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Prevalence of an Unidentified *Helicobacter* Species in Laboratory Mice and its Distribution in the Hepatobiliary System and Gastrointestinal Tract

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Abstract: An unidentified *Helicobacter* species, strain MIT 01-6451, was frequently detected in mice obtained from domestic commercial and academic institutions in Japan. To partially characterize this strain, its distributions in the gastrointestinal tract and hepatobiliary system of mice were investigated. In gastrointestinal tissues, this strain was detected in all cecum, colon, and feces samples tested, whereas fewer mice were positive in the ileum, jejunum, and duodenum. Interestingly, strain MIT 01-6451 was also detected in most stomach samples and in 33% of gallbladder samples. One mouse was found to be infected with multiple *Helicobacter* species. Fourteen copies of 16S rRNA genes were cloned from the tissues of this mouse. One had the highest level of sequence homology with *H. canadensis*, while 13 had the highest level of homology with the *H. ganmani* type strain or strain MIT 01-6451. Twelve of these 13 16S rRNA genes were mosaic sequences, being partially derived from *H. ganmani* and strain MIT 01-6451. These results suggest that *H. ganmani* and *Helicobacter* sp. MIT 01-6451 are prevalent in specific-pathogen-free mouse colonies in Japan and that lateral gene transfer probably occurs among *Helicobacter* species during coinfection.
Key words: *Helicobacter* sp. MIT 01-6451, laboratory mice, prevalence, organ distribution.

Introduction

Helicobacters are Gram-negative and spiral shaped bacteria. Novel species of *Helicobacter* are frequently detected in various species of mammals [1, 2, 7, 15, 16]. At present, the *Helicobacter* genus includes 33 formally named species (http://www.bacterio.cict.fr/h/helicobacter.html). The genus can be subdivided in two groups, gastric *Helicobacter*, which preferentially colonizes the stomach, and enterohepatic *Helicobacter*, which preferentially colonizes the intestinal and hepatobiliary system [6, 7]. One of the best-characterized gastric species, *H. pylori*, is a pathogen of the gastric mucosa, causing inflammation, ulcers, and neoplasia in humans [15, 20]. Enterohepatic *Helicobacter* species also known to cause diseases, such as *H. hepaticus* and *H. bilis*, have been associated with hepatitis and intestinal diseases in certain strains of mice and rats [1, 5, 10, 17, 18, 22, 28]. Furthermore, some other *Helicobacter* species including *H. felis*, classified in the gastric group, and *H. pullorum*, classified in the enterohepatic group, have been detected in both humans and animals and are recognized as zoo-

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notic pathogens [5, 22]. However, in contrast to the abundant reports of cases of these *Helicobacter* species, there are many species that have not yet been studied in detail with regard to the mechanisms of pathogenesis.

Recently, there have been reports describing the high prevalence of *Helicobacter* species in experimental animals from commercial and academic institutions in Europe and North America [3, 19, 31]. Furthermore, both previously identified and novel unclassified *Helicobacter* species including MIT 01-6451 have been detected at high frequency in the experimental animals kept at research facilities in Japan [14, 31]. Although most commercial laboratory rodents in Japan are kept under specific-pathogen-free (SPF) conditions that include monitoring for two *Helicobacter* species, *H. hepaticus* and *H. bilis*, other *Helicobacter* species can still be detected in SPF laboratory animals. Because the presence of these *Helicobacter* species in experimental animals may interfere with research and threaten the health of animal care and research personnel, the detection and characterization of these unidentified *Helicobacter* species is important.

To detect *Helicobacter* species infections, serologic tests, immunohistochemistry, and polymerase chain reaction (PCR) have been used [4, 21, 23, 33]. Culture-based diagnostic tests are limited since, in general, *Helicobacter* species are slow-growing bacteria requiring more than 3 days of incubation for visible colony formation in a microaerobic atmosphere and culture conditions vary for different species [24, 27]. Therefore, PCR detection and sequence analysis of the 16S rRNA gene has proven useful and most cost-effective for both detection and strain identification, although there is discordance between 16S and 23S rRNA gene phylogenies for the genus *Helicobacter* [6].

In the present study, the prevalence of *Helicobacter* species in SPF mice delivered to the Biomedical Research Center of Nagasaki University was investigated. *Helicobacter* sp. MIT 01-6451 was frequently detected in mice from domestic animal facilities in Japan by PCR of the 16S gene. Since there is little information on this strain, characteristics of MIT 01-6451 strain such as the distribution in the gastrointestinal tract and hepatobiliary system in naturally infected mice were examined. Simultaneously, other unidentified *Helicobacter* species were also detected, and BLAST searches with the sequence of *Helicobacter* 16S rRNA genes deposited in GenBank were performed.

Materials and Methods

Animals and sampling

Mice delivered to the Biomedical Research Center of Nagasaki University from both domestic or overseas academic institutions and from commercial sources were quarantined and tested for *Helicobacter* infection. Specimens (n=205) were fecal pellets obtained from cages immediately after delivery between April 2010 and December 2011.

For determination of the distributions of *Helicobacter* sp. MIT 01-6451 in the hepatobiliary system and gastrointestinal tract, nine naturally infected mice (human-*PRNP* transgenic mice, C57BL/6 background, 3 males and 6 females, ages 45 and 94 weeks) obtained prior to April 2010 were used. Mice were euthanized, and the stomach, duodenum, jejunum, ileum, cecum, colon, liver, and gallbladder were collected. After removal of intestinal tract contents, all tissue samples were stored at −30°C until DNA extraction. Animal care and experimental procedures were performed in accordance with the Regulations and Guidelines for Animal Experimentation of Nagasaki University and with approval of the Institutional Animal Care and Use Committee.

DNA extraction

DNA was extracted from 20 mg of tissue samples using a LaboPass^{TM} Tissue Mini kit (Cosmo Genetech Co., Ltd., Seoul, 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.

PCR amplification

In quarantine, detection of 16S rRNA genes of *Helicobacter* species was performed by single-round PCR assays using the following primer sets: He16rF3 and He16rR6 and He16rF5 and He16rR5 to detect *Helicobacter*-genus gene or HeH16rF6 and HeH16rR7 to detect the *H. hepaticus* gene as listed in Table 1. All reactions were performed in a final reaction volume of 20 μl containing 0.5 μl of extracted fecal DNA, 10 μl of EmeraldAmp^{TM} PCR Master Mix (Takara Bio Inc., Otsu, Shiga, Japan), and 1 μM of each primer. The PCR reactions using primers He16rF3 and He16rR6 were preheated at 95°C for 5 min and then subjected to 40 cycles consist-

Table 1. Primers used for 16S rRNA gene PCR amplification

Purposes	Primer	Sequence (5'–3')	Positions[a)]	Ref.
Quarantine and 1st PCR	He16rF3[b)]	CCAAGGCTATGACGGGTATC	247–266	[26]
(*Helicobacter*-genus specific)	He16rR6[b)]	ACTTCACCCCAGTCGCTG	1,424–1,441	[26]
Quarantine and nested PCR	He16rF5[b)]	AGGGAATATTGCTCAATGGG	335–354	[14]
(*Helicobacter*-genus specific)	He16rR5[b)]	TCGCCTTCGCAATGAGTATT	666–685	[14]
Quarantine	HeH16rF6	CATTTGAAACTGTTACTCTG	585–604	[13]
(*H. hepaticus* specific)	HeH16rR7	TCAAGCTCCCCGAAGGG	978–994	[13]
Cloning	He16rF1[b)]	AGAGTTTGATCCTGGCTCAGA	–1–21	In this study
Sequence analysis	He16rR3	CCATTGTAGCACGTGTGTAGC	1,174–1,194	In this study

[a)]Positions of primers within the *H. hepaticus* ATCC 51448^T 16S rRNA sequence (U07574) that correspond to the 5' to 3' ends of each primer are shown. [b)]This primer was used for sequence analysis.

ing of denaturation at 95°C for 30 s, primer annealing at 65°C for 30 s, and extension at 72°C for 1.5 min, with final extension at 72°C for 7 min. The other two PCR reactions using primers He16rF5 and He16rR5 and HeH16rF6 and HeH16rR7 were subjected after preheating to 40 cycles consisting of denaturation at 95°C for 30 s, primer annealing at 60°C for 30 s, and extension at 72°C for 30 s. Detection of 16S rRNA genes of *Helicobacter* spp. in tissues was performed by nested PCR using a final reaction volume of 20 μl containing 1 μl of extracted DNA. After the first round of PCR using primers He16rF3 and He16rR6, 1 μl of the reaction product diluted 1:5 in ultrapure distilled water was used as template in a nested PCR using the internal primer pair He16rF5 and He16rR5. The nested PCR was performed twice repeatedly to exclude false results. For cloning PCR products, the PCR reactions using primers He16rF1 and He16rR6 were performed to determine the longer sequences (more than 1,400 bp). PCR protocols are as described above. The specific PCR products were visualized on 1.5% agarose gels.

16S rRNA gene cloning and sequence analysis

All positive PCR products were purified using a LaboPass^{TM} Gel kit (Cosmo Genetech Co., Ltd., Seoul, Korea) and the sequences were analyzed using the primers shown in Table 1 and ABI PRISM BigDye^{TM} Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Carlsbad, CA, USA). The PCR products showing multi-infection with *Helicobacter* species were cloned into pCR 2.1-TOPO vector (TOPO TA cloning Kit, Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions, and introduced into *Escherichia coli* DH5-α cells. Plasmid DNA from DH5-α cells cultured in 2 ml of LB media was purified using a LaboPass^{TM} Mini kit (Cosmo Genetech Co., Ltd., Seoul, Korea) and DNA sequences of the recombinant plasmids were determined as described above. Novel 16S rRNA gene sequences as shown in Table 5 were deposited in GenBank (accession numbers AB693132-AB693144, AB693948, and AB693949). The identity of the partial 16S rRNA gene sequences was verified by comparison to the sequences in the NCBI database (http://www.ncbi.nlm.nih.gov/BLAST).

Results

Higher detection rate of Helicobacter sp. MIT 01-6451 in mice delivered from the domestic animal facilities

All mice delivered to the Biomedical Research Center of Nagasaki University from other academic institutions or from commercial suppliers of genetically engineered animals between April 2010 and December 2011 were tested for *Helicobacter* infection. All samples that were positive by this diagnostic PCR test were further analyzed by amplification and sequencing of the 16S rRNA gene. The identity of the *Helicobacter* species present in positive samples was then determined by BLAST search. As shown in Table 2, in mice obtained from domestic animal facilities, *Helicobacter* sp. MIT 01-6451 was detected at the highest rate [48/167 (28.7%) samples positive] followed by *H. ganmani* [30/167 (18.0%) samples positive]. Other *Helicobacter* species such as *H. mastomyrinus*, *H. hepaticus*, and *H. rodentium* were also detected at lower frequency [5/167 (3.0%), 2/167 (1.2%), and 2/167 (1.2%), respectively]. Other unidentified *Helicobacter* species were also detected in 20/167 (12.0%) samples. Although *H. ganmani* genes were detected in 2 samples from the European animal facilities, *Helicobacter* sp. MIT 01-6451 was not detected in

Table 2. Detection of *Helicobacter* species in mice received from animal facilities in Japan

Source (no. of institutions/total)	No. of mice tested	No. of positive mice (%)					
		H. sp. MIT 01-6451	*H. ganmani*	*H. mastomyrinus*	*H. hepaticus*	*H. rodentium*	Others[a]
Academic institution[b] (15/31)	102	21 (20.6)	27 (26.5)	5 (4.9)	2 (2.0)	2 (2.0)	14 (13.7)
Institution 1	4	0	3	0	0	0	1
Institution 2	12	0	0	0	0	0	0
Institution 3	4	0	0	0	0	0	0
Institution 4	8	1	0	0	0	0	4
Institution 5	23	10	0	2	0	2	1
Institution 6	6	0	1	2	0	0	1
Institution 7	2	0	1	0	0	0	1
Institution 8	4	2	2	0	0	0	0
Institution 9	10	2	6	0	0	0	2
Institution 10	4	1	3	0	0	0	0
Institution 11	7	1	4	0	0	0	2
Institution 12	4	0	0	0	0	0	0
Institution 13	8	0	7	1	2[c]	0	0
Institution 14	4	2	0	0	0	0	2
Institution 15	2	2	0	0	0	0	0
Commercial company (2/16)	65	27 (41.5)	3 (4.6)	0 (0)	0 (0)	0 (0)	6 (9.2)
Company 1	40	8	0	0	0	0	3
Company 2	25	19	3	0	0	0	3
Total	167	48 (28.7)	30 (18.0)	5 (3.0)	2 (1.2)	2 (1.2)	20 (12.0)

[a]Including the 16S rRNA gene sequences matched with those of formally unnamed *Helicobacter* species and those different from any *Helicobacter* genes deposited in GenBank. [b]No test results for any *Helicobacter* spp. contamination were provided from institutions 2, 6, 8, 10, 13, 14, and 15. [c]Detection of coinfection with *H. ganmani*.

Table 3. Detection of *Helicobacter* species in mice received from animal facilities in the United States and Europe

Source	No. of mice tested	No. of positive mice	*Helicobacter* species identified (No. of mice)
USA			
Academic			
Institution 1	3	0	
Institution 2	2	1	Unidentified species[a] (1)
Commercial			
Company 1	24	0	
Company 2	2	0	
Europe			
Academic			
Institution 1	3	3	*H. hepaticus* + *H. ganmani* (2) *H. hepaticus* + unidentified species[a] (1)
Institution 2	4	0	

[a]The 16S rRNA gene sequences were different from any *Helicobacter* genes deposited in GenBank.

mice from animal facilities in the United States or Europe (Table 3).

Organ distribution of Helicobacter sp. MIT 01-6451 in mice

To determine if *Helicobacter* sp. MIT 01-6451 is present in the gastrointestinal tract and/or hepatobiliary system of mice, nested PCR was performed using the He16rF3 and He16rR6 and He16rF5 and He16rR5 primer pairs. C57BL/6 background transgenic mice that were purchased from a commercial vendor ("Company 1" in Table 2) prior to April 2010 and naturally infected with MIT 01-6451 were used. No macroscopic lesions were observed in any organs. However, *Helicobacter* sp. MIT 01-6451 was detected in organs by nested PCR as summarized in Table 4. In the gastrointestinal tract, MIT

Table 4. Detection of *Helicobacter* sp. MIT 01-6451 DNA in the tissue samples of 9 mice by single and nested PCR (results represented as "single PCR/nested PCR")

Tissue	Male			Female					
	1	2	3	1	2	3	4	5	6
Stomach	−/+ [a)]	−/+	−/−	−/+	−/+	−/+	−/+	−/+	−/+
Duodenum	−/−	−/+	−/−	−/−	−/−	−/−	−/−	−/−	−/+
Jejunum	−/−	−/+	−/−	−/−	−/−	−/−	−/−	−/−	−/−
Ileum	−/+	−/+	−/−	−/+	−/+	−/+	−/−	−/−	−/+
Cecum	+ [a)]/nd	+/nd	+/nd	+/nd	+/nd	+/nd	+/nd	+/nd	+/nd
Colon	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+
Liver	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−
Gallbladder	−/+	−/−	−/−	−/−	−/+	−/+	−/−	−/−	−/−
Feces	+ [a)]/nd	+/nd	+/nd	+/nd	+/nd	+/nd	+/nd	+/nd	+/nd

−, negative; +, positive; nd, not done. [a)]Multiple infections with *Helicobacter* species were expected by sequencing analysis.

Table 5. The highest similarities of 16S rRNA sequences between clones 1–14 detected in the mouse (#1, male) and *Helicobacter* species deposited in GenBank by BLAST analysis

Clone [a)]	Size (bp)	Highest similarity (GenBank accession no.)	Identity (%)	Gap (%)
1	1158	*Helicobacter* sp. MIT 01-6451 (EF373968)	98.0	0.17
2	1159	*H. ganmani* CMRI H02^T (AF000221)	98.4	0
3	1159	*H. canadensis* CCUG 47163^T (AM998803)	97.8	0
4	1158	*Helicobacter* sp. MIT 01-6451 (EF373968)	99.5	0
5	1403	*H. ganmani* CMRI H02^T (AF000221)	99.4	0
6	1404	*Helicobacter* sp. MIT 01-6451 (EF373968)	99.5	0
7	1404	*Helicobacter* sp. MIT 01-6451 (EF373968)	98.0	0
8	1403	*H. ganmani* CMRI H02^T (AF000221)	100	0
9	1403	*H. ganmani* CMRI H02^T (AF000221)	98.6	0.14
10	1402	*H. ganmani* CMRI H02^T (AF000221)	99.0	0.36
11	1403	*H. ganmani* CMRI H02^T (AF000221)	98.4	0.14
12	1405	*H. canis* (AF177475)	96.9	0.07
		H. ganmani CMRI H02^T (AF000221)	96.9	0.14
13	1405	*H. ganmani* CMRI H02^T (AF000221)	96.2	0.14
14	1403	*H. ganmani* CMRI H02^T (AF000221)	99.1	0.14

[a)]Detection in cecum (clones 1–9) and feces (clones 10–14) samples.

01-6451 was detected in feces and the large intestine (cecum and colon) in the first round PCR reaction. In contrast, MIT 01-6451 was only detected in the stomach and small intestine (duodenum, jejunum, and ileum) by nested PCR. Although almost all stomach samples were positive, the frequency of detection was less in the duodenum, jejunum, and ileum. In the hepatobiliary system, although no liver samples were positive for *Helicobacter* sp. MIT 01-6451, nested PCR yielded positive results in 3 out of 9 gallbladders.

Detection of multiple Helicobacter spp. in an MIT 01-6451-infected mouse

Infection with multiple *Helicobacter* species was suspected in male mouse #1 because of multiple sequence signals from PCR products obtained from stomach, cecum, and fecal samples. Therefore, PCR products of cecum and fecal samples were cloned, and multiple clones from each sample were sequenced. In addition to the sequences that were identical to MIT 01-6451, 16S rRNA *Helicobacter* sequences that were different from that of strain MIT 01-6451 were detected at least 9 (clones 1–9) and 5 clones (clones 10–14) in cecum and fecal samples, respectively. The sequence of clone 8 matched 100% with that of the *H. ganmani* type strain. Ten out of 14 clones (clones 5–14) were determined more than 1,400 bp sequences and these clones lacked any intervening sequences (IVS), as occurs in *H. bilis* [8] and *H. typhlonius* [9]. BLAST (blastn) searches between these 14 clones and the sequence of *Helicobacter* 16S rRNA genes deposited in GenBank were performed, and the results are shown in Table 5. With the exception of clones 3 and 12, clones had the greatest homology with *Helicobacter* sp. MIT 01-6451 or *H. ganmani*. Furthermore, as shown

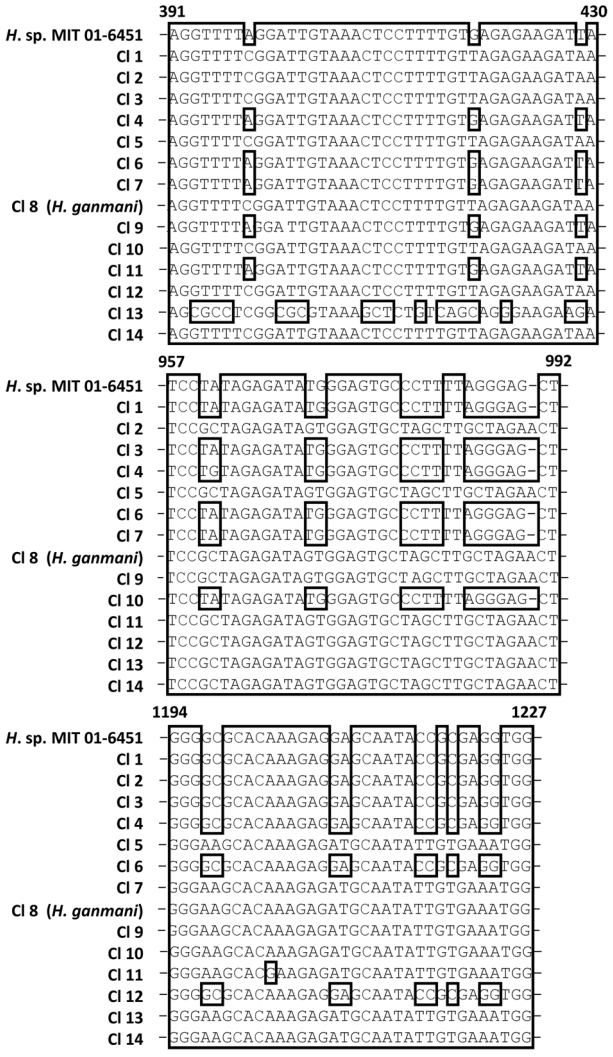

Fig. 1. Comparisons between *Helicobacter* sp. MIT 01-6451 and 14 cloned 16S rRNA gene sequences including an *H. ganmani* gene sequence (clone 8). The numbers at the top of each panel represent the coordinates of the 16S rRNA gene within the strain MIT 01-6451 gene sequence (EF373968) where the three domains are located. The parts of sequences from other major sequences are grouped with solid lines.

in Fig. 1, these cloned 16S rRNA sequences, except for the forward region of clone 13 having multiple single mutations, showed as mosaics containing some part of each *H. ganmani* and strain MIT 01-6451 sequence.

Discussion

The fastidious nature of most *Helicobacter* species and the coexisting intestinal flora hinder their routine isolation from the contents of the intestine. Although histological methods such as immunohistochemistry or fluorescence in situ hybridization (FISH) are options for detection of *Helicobacter* species to visualize morphologically intact microorganisms and their spatial distribution within the gastrointestinal tract [4, 23], these methods are laborious and suffer from cross-reactivity with closely related bacteria such as *Campylobacter* species. There is however a positive correlation between the results of PCR detection of the 16S rRNA gene and detection of organisms by immunohistochemistry or FISH [23]. We therefore selected a culture- and histological-independent approach, using PCR detection of the 16S rRNA gene, to detect *Helicobacter* species in this study.

Our results showed that *Helicobacter* species MIT 01-6451 and *H. ganmani* were present in mice received from domestic animal facilities at a high frequency. Goto *et al.* surveyed mice kept at academic and commercial animal facilities in Japan from 1998 to 1999 for *Helicobacter* infection and showed that, in addition to *H. hepaticus* and *H. rodentium*, *Helicobacter* strain MIT 95-2011, identified later as *H. ganmani*, and *H. westmeadii*-like organism were frequently present in the mice [14]. *H. westmeadii*-like organism might be strain MIT 01-6451, since the partial sequence of *H. westmeadii*-like organism shown in that report is different by only 1 nucleotide from that of MIT 01-6451. Furthermore, Taylor *et al.* indicated that only the mice obtained from animal facilities in Japan were infected with *Helicobacter* sp. MIT 01-6451 [31]. Consistent with these results, we detected strain MIT 01-6451 in only mice received from domestic animal facilities. Therefore *H. ganmani* and strain MIT 01-6451 appear to be the predominant *Helicobacter* strains infecting mice in Japanese animal facilities.

To determine some characteristics of strain MIT 01-6451, its distribution in infected mice was investigated. Strain MIT 01-6451 was abundantly detected in the colon and cecum of all mice, while lower amounts were present in the stomach, gallbladder, and other regions of the intestinal tract of some mice. These results suggest that the stomach and small intestine are more likely regarded as transition sites related to coprophagy rather than active colonization sites for MIT 01-6451. Since urease activity plays a crucial role in colonization of Helicobacters in low pH environments and the hepatobiliary system [12], further studies with pure culture are needed to determine the biochemical and morphological characteristics of this strain in detail. On the other hand, no macroscopic lesions in any organs of strain MIT 01-6451-infected mice were observed. Taylor *et al.*

similarly reported no apparent histopathology by strain MIT 01-6451 in the liver, gallbladder, or intestinal tract of infected mice [31]. However, both studies used C57BL/6 mice, a strain known to be resistant to the pathology caused by *Helicobacter* species. Furthermore, Ge *et al.* reported that coinfection of mice with enterohepatic *Helicobacter* species (*H. hepaticus* or *H. muridarum*) enhanced or attenuated the *H. pylori* gastric pathology by modulation of the host immune responses [11]. According to their results, the pathogenicity of *Helicobacter* sp. MIT 01-6451 also may modulate the pathology when mice are coinfected with other *Helicobacter* species. It is also possible that *Helicobacter* pathology could be caused by infection with other pathogenic microorganisms. Further infection studies are therefore needed to determine what the pathogenicity of strain MIT 01-6451 really is and what effect host immunity has on its ability to produce pathology.

The results of this study also show the detection of multiple unidentified *Helicobacter* species genes within a single mouse. It has been reported that sequence variation in the 16S rRNA gene can vary among isolates of a single species by up to 3–4% [32]. However, the cloned 16S rRNA gene sequences appear to be mosaics containing some part of each *H. ganmani* and strain MIT 01-6451 sequence. Furthermore, the forward region sequence of clone 13 showed the highest homology with the 16S rRNA gene sequence of a commensal bacterium, *Lachnospiraceae* bacterium, rather than that of *Helicobacter* species (data not shown). In *H. pylori*, recombination frequently occurs with type IV secretion systems and the nuclease components [29, 30]. In other bacterial species such as *Streptococcus anginosus*, lateral gene transfer has been demonstrated to occur [25]. These mosaic genes therefore might be created by lateral gene transfer of short gene segments either within *Helicobacter* species or between *Helicobacter* species and other bacterial species and represent the generation of *Helicobacter* subspecies via gene transfer and recombination among these bacteria in coinfected mice.

In conclusion, we have shown that strain MIT 01-6451 is the predominant strain infecting mice kept in animal facilities in Japan. This strain could be detected in the gastric and hepatobiliary systems as well as the lower intestinal tract. Furthermore, we detected multiple unidentified 16S *Helicobacter* species genes, although given the high level of sequence variation that can occur in the 16S rRNA gene, these may be sub-strains of *H. ganmani* and/or strain MIT 01-6451. These results suggest that *Helicobacter* species including unidentified species are still prevalent in SPF mice.

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