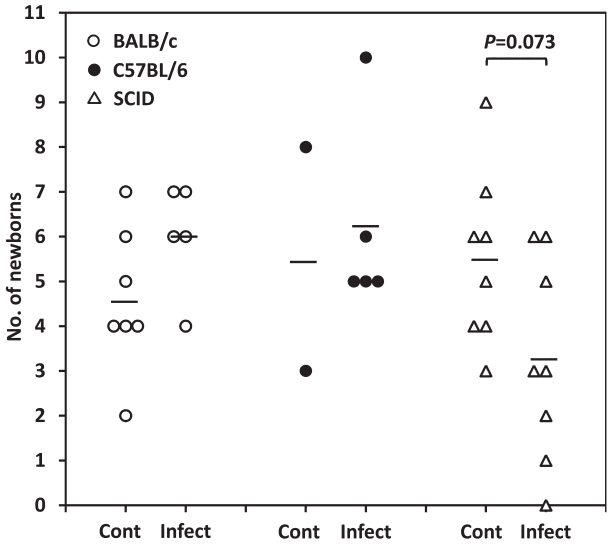

Fig. 1. The effects of MIT 01-6451 infection on pregnancy in immunocompetent (BALB/c and C57BL/6) and immunodeficient (SCID) mouse strains. Individual numbers of newborn mice per litter of uninfected controls (cont) and MIT 01-6451-infected (infect) groups are shown. Bars represent the mean number of newborn mice per litter.

newborn pups per litter was not evident between infected and uninfected SCID dams, stillbirths occurred in 3 of 6 SCID dams, and the birth rate tended to be lower in infected SCID dams compared with uninfected dams (Fig. 1 and Table 4). In particular, all littermates of SCID dam #2 were born dead, and the dam also died during delivery. Furthermore, the placentas of these stillbirths were all positive for MIT 01-6451 in the first round of PCR reaction.

Higher IgA and IgG antibody titers to MIT 01-6451 in infected mice

Since maternal antibodies to MIT 01-6451 could affect the timing of infection of newborn mice, sera were collected from both BALB/c and C57BL/6 female mice that were uninfected or infected with MIT 01-6451. The titers of IgA and IgG to MIT 01-6451 in sera were determined by ELISA. As shown in Fig. 2, although nonspecific reactions that were likely due to use of whole cell proteins of MIT 01-6451 as antigens were detected in normal sera, the anti-MIT 01-6451 IgG titers in sera of both infected mouse strains were significantly higher than those in sera of uninfected mice ($P<0.0003$ in BALB/c and $P=0.024$ in C57BL/6 mice), but only BALB/c mice developed serum IgA titers ($P<0.0003$). Furthermore, both the serum IgA and IgG titers to MIT 01-6451 in BALB/c mice were significantly higher than those in C57BL/6 mice ($P<0.003$ and $P<0.04$, respectively), while significant differences in IgG titers were also detected in normal sera between the two mouse strains ($P<0.006$).

Discussion

The results of the present study showed that the colonization sites for *Helicobacter* sp. MIT 01-6451 in immunocompetent mice did not include reproductive organs (vagina, uterus, and mammary gland); the bacteria were restricted to the gastrointestinal tract and hepatobiliary system. However, the results also showed that MIT 01-6451 was present in reproductive organs in 50% of immunodeficient mice. Another *Helicobacter* species (*H. typhlonius*) was detected transiently in reproductive organs of both immunodeficient and immunocompetent

Table 4. Detection of *Helicobacter* sp. MIT 01-6451 DNA in newborn tissue samples of SCID mice by single and nested PCR

Tissue	SCID female dams							
	1 [a]		2	3 [a]		4	5	6
No. of newborns (No. of stillbirths)	3	4	0 (7)	2 (1)	6	3	6	1 (1)
Placenta	nd	nd	+/+ (n=4)	nd	nd	nd	nd	nd
Newborn								
Day 0-6	Day 4 −/− (n=3)	nd	nd	nd	Day 6 −/± [c] (n=3)	nd	Day 4 −/− (n=2)	Day 0 −/− (n=2)
Day 10-14	nd	Day 10 −/± [b] (n=4)	nd	nd	Day 14 +/+ (n=3)	Day 10 −/± [c] (n=3)	Day 10 ±/± [c] (n=3)	nd

The results are presented as single PCR/nested PCR. –, negative; +, positive; nd, not done. [a]These dams were mated twice, and the 1st and 2nd pregnancy are shown in the left and right column, respectively. [b]Half of the newborns were positive. [c]One out of 3 newborns were positive.

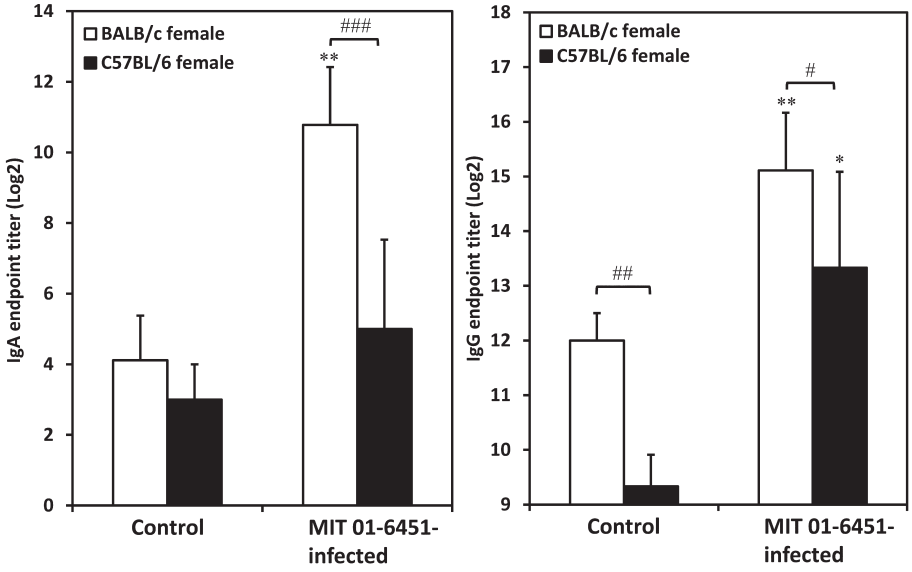

Fig. 2. The IgA and IgG titers to MIT 01-6451 in sera of infected and uninfected mice. The data are presented as the mean of endpoint titers ± standard deviation. Significant differences between control and infected mice (**P*=0.024; ***P*<0.0003) and between BALB/c and C57BL/6 mice ($^{\#}P$<0.04; $^{\#\#}P$<0.006; $^{\#\#\#}P$<0.003) are indicated.

mice in a previous study [21], and *H. cinaedi*, *H. fennelliae*, and *F. rappini* colonized the gastrointestinal tract and produced bacteremia in humans [11, 25, 27, 28]. Therefore, it is possible that MIT 01-6451 may also produce transient bacteremia, particularly in immunodeficient mice. Indeed, as shown in Table 4, SCID dam #3 showed abnormal delivery in the first pregnancy but not in the second. Although invasion of MIT 01-6451 through intestinal epithelial cells into the bloodstream may be prevented by the optimal immune responses in immunocompetent mice, immunodeficient mice might sustain the bacteremia, resulting in colonization of the reproductive organs. This opportunistic translocation may cause the occurrence of pregnancy-related disorders such as miscarriage and dystocia, since both low birth rates and stillbirths were observed in SCID mice infected with MIT 01-6451.

Vertical transmission of MIT 01-6451 via the placenta, birth canal, and/or breast milk was not observed in either immunocompetent or immunodeficient mouse strains. In addition, although infection with MIT 01-6451 did not have negative influences on pregnancy and delivery in immunocompetent mice, low birth rates and stillbirths were observed in immunocompromised mice. The mean number of fetuses per litter in infected SCID dams, even infected dams with MIT 01-6451 detected in reproductive organs, except for one dam (#3 in Table 2), was comparable to that of newborn pups of MIT 01-6451-free SCID dams. MIT 01-6451 infection might exhibit opportunistically negative effects on delivery rather than maintenance of pregnancy. In contrast, *H. hepaticus* has been reported to be vertically transmitted to fetuses via the placenta in SCID mice [14], and it has been reported that guinea pigs experimentally infected with *Flexispira rappini* transmitted the agent vertically via placenta and that this induced abortion [2]. Furthermore, infection of immunocompetent or immunodeficient mouse strains with *H. rodentium*, *H. typhlonius*, or *H. pylori* had a negative effect on both pregnancy rate and number of pups per litter [20, 22]. Therefore MIT 01-6451 may have a comparable or even lower potential to induce pregnancy-related disorders compared with other *Helicobacter* species. Also, since infection of mice with multiple *Helicobacter* species (e.g., *H. rodentium* plus *H. typhlonius* and *H. rodentium* plus *H. hepaticus* or *H. bilis*) showed more severe inflammation with negative effects on pregnancy and newborn mice as compared with mice infected with single strains [9, 17, 22, 23]. Additional studies on the pathogenicity of MIT 01-6451 during pregnancy are required.

Although no infection with MIT 01-6451 was detected at birth in newborn mice delivered from infected BALB/c or C57BL/6 dams, the times of infection after birth were different between BALB/c and C57BL/6 newborn mice; infection was delayed in BALB/c newborn mice compared

with C57BL/6 newborn mice. BALB/c and C57BL/6 mice are well known as predominant type 2 and type 1 helper T cell (Th) responders, respectively [10]. The present results also showed that specific serum antibody titers to MIT 01-6451 in sera of BALB/c female mice were significantly higher than in C57BL/6 mice, so it is possible that higher levels of maternal antibodies may play a role in protecting newborn mice against infection. Infection with *Helicobacter* species in newborn mice is considered to occur through oral-oral and/or fecal-oral contact. Previous reports also indicated that active immunity protected against colonization by *Helicobacter* species [1, 13] and that passive immunity (IgG and IgA in breast milk) protected against *Helicobacter* infection in newborn rodents and humans [7, 19, 29]. In addition, whereas newborn pups suckled by dams infected with *Helicobacter* species were infected 13–16 days after birth, newborn pups fostered by *Helicobacter*-free mice after having contact with an infected dam for more than 24 h were already infected with *Helicobacter* species in their intestinal tracts [24, 30]. Therefore, although it depends on the frequency of contact between dams and newborn pups and the possibly coprophagous behavior of newborn pups, a specific antibody in breast milk of BALB/c dams might delay infection in newborn mice either by neutralization or excretion of bacteria with intestinal contents.

Differences in sensitivity to *Helicobacter* species (both gastric and enterohepatic species) have been observed among different mouse strains, and sensitive mouse strains exhibited substantial colonization in usual sites as well as severe gastritis, hepatitis, or inflammatory bowel disease [16, 21, 31, 32]. The BALB/c mouse strain is more sensitive to *Helicobacter* species, especially enterohepatic species such as *H. hepaticus*, than C57BL/6 mice [31, 32]. The present study demonstrates that the antibody response of BALB/c mice to MIT 01-6451 was higher than that of C57BL/6 mice. While these different antibody responses between the two mouse strains might be caused only by predominant Th responses, our present results also suggest that mouse strains differ in their sensitivity to MIT 01-6451 as well as to the other *Helicobacter* species. Additional studies are required to clarify the pathogenicity of MIT 01-6451 using various mouse strains having differing susceptibilities to *Helicobacter* species.

In conclusion, our results suggest that *Helicobacter* sp. MIT 01-6451 infects newborn mice after birth via the oral route rather than by vertical transmission. Also, infection during neonatal life appears to be influenced by the maternal immune response, possibly by antibodies present in breast milk. Furthermore, although MIT 01-6451 normally colonizes the large intestines, it can opportunistically infect reproductive organs and possibly cause pregnancy-related disorders in mice. Many *Helicobacter* species have the potential to induce colitis in immunodeficient and even immunocompetent mice. Further studies are required to fully clarify the pathogenicity of MIT 01-6451 not only in reproductive tissue but also in other colonization sites such as the cecum, colon, and rectum.

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