72°C for 1 min 30 s). The amplified DNA was analyzed following electrophoresis on a 2% agarose gel.

Teratogenicity and reproduction

The teratogenicity and reproductive toxicity study was carried out in accordance with internationally accepted guidelines[33] and in accordance with good laboratory practices. The studies were conducted by the research laboratory of Miyarisan Pharmaceutical Co., Ltd with technical support from Japan's Central Institute for Experimental Animals. All mice were acclimated to the rearing environment for 14–30 days and evaluated during the acclimation period. The housing conditions were 23 $\pm$ 2°C, 50 $\pm$ 10% humidity, 10–13 times/h air circulation and 12 h light/dark cycle (8:00 a.m. to 8:00 p.m.). Feed (CA-1, purchased from CLEA Japan Inc., Tokyo, Japan) and sterilized tap water were provided ad libitum.

A concentrated powder preparation of CBM 588® (Miyarisan Pharmaceutical Co., Ltd; lot no. 011, 2.4×10^9 colony-forming unit (CFU)/g) was used as the test substance. CBM® powder also contains pharmaceutical-grade lactose and corn starch as excipients and is manufactured under WHO good manufacturing practice (GMP) conditions. The powder was freshly suspended in water with 0.5% (w/v) carboxymethyl cellulose (CMC) salt prior to each administration.

Teratology. The teratology study was conducted with healthy 10-week-old male and 8-week-old nulliparous female ICR mice. Males (F_0) and females (F_0) were pair mated, and females were examined daily for evidence of a vaginal plug. Detection of the vaginal plug was determined as day 0 of pregnancy. Pregnant mice were separated, weighed, and randomly distributed into one of four feeding groups (37–38 females/feeding group).

Females in each feeding group were administered 0, 24, 240, or 4800 mg/kg BW of the CBM powder by oral gavage, daily, from days 6–15 of gestation. Total volume administered was adjusted for BW (0.15 mL/10 g BW). The mice were weighed daily, from gestational day 0, days 6–18, and then on postpartum days 0, 7, 14, and 21. Two-thirds of the pregnant F_0 mice (26 females/feeding group) were anesthetized and killed on day 18 of gestation. The abdominal wall was cut along the medioventral line to expose the uterine horn. The numbers of implantations, live fetuses, dead fetuses, and absorbed fetuses were recorded. The uterine horn was removed, and the viable fetuses were examined for external abnormalities, weighed, and sexed. A gross visceral examination of the major thoracic/abdominal organs (lung, liver, spleen, kidneys, adrenal gland, testis, and ovaries) was conducted in the viable F_1 fetuses, followed by fixation with 95% ethanol and alizarin red staining[34] to examine the skeletal structures.

The remaining one-third of the pregnant F_0 mice (11–12 females/feeding group) were continuously

Table 1. CBM588® antibiotic susceptibility.

Antibiotic	MIC (mg/L)
Penicillins	
Benzyl penicillin	0.25
Carbenicillin	4.0
Cephamycins	
Cefmetazole	2.0
Quinolones (generation)	
Nalidixic acid (1st)	32.0
Cinoxacin (1st)	64.0
Norfloxacin (2nd)	4.0
Ofloxacin (2nd)	1.0
Enoxacin (2nd)	2.0
Ciprofloxacin (2nd)	1.0
Lomefloxacin (2nd)	4.0
Tosufloxacin (3rd)	0.25
Lincomycins	
Lincomycin	4.0
Polypeptides	
Colistin	>256.0
Bacitracin	4.0
Carbapenems	
Imipenem	1.0
Cephalosporins (generation)	
Cephalothin (1st)	4.0
Cefazolin (1st)	1.0
Cefoperazone (3rd)	0.5
Ceftazidime (3rd)	32.0
Tetracyclines	
Chlortetracycline	0.06
Oxytetracycline	0.06
Doxycycline	0.03
Minocycline	0.015
Aminoglycosides	
Neomycin	128.0
Paromomycin	128.0
Amikacin	128.0
Macrolides	
Spiramycin	2.0
Others	
Fosfomycin	32.0

CBM 588®: *Clostridium butyricum* MIYAIRI 588®; MIC: minimum inhibitory concentration.

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Table 2. Sensitivity of CBM 588® to clinically relevant antibiotics.

	MIC (mg/L)			
	C. butyricum MIYAIRI 588®			
	Working cell bank: vial no. 191	Deposited strain: no. FERM BP-2789	*C. butyricum* ATCC 19398^T	EFSA 2012 break points for G+ bacteria
Ampicillin	0.25	0.25	0.25	1.0
Chloramphenicol	2.0	2.0	2.0	2.0
Clindamycin	1.0	1.0	0.5	0.25
Erythromycin	1.0	1.0	1.0	0.5
Gentamicin[a]	64.0	64.0	64.0	4.0
Kanamycin[a]	128	64.0	128.0	16.0
Metronidazole	1.0	2.0	1.0	4.0[b]
Streptomycin[a]	>256	256	256	8.0
Tetracycline	0.06	0.06	0.03	2.0
Vancomycin	1.0	1.0	1.0	2.0

G+: Gram positive; MIC: minimum inhibitory concentration; EFSA: European Food Safety Authority; EUCAST: European Committee on Antimicrobial Susceptibility Testing.
[a]Aminoglycoside antibiotics.
[b]No EFSA break point[36] exists for metronidazole, so EUCAST[37] values are given.

reared for spontaneous delivery in order to collect data on postnatal development in live fetuses, delivery conditions, nursing behavior, implantation site counts, and reproductive performance. All F_1 live mice were nursed through postpartum day 4. On postpartum day 4, any F_1 pups with external abnormalities were euthanized and subjected to a gross observation of thoracic/abdominal organs. The remaining F_1 pups were weighed daily and notes on general condition were taken. Examinations of pinna detachment (day 4), dorsal hair growth (day 11), tooth germ eruption (day 12), external acoustic pore (day 14), and eyelid opening (day 15) were conducted. Additionally, one male and one female were randomly selected from each litter to undergo observation for motor development. Mice not part of the reproductive study were euthanized on postpartum day 22. Gross observations were made of the same major thoracic/abdominal organs as in the F_1 fetuses.

Reproduction studies. A single male and female F_1 mouse from each litter were randomly selected and continuously reared for the F_1 reproductive examination. Non-siblings were pair mated, and females were monitored for the presence of a vaginal plug. F_1 dams were weighed, and feed consumption was recorded on days 0, 6, 15, 18, and 19. All established pregnancies were spontaneously delivered. Delivery conditions, nursing behavior, number of live births, and number of dead fetuses were examined in the F_2 generation. All live F_2 offspring were allowed to nurse until postpartum day 7 at which point all F_2 pups and remaining F_1 mice were euthanized, and gross observations were made of the major thoracic/abdominal organs.

Statistical analysis

Means and standard deviations were calculated for all data. Data were analyzed using a Student's *t*-test between each groups. In addition, χ^2 test was used for the parameters that were recorded as frequency of occurrence.

Results

CBM 588® lacks acquired antibacterial resistance

First, the MIC of several antimicrobials were determined for CBM 588® (Table 1). CBM 588® was susceptible to most antibiotics in all of the antimicrobial classes except the aminoglycosides. Additionally, there were a few antibiotics that also had high MIC values, such as cinoxacin (64 mg/L) and colistin (>256 mg/L). However, cinoxacin has been discontinued in the United States, and colistin is used primarily against respiratory infections in cystic fibrosis patients.[35]

Next, the susceptibility of CBM 588® to antibiotics in clinical use was determined (Table 2). CBM 588® was susceptible to all of the clinically

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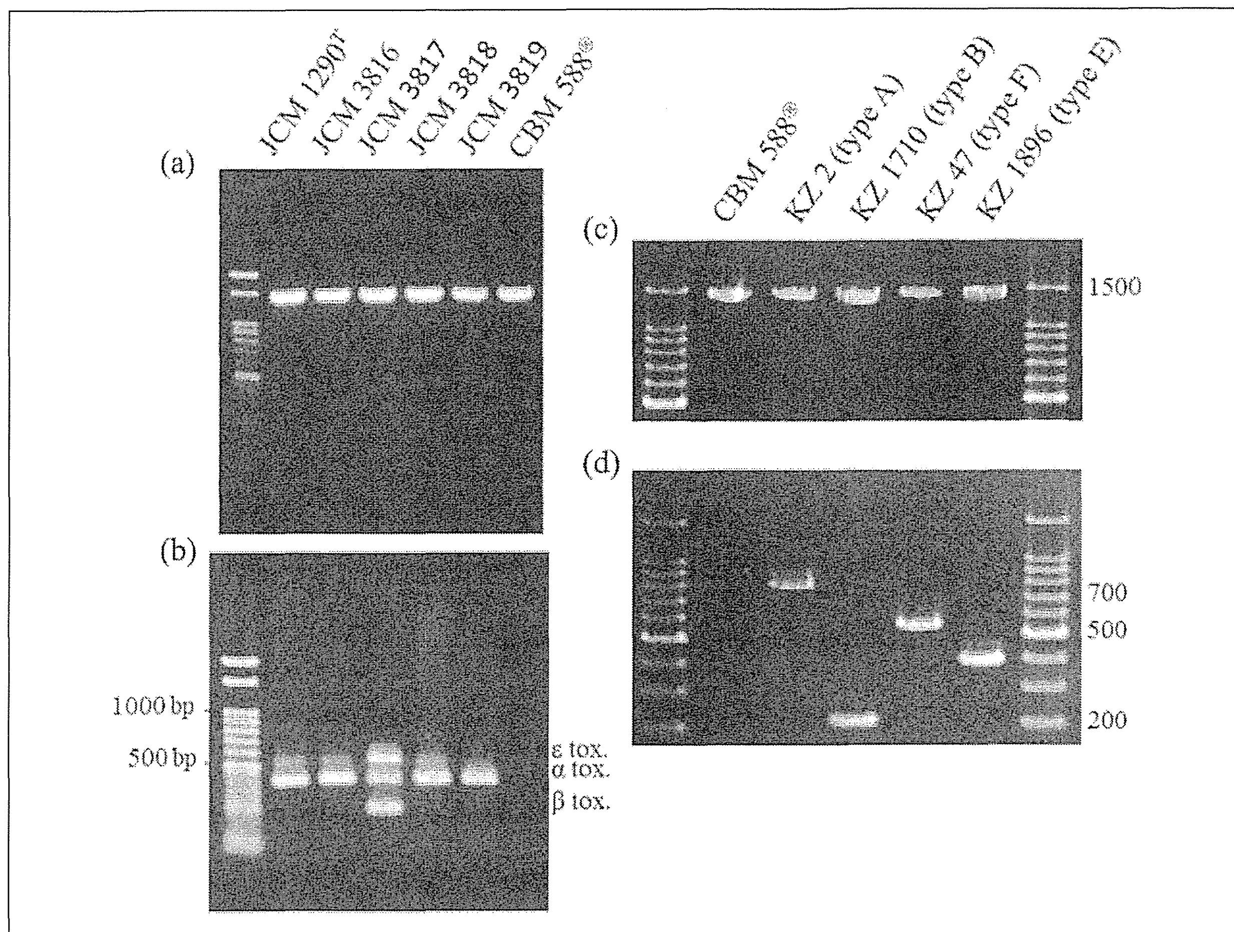

Figure 1. Demonstration of absence of genes encoding *Clostridium* toxins in CBM 588®. (a) Determination of 16S rRNA gene for *C. perfringens* with universal primers. (b) Detection of *C. perfringens* α, β, and ε toxins. All JCM strains show the presence of toxin genes, but CBM 588® does not. (c) Determination of 16S rRNA gene for *C. botulinum* with universal primers. (d) Detection of BoNTs: A, B, E, and F. All KZ strains show the presence of toxin genes, but CBM 588® does not. CBM 588®: *Clostridium butyricum* MIYAIRI 588®; rRNA: ribosomal RNA; JCM: Japan Collection of Microorganisms; KZ: Kanazawa University; BoNT: botulinum neurotoxin.

relevant antimicrobials used, with the exception of the aminoglycoside antibiotics (i.e. gentamicin, kanamycin, and streptomycin). European Food Safety Authority break points[36] for gentamicin, kanamycin, and streptomycin for Gram-positive bacteria are 4.0, 16.0, and 8.0 mg/L, respectively. The MIC values for CBM 588® against these antibiotics were 64.0, 128, and >256 mg/L, respectively. To determine whether these high MIC values were unique to the CBM 588® strain, the cryopreserved, deposited sample (FERM BP-2789) of CBM 588® as well as *C. butyricum* ATCC 19398^T were tested. Both the cryopreserved CBM 588® and the ATCC strain of *C. butyricum* were resistant to aminoglycosides, indicating that aminoglycoside resistance is not a specific trait of CBM 588®.

CBM 588® lacks Clostridium *toxins*

CBM 588® has thus far shown no evidence of pathogenicity, although the presence of toxins produced by other *Clostridium* species has not been fully assessed. Therefore, the genome of CBM 588® was analyzed for the presence of *Clostridium* toxins (Figure 1(b) and (d)). 16S rRNA[30] gene was amplified as an internal control for all PCR reactions (Figure 1(a) and (c)). The primers for all reactions are listed in Table 3.

Many strains of *Clostridium perfringens* produce α, β, and ε toxins and are known virulence factors. The *C. perfringens* JCM strains 1290^T, 3816, 3817, 3818, and 3819 were used as positive controls for α, β and ε toxin genes (Figure 1(b)). Sequence heterogeneity has been shown for α toxin gene that may affect the

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Table 3. Nucleotide sequences of primers.

Primer	Sequence	References
CPA (α toxin)		31
Forward	5′-GTTGATAGCGCAGGA CATGTTAAG-3′	
Reverse	5′-CATGTAGTCATCTGTT CCAGCATC-3′	
CPB (β toxin)		31
Forward	5′-ACTATACAGACAGATC ATTCAACC-3′	
Reverse	5′-TTAGGAGCAGTTAGAA CTACAGAC-3′	
CPE (ε toxin)		31
Forward	5′-ACTGCAACTACTACTC ATACTGTG-3′	
Reverse	5′-CTGGTGCCTTAATAGA AAGACTCC-3′	
Type A		32
CBMLA1	5′-AGCTACGGAGGCAGCT ATGTT-3′	
CBMLA2	5′-CGTATTTGGAAAGCTGA AAAGG-3′	
Type B		32
CBMLB1	5′-CAGGAGAAGTGGAGC GAAAA-3′	
CBMLB2	5′-CTTGCGCCTTTGTTTT CTTG-3′	
Type E		32
CBMLE1	5′-CCAAGATTTTCATCCG CCTA-3′	
CBMLE2	5′-GCTATTGATCCAAAAC GGTGA-3′	
Type F		32
CBMLF1	5′-CGGCTTCATTAGAGAA CGGA-3′	
CBMLF2	5′-TAACTCCCCTAGCCCC GTAT-3′	
16S rRNA gene		30
27F	5′-AGAGTTTGATCCTGGC TCAG-3′	
1492R	5′-GGTTACCTTGTTACGA CTT-3′	

rRNA: ribosomal RNA.

binding of the PCR primers. We confirmed the absence of α toxin gene using additional primers described by Siqueira et al.[38] (data not shown). The presence of α, β, or ε toxin genes was not detected in the genome of CBM 588®.

BoNT is a group of seven, serologically distinct toxins produced by many members of the *Clostridium* genus. Of these, A, B, E, and occasionally F are the main causes of botulinum poisoning in humans. The genomes of the positive control *C. botulinum* strains (*C. botulinum* KZ 2, KZ 1710, KZ 47, and KZ 1896) showed the genes encoding their respective toxins (toxin A, B, F, and E; Figure 1(d)). CBM 588®, however, did not contain any genes encoding for BoNT A, B, E, or F toxins. Additionally, nontoxic non-hemagglutinin (NTNH) protein is also present in all BoNT-producing bacteria.[39] The highly conserved NTNH gene is located directly upstream from the BoNT genes. The four positive control *C. botulinum* strains and CBM 588® were examined for the NTNH gene. The *C. botulinum* strains producing BoNTs also encoded for the NTNH gene, while CBM 588® lacked genes for BoNT A, B, E, or F as well as NTNH (data not shown). These results indicate that CBM 588® lacks *Clostridium* toxin virulence factors α, β, and ε toxins; types A, B, E, or F neurotoxins; and NTNH.

CBM 588® does not adversely affect reproduction or development

Dams were administered the CBM 588® via gavage at 0, 24, 240, or 4800 mg/kg BW, daily through days 6–15 of gestation. Evaluation of fetuses for gross visceral and skeletal malformations at day 18 of gestation along with monitoring live pups for survival, growth, and development were carried out on the resulting F_1 litters. Additionally, 10–11 mating pairs from the F_1 generation were followed to determine whether CBM 588® had any adverse effects on second generation development.

A total of three F_0 dams died during the experiment (Table 4). Two were in the 4800 mg/kg BW group and died due to technical error on days 10 and 15 of gestation. Tracheal damage and thoracic retention of the test substance were observed and the cause of death was determined to be intrathoracic pressure due to incorrect administration of the test substance. A control animal died on day 16 postpartum due to unknown causes. None of these deaths were attributed to CBM 588®. One dam was killed for necropsy since all live offspring were deceased on postpartum day 9. Body weights and feed intake were not affected by CBM 588® consumption.

CBM 588® did not produce any dose-related adverse effects on F_1 fetal survival. At 18 days gestation (approximately 1 day before expected delivery), no differences in placental remnants, reabsorption sites, sex ratio, dead embryos, or gross external or organ malformations were seen in CBM 588®-treated animals compared with the controls (Table 5). The dams receiving CBM588® at 240 mg/kg BW had

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Table 4. Effects of CBM 588® powder on maternal growth and survival.[a]

	Administration of CBM 588® powder			
	Control	24 mg/kg BW	240 mg/kg BW	4800 mg/kg BW
Pregnant female at initiation	37	38	37	37
Pregnant female at day 18	37	38	37	35
Parturition rate (*n*)	100% (11)	100% (12)	100% (11)	100% (11)
Dams at weaning[b]	10/11	11/12	11/11	11/11
Body weight (g)—day 0 (*n*)	28.6 ± 1.1 (26)	28.2 ± 1.0 (26)	28.4 ± 0.7 (26)	28.0 ± 1.4 (26)
Body weight (g)—day 0 (*n*)	27.8 ± 1.4 (11)	28.6 ± 1.2 (12)	28.5 ± 1.0 (11)	28.2 ± 0.8 (11)
Body weight (g)—day 18 (*n*)	60.0 ± 3.8 (26)	59.4 ± 3.0 (26)	59.0 ± 3.1 (26)	61.2 ± 4.4 (24)
Body weight (g)—day 18 (*n*)	56.6 ± 5.6 (11)	58.9 ± 1.8 (12)	57.8 ± 4.3 (11)	57.4 ± 3.6 (11)
Feed intake (g/day):				
Days 6–11	4.4 ± 0.7 (26)	4.7 ± 0.7 (26)	4.8 ± 0.6 (26)	4.7 ± 0.6 (26)
	4.1 ± 0.1 (11)	4.2 ± 0.3 (12)	4.1 ± 0.2 (11)	4.0 ± 0.3(11)
Days 11–15	5.3 ± 0.4 (26)	5.6 ± 0.4 (26)	5.9 ± 0.8 (26)	5.9 ± 0.8 (24)
	5.3 ± 0.2 (11)	5.3 ± 0.2 (12)	5.0 ± 0.2[c] (11)	4.9 ± 0.3[d] (11)
Days 15–18	6.3 ± 0.6 (26)	7.3 ± 0.8 (26)	6.6 ± 0.4 (26)	7.0 ± 0.6 (24)
	6.8 ± 1.2 (11)	6.4 ± 0.7 (12)	6.5 ± 0.8 (11)	6.3 ± 1.0 (11)
Body weight (g) postpartum:				
Day 0 (*n*)	34.7 ± 2.3 (11)	33.9 ± 2.0 (12)	33.7 ± 2.3 (11)	32.8 ± 1.8[c] (11)
Day 21 (*n*)	37.0 ± 3.7 (10)	34.8 ± 3.2 (11)	36.5 ± 5.2 (11)	38.8 ± 3.8 (11)

CBM 588®: *Clostridium butyricum* MIYAIRI 588®; BW: body weight; CFU: colony-forming unit; SD: standard deviation.
[a]Approximately 24 mg/kg BW: 5.8×10^7 CFU/kg BW; 240 mg/kg BW: 5.8×10^8 CFU/kg BW; 4800 mg/kg BW: 1.2×10^{10} CFU/kg BW.
[a]Number of live females/number of females allowed to carry litters to term.
[b]$p < 0.05$.
[c]$p < 0.01$.

significantly fewer live pups/litter ($p < 0.05$) as well as fewer corpora lutea and implantations/dam compared with the control ($p < 0.01$). A small but significant ($p < 0.01$) increase in the weight of the live fetuses was seen in the litters from the dams receiving CBM 588® at 24 and 240 mg/kg BW. However, there were no significant adverse effects in any of these parameters in fetuses from dams receiving the highest dose, 4800 mg/kg BW CBM 588®.

CBM 588® did not negatively affect the development of the F_1 fetuses examined at day 18 of gestation (Table 5). The weight of viable fetuses, both male and female, was slightly higher in the litters from dams given 24 or 240 mg/kg BW CBM 588® ($p < 0.05$–0.01) compared with the controls. The skeletal malformation rate did not differ significantly in treated groups compared with the control group, with the exception of a statistically significant increase in the incidence of extra cervical ribs noted in F_1 fetuses from the group receiving CBM 588® at 4800 mg/kg BW compared with the control. Additionally, the number of ossified caudal vertebrae was higher in the fetuses in the 240 mg/kg group compared with the control group.

CBM 588® had no dose-related adverse effect on the survival or physical and behavioral development of the F_1 mice evaluated up to 21 days postnatal age (Table 6). No effect was seen on gestational length, live birth index, stillborn count, sex ratio, external abnormalities, day 4 survival rate, weaning rate, and physical or behavioral development. There was a significant reduction in BW of both male and female pups at day 21 from dams receiving CBM 588® at 240 and 4800 mg/kg BW compared with the control group (Table 6).

CBM 588® did not produce any dose-related adverse effects on the reproductive capabilities of the F_1 generation or the survival and growth of the F_2 generation (Table 7). There was no significant difference in gestation length, implantations, and number of live pups/litter in all test groups compared to control. F_1 dams fed with CBM 588® gained more weight between 3 and 11 weeks than the F_1 dams in the control group. All mating pairs copulated, but the fertility rate for animals receiving CBM 588® at 24 and 4800 mg/kg BW was only 90% compared to 100% for the control and 240 mg/kg BW CBM 588®. Additionally, all confirmed pregnancies were delivered, with the exception of one litter in the 240 mg/kg BW group.

CBM 588® did not alter the survival of the F_2 pups (Table 7) through day 7. One F_2 litter in the 4800mg/kg BW group was lost on postpartum day 5

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Table 5. Effects of CBM 588® powder on survival, growth, and development of F_1 fetuses.[a]

	Administration of CBM 588® powder			
	Control	24 mg/kg BW	240 mg/kg BW	4800 mg/kg BW
Corpora lutea (per litter)	16.0	15.6	15.0[b]	15.9
Implantations (per litter)	15.4	15.1	14.2[b]	15.3
Placental remnants	6	7	6	7
Resorption sites	5	6	7	6
Dead embryos	4	2	1	4
Fetal loss rate (%)	3.8	3.8	3.7	4.5
Live fetuses				
Total	386	377	355	351
Per litter	14.8	14.5	13.6[c]	14.6
Fetal sex ratio (female/total)	0.49	0.50	0.47	0.48
Viable fetal weight[d] (g):				
Female	1.35 ± 0.05	1.39 ± 0.08[c]	1.44 ± 0.07[b]	1.38 ± 0.10
Male	1.41 ± 0.05	1.48 ± 0.08[b]	1.52 ± 0.09[b]	1.45 ± 0.10
Skeletal malformation (per litter)				
Fusion of bone[e]	0	0	0.4%	0.4%
Cervical ribs	0.4%	0.4%	1.3%	6.0%[b]
14th rib	33.9%	37.6%	29.5%	41.7%
Decrease of presacral vertebra (19–25 vs. 26)	0.8%	1.2%	0.4%	2.7%
Skeletal variations (per litter)				
Delayed ossification of metacarpus	0	0	0	0
Delayed ossification of metatarsus	0	0	0	0
Number of ossified caudal vertebra	9.2 ± 1.3	9.8 ± 1.1	10.3 ± 1.2[c]	9.4 ± 1.5

CBM 588®: *Clostridium butyricum* MIYAIRI 588®; BW: body weight; CFU: colony-forming unit; SD: standard deviation.
[a]Approximately 24 mg/kg BW: 5.8×10^7 CFU/kg BW; 240 mg/kg BW: 5.8×10^8 CFU/kg BW; 4800 mg/kg BW: 1.2×10^{10} CFU/kg BW.
[b]$p < 0.01$: compared with the control group.
[c]$p < 0.05$: compared with the control group.
[d]Average and SD values of female and male F_1 mice in each group were calculated with the average BW of two to six mice randomly selected per litter.
[e]Fusion of occipital bone with atlas or 7th cervical vertebra with 1st thoracic vertebra.

due to neglect by the F_1 dam. No statistically significant dose-related malformations were noted in the F_2 pups. BWs of male and female F_2 pups were also unaffected by CBM 588® administration.

Discussion

CBM 588® is used as a probiotic in Japan for both humans and animals and is approved as a probiotic for animal feed[40–42] and as a novel food[22] in Europe. CBM 588® is the first *Clostridium* species and strain to be used effectively as a probiotic.[17] However, because there are many pathogenic *Clostridium* species, it is essential that the safety and toxicity of CBM 588® is thoroughly assessed.

The increasing prevalence of antibiotic resistant strains of bacteria is a global public health concern. Probiotics should have minimal antibiotic resistance for clinically relevant antibiotics. The MICs of several antibiotics were determined (Table 1), and CBM 588® is susceptible to most classes of antibiotics with the exception of aminoglycosides. However, the resistance to aminoglycosides, in anaerobic bacteria, is a physical, intrinsic feature rather than genetic or metabolic property of anaerobic bacteria due to the inability of anaerobes to transport these antibiotics across the microbial cell membrane.[43] In addition to the aminoglycosides, there were a few specific antibiotics with elevated MICs. Cinoxacin (MIC = 64 mg/L) has been discontinued in the United States. Colistin, also known as polymixin E, (MIC = >256 mg/L) is used intravenously, primarily as a drug of last resort in multidrug-resistant, Gram negative bacterial infections in the respiratory tract of cystic fibrosis patients.[35] The susceptibility of CBM 588® to clinically relevant antibiotics was also assessed (Table 2). CBM 588® was resistant to gentamicin, kanamycin, and streptomycin, all aminoglycosides. The nonpathogenic

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Table 6. Effects of CBM 588® powder on survival, growth development of F_1 mice.[a]

	Administration of CBM 588® powder			
	Control	24 mg/kg BW (low)	240 mg/kg BW (medium)	4800 mg/kg BW (high)
Survival				
Length of gestation (day)	18.4 ± 0.5	18.4 ± 0.5	18.4 ± 0.5	18.6 ± 0.5
Implantations per litter	14.4 ± 1.6	15.8 ± 1.9	15.9 ± 1.6[b]	15.4 ± 0.8
Total live newborns per litter	13.5 ± 1.9	14.6 ± 1.9	15.2 ± 1.4[b]	14.4 ± 1.6
Dead pups at birth	0.0 ± 0.0	0.6 ± 1.0	0.1 ± 0.3	0.0 ± 0.0
Live newborn rate (%)	93.6 ± 6.5	92.8 ± 7.2	95.6 ± 4.6	92.3 ± 7.1
Sex ratio (female/total)	0.57 ± 0.18	0.46 ± 0.15	0.50 ± 0.16	0.46 ± 0.10
Live newborns nursed per litter	13.5 ± 1.9	14.5 ± 1.7	15.2 ± 1.4[b]	14.3 ± 1.7
Survival rate—postnatal day 4 (%)[c]	94.3 ± 9.8	94.8 ± 12.2	95.7 ± 5.5	94.2 ± 11.1
Survival rate—weaning (%)[d]	96.2 ± 8.4	86.4 ± 30.8	92.0 ± 12.8	96.6 ± 8.1
Growth and physical developments				
Number of F_1 litters:				
Postnatal day 0	11	12	11	11
Postnatal day 21	10	11	11	11
Female body weight day 0 (g)[e]	1.49 ± 0.12	1.41 ± 0.12	1.44 ± 0.10	1.42 ± 0.12
Male body weight day 0 (g)[e]	1.57 ± 0.12	1.50 ± 0.11	1.50 ± 0.11	1.54 ± 0.12
Female body weight day 21(g)[e]	10.57 ± 0.64	9.78 ± 1.30	8.48 ± 1.53[b]	8.68 ± 1.21[f]
Male body weight day 21(g)[e]	10.84 ± 0.84	10.32 ± 1.31	9.08 ± 1.80[f]	9.06 ± 1.06[b]
Pinna detachment—day 4 (%)	86.7 ± 19.2	72.9 ± 41.9	80.7 ± 40.1	90.9 ± 26.3
Hair growth—day 11 (%)	100.0	100.0	100.0	100.0
Tooth eruption—day 12 (%)	100.0	100.0	100.0	100.0
Opening of external auditory canals—day 14 (%)	98.9 ± 3.8	100.0	100.0	100.0 ± 0.0
Opening of eyes—day 15 (%)	100.0	97.7 ± 7.5	97.7 ± 5.0	97.7 ± 7.5
Behavioral developments/pups examined (female, male)				
Pivoting—day 4	11/11, 10/11	12/12, 12/12	11/11, 11/11	11/11, 11/11
Righting reflex—day 6	11/11, 11/11	12/12, 12/12	11/11, 10/11	11/11, 11/11
Crawling—day 7	11/11, 11/11	11/11, 11/11	11/11, 11/11	11/11, 11/11
Walking—day 10	11/11, 11/11	11/11, 11/11	11/11, 11/11	11/11, 11/11
Cliff avoidanceday 11	11/11, 11/11	11/11, 11/11	11/11, 11/11	11/11, 11/11
25° slant negative geotaxis—day 8	11/11, 11/11	11/11, 11/11	11/11, 11/11	11/11, 11/11
45° slant negative geotaxis—day 13	11/11, 11/11	11/11, 11/11	11/11, 11/11	11/11, 11/11

CBM 588®: *Clostridium butyricum* MIYAIRI 588®; BW: body weight; CFU: colony-forming unit; SD: standard deviation.
[a]Approximately 24 mg/kg BW: 5.8×10^7 CFU/kg BW; 240 mg/kg BW: 5.8×10^8 CFU/kg BW; 4800 mg/kg BW: 1.2×10^{10} CFU/kg BW.
[b]$p < 0.05$: compared with the control group.
[c]Live F_1/live newborn nursed.
[d]Live pups at postnatal day 22/live pups at postnatal day 4.
[e]Average and SD values of female and male F_1 mice in each group were calculated with the average BW of two to six mice randomly selected per litter.
[f]$p < 0.01$: compared with the control group.

C. butyricum ATCC 19398ᵀ strain was also resistant to these antibiotics, indicating that the resistance is not specific to CBM 588® but similar across multiple *C. butyricum* strains. Aside from the aminoglycosides, CBM 588® does not show resistance to any class of antibiotics.

CBM 588® does not produce α, β, or ε toxins commonly associated with *C. perfringens*, or BoNTs A, B, E, or F (Figure 1). While it is known that some toxin genes are located on plasmids, it has been reported that CBM 588 has only one plasmid and critical genes, such as toxin genes or antibiotic resistance genes, relevant to safety were not found.[44] The only toxin *C. butyricum* has been reported to produce is BoNT type E.[45,46] BoNT type E producing *C. butyricum* have been isolated from soil[47] and suspect food

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Table 7. Effects of CBM 588® powder on F_1 reproduction and survival and growth of F_2 mice.[a]

	Administration of CBM 588® powder			
	Control	24 mg/kg BW (low)	240 mg/kg BW (medium)	4800 mg/kg BW (high)
Reproductive performance of F_1 mice				
Female weight gain (from 3–11 weeks, %)	153.3 ± 16.0	174.8 ± 25.2[b]	228.5 ± 89.0[b]	230.9 ± 56.7[c]
Length of gestation (days)	18.7 ± 0.5	18.7 ± 0.5	18.7 ± 0.5	18.6 ± 0.5
Fertility rate (%)	100	90	100	90
Parturition rate (%)	100	100	90	100
Implantations per litter	16.4 ± 2.2	15.2 ± 3.0	15.0 ± 1.9	15.6 ± 1.3
Live newborns per litter				
Total	14.9 ± 2.6	14.4 ± 3.1	13.7 ± 2.2	15.0 ± 1.4
Female	7.6 ± 1.5	7.2 ± 3.4	6.4 ± 2.3	8.7 ± 1.6
Male	7.3 ± 2.0	7.2 ± 3.1	7.2 ± 2.4	6.3 ± 2.0
Newborn sex ratio (female/total)	0.51 ± 0.08	0.50 ± 0.20	0.47 ± 0.15	0.58 ± 0.12
Dead pups at birth	0	0	0.3 ± 0.5[b]	0.2 ± 0.4
Malformed live newborns (open eyelid)	1 (0.7%)	0	2 (1.5%)	1 (0.8%)
Live newborn rate (live newborn/implantations) (%)	91.0 ± 11.9	94.4 ± 5.0	92.0 ± 5.4	96.5 ± 5.4
Postnatal observations in F_2				
No. of live newborns nursed	14.8 ± 2.7	14.4 ± 3.1	13.4 ± 2.1	14.9 ± 1.6
No. of live pups—postnatal day 7:				
Total	14.4 ± 2.8	13.9 ± 3.0	13.4 ± 2.1	12.9 ± 5.2
Female	7.3 ± 2.0	6.9 ± 3.4	6.4 ± 2.3	7.3 ± 3.2
Male	7.1 ± 1.8	7.0 ± 3.2	7.0 ± 2.3	5.6 ± 2.8
No. of dead in postnatal day 1-7	0.4 ± 1.0	0.6 ± 0.5	0.0 ± 0.0	2.0 ± 4.6
Body weight of F_2 mice				
Female body weight at birth (g)[d]:	1.62 ± 0.12	1.60 ± 0.17	1.62 ± 0.10	1.64 ± 0.05
Male body weight at birth (g)[d]:	1.70 ± 0.13	1.69 ± 0.18	1.73 ± 0.12	1.71 ± 0.08
Female body weight (g)—postnatal day 7[d]	3.50 ± 0.61	3.65 ± 1.00	3.69 ± 0.61	3.28 ± 0.38[e]
Male body weight (g)—postnatal day 7[d]	3.98 ± 0.92	3.70 ± 0.92	3.97 ± 0.52	3.52 ± 0.42[e]

CBM 588®: *Clostridium butyricum* MIYAIRI 588®; BW: body weight; CFU: colony-forming unit; SD: standard deviation.
[a]Approximately 24 mg/kg BW: 5.8×10^7 CFU/kg BW; 240 mg/kg BW: 5.8×10^8 CFU/kg BW; 4800 mg/kg BW: 1.2×10^{10} CFU/kg BW.
[b]$p < 0.05$: compared with the control group.
[c]$p < 0.01$: compared with the control group.
[d]Average and SD values of female and male each group were calculated with the average BW of all live pups per litter.
[e]Data were calculated based on eight litters since all pups from one litter died by postnatal day 5 due to maternal neglect.

in a botulism outbreak in China[48] as well as from the feces of two infants suffering from botulism in Italy.[49–51] The strains producing the type E toxin were confirmed as *C. butyricum* by 16S rRNA gene sequencing.[52] Due to the sequence homology (97%) of the type E toxin produced by *C. butyricum* and *C. botulinum*,[53] it is likely that *C. butyricum* acquired the toxin gene (*bont*/E) through horizontal gene transfer. However, genetic analysis of the toxins from the C. *butyricum* isolated from Italy and China represent two distinctive subtypes, indicating that two distinct horizontal gene transfer events occurred.[54–56]

Although possible, horizontal genetic transfer of type E toxin has proven to be very difficult to induce in a laboratory setting since it is well known that *Bont*/E is chromosomally located. However, one study has shown type E toxin can be stably transferred from a toxigenic *C. butyricum* BL 5839 strain to a nontoxigenic *C. botulinum* type E-like S-5 in the presence of a bacteriophage transducing agent and the "helper" strain, nontoxigenic *C. butyricum* ATCC 19398^T. The transfer rate was 1 in 10.[4,57] The transfer appears to be facilitated by a temperate prophage. Mature bacteriophages have been implicated in toxins C and D transfer between *C. botulinum* strains and have resulted in much higher transfer rates.[58] The low in vitro transfer rates, combined with the low frequency of type E producing *C. butyricum* isolated

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from food and soil, suggests that the chances of CBM 588® becoming toxigenic through horizontal gene transfer is very low.

The developmental toxicity of CBM 588® was assessed through two generations. F_0 dams were administered concentrated CBM 588® powder (2.4×10^9 CFU/g) at 0, 24, 240, or 4800 mg/kg BW, daily during days 6–15 of gestation. No dose-related adverse effects related to CBM 588® administration were noted. The resulting F_1 fetuses and live pups from dams given CBM 588® showed no adverse effects on physical or behavioral development. CBM 588® administration in the F_0 generation did not affect the survival and growth of the F_2 generation. The skeletal malformation rate did not differ significantly in treated groups compared with the control group, with the exception of a statistically significant increase in the incidence of extra cervical ribs noted in F_1 fetuses from the group receiving CBM 588® at 4800 mg/kg BW compared with the control. Supernumerary or accessory ribs (SNR), either lumbar or cervical (CR), are a common finding in standard developmental toxicology bioassays. The biological significance of these anomalies has been the subject of some debate in the literature. In rodents, the spontaneous incidence of SNR is species and strain related and ranges from <1% to >30%.[59] In this study, the incidence of CR was within reported historical range, and there were no associated changes in any other skeletal parameters supporting the conclusion that this finding is not related to test article administration.

Conclusion

These data support the safe use of CBM 588® as a probiotic for use in food supplements and animal feeds. CBM 588® is susceptible to most antibiotics, with the exception of amnioglycosides, which are known to have limited effect on anaerobes. The genome of CBM 588® lacks genes encoding known *Clostridium* toxins. Additionally, CBM 588® does not produce developmental or reproductive toxicity in mice.

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Authors' Note

Authors KI and KO contributed equally to this work.

Conflict of interest

KI, KO, MS, KW, SN, MT and MT are employees of Miyarisan Pharmaceutical Co. Ltd., Tokyo, Japan.

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Low Prevalence and Incidence of *Helicobacter pylori* Infection in Children: A Population-Based Study in Japan

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Keywords
children, epidemiology, *Helicobacter pylori* infection, family.

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Abstract

Background: Infection of *Helicobacter pylori* mainly occurs in childhood. In Japan, incidence of gastric cancer is still high in the senior citizen population, but little is known about the current *H. pylori* infection status among children or their family members.
Methods: As a population-based study, the prevalence of *H. pylori* infection and change in infection status over a 1-year interval in children were determined. Family members of some participants were also invited to participate in the study to determine their infection status. All children of specific ages attending 16 schools in Sasayama, Hyogo Prefecture, were invited to participate. *H. pylori* infection was determined by the stool antigen test and diagnosis confirmed by polymerase chain reaction and the urea breath test.
Results: *Helicobacter pylori* prevalence was 1.9% among 689 children aged 0–8 years in 2010 and 1.8% among 835 children aged 0–11 in 2011. No feco-conversion was observed in 430 children aged 0–8 years (170 were aged 0–4 years) who provided follow-up stool samples after 1 year. The prevalence of infection was 6% (2 of 33) and 38% (6 of 16) in mothers of negative and positive probands ($p = .04$), respectively, and 12% (3 of 25) and 50% (8 of 16) ($p = .01$), respectively, in fathers.
Conclusion: *Helicobacter pylori* prevalence in Japanese children is approximately 1.8%, which is much lower than that reported in Japanese adults. New infection may be rare. Parent-to-child infection is thought to be the main infection route of the infrequent infection for children in Japan.

Helicobacter pylori is a major risk factor for gastric cancer. Subjects with *H. pylori* infection have more than a 20-fold increased risk of gastric cancer compared with those with no history of infection [1,2]. East Asian-type *H. pylori*, which has both strong pathogenicity and carcinogenicity, has a prevalence of more than 96% in the Japanese population [3], where gastric cancer is the second leading cause of cancer deaths.

Helicobacter pylori infection occurs mainly during childhood, especially under the age of 5 years [4–6], and *H. pylori* prevalence in adulthood depends on infection in childhood [7]. It is important to determine the status of current *H. pylori* infection in children including prevalence, incidence, and origin of infection, because such evidence can be used to expect the incidences of *H. pylori* related diseases including gastric cancer in future and can also be incorporated into a prevention strategy for gastric cancer that includes interrupting the infection to children.

The Sasayama study is a population-based project to investigate current *H. pylori* infection status in children and their families in one area of Japan. The local government and our research group are carrying out the study.

Subjects and Methods

The study was carried out in Sasayama, which is 60 km north–northwest of Osaka, the second largest city in

Japan and has a population of about 50,000. Some residents commute to the Osaka–Kobe area. Agriculture and tourism are the main industries, and the area is typical of a rural area in Japan.

Two cross-sectional studies with a 1-year interval were conducted. In 2010, children attending 16 schools (seven elementary schools, six kindergartens, and three nursery schools) in the area who were aged 0–8 years were all invited to participate in the study. Of 1299 invited children, 689 (53%) participated in the study. The participants were asked to provide stool samples in November to December. In 2011, a similar cross-sectional study was conducted. Children attending the same 16 schools who were aged 0–11 years were all asked to provide stool samples in November to December, irrespective of their participation in 2010. Of 1909 invited, 835 (44%) children participated. As the school grade in Japan is traditionally decided by the child's age (years) on April the 2nd, the age of the child was expressed as the age on that day in each year. The prevalence of *H. pylori* infection was determined using the stool samples.

As 439 children provided stool samples both in 2010 and 2011, their *H. pylori* infection status was analyzed as a cohort study with 1-year observation period.

To investigate the infection status of family members, all members of some families of children who provided stool samples were asked to participate in the study according to the results of the cross-sectional studies. All families with positive-index children were invited. Two to four families with negative probands were selected as control families for each family of a positive proband, so that the probands were in the same class or in the same grade and school. The sample collection was carried out a few months after the cross-sectional studies. In 12 families who agreed to join the study, a sibling of a positive proband and 16 siblings of negative proband provided stool samples not for the family study but for either of the cross-sectional studies. They were included in the analyses.

Written informed consent was obtained from parents or guardians of all participants, and the study was approved by the ethical committees of the three medical schools, to which the researchers belonged.

Diagnosis of *Helicobacter pylori* Infection

Stool samples were obtained from each subject for determination of the *H. pylori* antigen using TestMate Pylori Antigen EIA (Wakamoto Pharmaceutical Co., Ltd., Tokyo, Japan). The cutoff value was 0.1 according to the manufacturer's instructions, and the kit showed 96.8% of sensitivity in 93 urea breath test (UBT) positive and 100% of specificity in 31 negative Japanese adults, when cutoff value was 0.1 [8]. In 2010, the detection of the *H. pylori* antigen for each stool sample was carried out twice using different extraction buffers, and diagnosis of *H. pylori* infection was carried out as detailed in the Fig. 1.

When both results of the stool antigen tests using two different buffers were <0.05, the test was defined as negative. In the other cases, *H. pylori* DNA was determined in the stool samples. The researcher who performed the analysis was blinded to the results of the stool antigen test. Total DNA was extracted from stool samples. A fecal sample (200 mg), 300 mg glass beads (GB-01; TOMY, Tokyo, Japan; diameter 0.1 mm), and 1400 μL buffer AE were mixed vigorously at 1500 rpm three times for 30 seconds using the Multi-Beads Shocker (MB755U; Yasui Kikai, Osaka, Japan) and incubated at 75 °C for 10 minutes. After incubation, the suspensions were mixed at 1500 rpm three times for 30 seconds, and DNA was extracted using the QIA Amp Stool kit (Qiagen, Venlo, the Netherlands) as described elsewhere [9]. The 16S rRNA gene of *H. pylori*-targeted primers was used for detection of *H. pylori* DNA by real-time polymerase chain reaction (PCR). PCR was performed in duplicate. Quantitative data were calculated from a standard curve generated by amplifying serial dilutions of a known quantity of amplicon. For this approach, the specificity of the PCR product was confirmed by a dissociation curve analysis (7500 quantification program; Applied Biosystems Inc.,

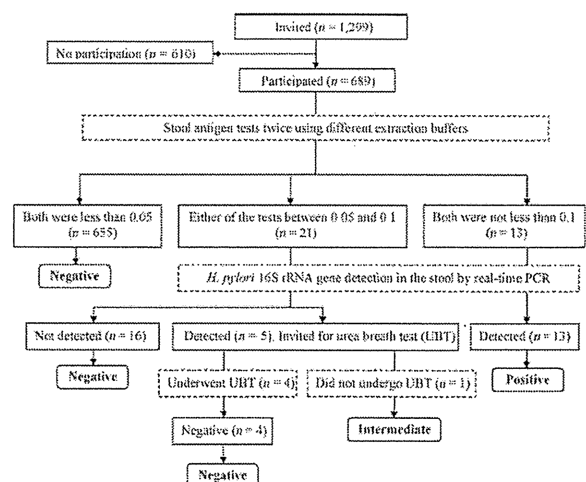

Figure 1 Among 1299 children invited to the study in 2010, 689 gave stool samples. The figure shows who underwent the further tests, which were measurement of *H. pylori* DNA in the stool samples and urea breath test. In consequence, 675 were *H. pylori* negative, 13 were positive and one was intermediate. See texts for more details.

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Carlsbad, CA, USA). *H. pylori* DNA was defined as present when an amount equivalent to not less than one bacterium per 1 g of stool sample was obtained.

When both results of the stool antigen tests were not <0.1, and *H. pylori* DNA was detected, the sample was defined as positive for *H. pylori*. When any of the stool antigen tests were <0.1, and *H. pylori* DNA was not detected, the sample was defined as negative for *H. pylori*. When any of the stool antigen tests were <0.1 and *H. pylori* DNA was detected, the children were invited to have a UBT, and diagnosis was decided according to the results of the UBT.

In 2011, the diagnostic procedure was the same as in 2010 except that the detection of the *H. pylori* antigen for each stool sample was carried out once using an extraction buffer that showed better concordance with final diagnosis in 2010. When the result of the stool antigen test was <0.05, it was defined as negative. In the other cases, *H. pylori* DNA was determined in the stool samples as before. In the stool antigen test, results not <0.1 were defined as positive, and results between 0.05 and 0.1 were defined as negative. When the stool antigen test was inconsistent with *H. pylori* DNA detection, the subject was invited for a UBT.

Statistical Analyses

The prevalence of *H. pylori* infection in each calendar year was calculated classifying children into 1-year age classes. The change in *H. pylori* infection status in subjects with a follow-up stool sample was also calculated. Fisher's exact test was used to determine the association between *H. pylori* infection in children and various family members.

Results

The Fig. 1 illustrates the results of the stool analysis in 2010, and the prevalence of *H. pylori* infection was 1.9% (13 of 688). In 2011, no sample gave results between 0.05 and 0.1 in the stool antigen test, and *H. pylori* DNA was detected in all stool samples that had *H. pylori* antigen test results not <0.1. The prevalence of infection was 1.8% (15 of 835). The prevalence of *H. pylori* infection in children according to age is shown in Table 1. No subject aged younger than 2 years was infected. The prevalence of infection in subjects aged 5 years and older was higher compared with that in children aged younger than 5 years.

Results of the analysis of 439 children who participated both in 2010 and 2011 are presented in Table 2. The majority (n = 430) were negative in 2010, one was intermediate, and eight were positive. No new infection was observed during the 1-year interval in the 430 noninfected children, including 170 aged 0–4 years. All eight children with a positive result in 2010 remained infected during the 1-year interval. Eight children (one 1-year-old, three 5-year-olds, and four 7- to 8-year-olds) who had negative stool antigen tests in both years had *H. pylori* -positive family members.

In the study on family members, 94 members of 20 families with *H. pylori* -positive probands were invited and 58 members of 17 families participated, while 331 ones of 68 families with negative probands were invited and 106 members of 35 families with negative probands participated. In detail, 17 (85%) mothers and 16 (80%) fathers of positive probands and 33 (49%) mothers and

Table 1 Prevalence of *Helicobacter pylori* in children

Age (years)	Prevalence in 2010		Prevalence in 2011	
0	0/19	0.0%	0/6	0.0%
1	0/29	0.0%	0/26	0.0%
2	0/36	0.0%	1/34	2.9%
3	0/62	0.0%	1/44	2.3%
4	1/120	0.8%	0/104	0.0%
5	5/134	3.7%	1/115	0.9%
6	2/89	2.2%	4/120	3.3%
7	2/109[a]	1.8%	1/71	1.4%
8	3/90	3.3%	1/100	1.0%
9			1/69	1.5%
10			4/82	4.9%
11			1/64	1.6%
Total	13/688[a]	1·9% (0.9%, 2.9%)[b]	15/835	1.8% (0.9%, 2.7%)

Data presented as number of positive subjects/total number of subjects and percentage.
[a]One subject with an intermediate result (see figure) was not included.
[b]95% confidence interval.

Table 2 Change in *Helicobacter pylori* infection status from 2010 to 2011 in children

Age (years)	Negative to negative	Intermediate to negative	Positive to positive
0	9	0	0
1	18	0	0
2	21	0	0
3	37	0	0
4	85	0	1
5	88	0	4
6	56	0	1
7	69	1	1
8	47	0	1
Total	430	1	8

25 (37%) fathers of negative probands participated. Twenty-one (88%) of invited 24 siblings of positive probands and 46 (49%) of invited 94 siblings of negative probands provided stool samples. No sample of the 164 family members gave results between 0.1 and 0.05, and *H. pylori* DNA was detected in all samples giving results not <0.1. The prevalence of *H. pylori* infection was significantly higher in mothers and fathers of positive-index children than in parents of those who were negative (Table 3). Such an association was not observed in siblings or grandparents.

Discussion

In this population-based study, the prevalence of *H. pylori* was 1.9% among those aged 0–8 years in 2010 and 1.8% among those aged 0–11 years in 2011. The prevalence was considerably lower than in the Japanese adult population (23% in those aged 20–29) [10,11], in children in other developing countries (17–63%) [12–15], and in East Asian countries (9–11%) [16,17]. The prevalence was lower than that determined in studies in Japanese children conducted several years ago: around 25% in Fukui in 1996–1997 [18], 9–17% in Aomori in 1999–2004 [19], and 4.0–6.7% in Tokyo in 2002–2003 [20].

The decline in the prevalence of infection may be because of the development of sanitary conditions [21]. In the Netherlands, the decline in *H. pylori* infection in children ceased at a prevalence of approximately 9% [22]. It is not clear whether the decline will continue or stop in Japan, but the prevalence in Japan has become considerably lower than in the Netherlands

Table 3 Association between *Helicobacter pylori* infection status in index children and their family members

Family member Index child	Positive/ tested (%)	Fisher's exact probability	Age (years) mean ± S.D.†	*p*-Value*
Mother				
Negative	2/33 (6.1)	0.041	37.7 ± 3.9	.979
Positive	6/16 (37.5)		37.8 ± 3.8	
Father				
Negative	3/25 (12.0)	0.012	39.7 ± 5.4	.697
Positive	8/16 (50.0)		40.4 ± 6.0	
Sibling				
Negative	1/46 (2.2)	0.548	7.0 ± 3.2	.968
Positive	2/20 (10.0)		7.1 ± 5.3	
Grandparent				
Negative	1/2 (50.0)	1.000	73.5 ± 2.1	.037
Positive	3/6 (50.0)		60.8 ± 6.2	

**p*-Value for the difference of mean of age by *t*-test.
†Standard deviation.

and may be the lowest of the publications to date with considerable sample sizes.

New infection was not observed during 1 year in 430 children aged 0–8 years, while all children with positive stool antigen tests also had positive results after 1 year. Transient infection has been reported in areas with a high prevalence of *H. pylori* infection [13,23] and even in Japan with a low prevalence [20,24], but no change in *H. pylori* infection status was observed in the current study. The reason of the contradictory results may be as follows. Basically, transient infection may occur in children aged under 3 or 5 years, when they were exposed to *H. pylori*. If *H. pylori* infection is not frequent, exposure to *H. pylori* may be infrequent and consequently transient infection is expected to be rare. Compared with the current study, prevalence of persistent *H. pylori* infection is higher in the two Japanese studies: around 3% by stool antigen in those aged 1–2 years [6] and around 6% by urine antibody in those aged 4 years [20]. If sample size of the current study had been larger, a few transient infection cases might have been observed. The results of the current study indicate that the spread of infection among children through child-to-child transmission is negligible, suggesting that eradication therapy for children is not necessary to prevent the spread of infection among children.

Helicobacter pylori infection in children was associated with infection in their mothers or fathers, but not in siblings or grandparents. This may partly arise because infection was rare in siblings and the sample size of grandparents was small, as fewer households in Japan now include both grandparents and children. In studies of intrafamilial infection, there is conflicting evidence on whether parents [24,25] or siblings are the main origin of infection [26,27]. In the current study, the prevalence of *H. pylori* infection among siblings was very low, and they were unlikely to be the origin of infection. Actually, plural infected siblings were observed in only one of 17 families with plural children including an infected child. Mothers and fathers may be the main origin of infection in this population. We have already reported that intrafamilial infection of *H. pylori* is possible by mother-to-child or father-to-child transmission in three families by multilocus sequencing type (MLST) analysis using fecal specimens. In two families, *H. pylori*-infected grandfathers were not the source of intrafamilial infection for children [9]. In developing countries, waterborne infection is the main infection route of *H. pylori* [28], while intrafamilial infection is the main infection route in developed countries [24–26]. In Japan, intrafamilial infection may also be the main infection route for children, as there are satisfactory water supplies and sewage systems, which

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would prevent waterborne diseases. *H. pylori* infection to children may be prevented by eradication therapy for the possible origins of infection, who are the infected family members, especially the parents.

A limitation of the current study is that the participation rate was rather low: 53% in 2010 and 44% in 2011. Although the reasons of nonparticipation were not clear, the subjects were asked not to give diarrheal stool as samples and infectious diarrhea cases were observed in the study area during the sample collection, which may be a reason. The low participation rates might have provoked sampling bias. Nevertheless, any effect of sampling bias may be small, because *H. pylori* prevalence did not differ between 2010 and 2011 and because those who were invited to take part in the study and their parents did not know their *H. pylori* infection status. Another limitation is the low participation rates in families with negative probands (37–49%), which indicates possibility of sampling bias. Those who were invited and participated in the study did not know their infection status, although they knew infection status of the probands. Siblings of negative probands showed similar *H. pylori* prevalence to that in cross-sectional analyses of the current study. In families with positive probands, participation rates (80–88%) were not low, which reinforces reliability of the results. Thus, the results of the current study are thought to reflect the *H. pylori* infection status in Japan.

As *H. pylori* prevalence did not increase among either children or adults in an 8-year birth cohort study [29], *H. pylori* prevalence in adulthood is determined by infection during childhood [7] also in Japan. Actually, gradual decrease in *H. pylori* prevalence with the birth years has been observed [30,31]. In the current study, 98% of children were negative for *H. pylori* infection, and their *H. pylori* prevalence is expected to change little for their lives. When Japan was a developing country, there was a high prevalence of *H. pylori* infection, and consequently, there is now a high incidence of gastric cancer in the population aged older than 70 years. A change from "most are infected" [30] to "most are not infected" seems to have occurred in Japan. It is expected that this change will exert several effects on health in Japan including a decrease in distal gastric cancer and peptic ulcer diseases. Although no increase in proximal gastric cancer or esophageal adenocarcinoma has been observed in Japan yet, attention should be paid to these, because these diseases increased with decrease of distal gastric cancer [32,33].

In conclusion, the prevalence of *H. pylori* in Japanese children may be around 1.8%, which is much lower than that reported in Japanese adults. New infection was not observed in children aged either 0–4 or 5–8 years over a 1-year period. The study indicated that parent-to-child transmission may be the main infection route of the infrequent infection for children in Japan.

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Author Contributions

The sponsors of the study had no role in the study design, data collection, data analysis, data interpretation, or writing of the report.

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Review Article

Biofilm Formation by *Helicobacter pylori* and Its Involvement for Antibiotic Resistance

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Bacterial biofilms are communities of microorganisms attached to a surface. Biofilm formation is critical not only for environmental survival but also for successful infection. *Helicobacter pylori* is one of the most common causes of bacterial infection in humans. Some studies demonstrated that this microorganism has biofilm forming ability in the environment and on human gastric mucosa epithelium as well as on *in vitro* abiotic surfaces. In the environment, *H. pylori* could be embedded in drinking water biofilms through water distribution system in developed and developing countries so that the drinking water may serve as a reservoir for *H. pylori* infection. In the human stomach, *H. pylori* forms biofilms on the surface of gastric mucosa, suggesting one possible explanation for eradication therapy failure. Finally, based on the results of *in vitro* analyses, *H. pylori* biofilm formation can decrease susceptibility to antibiotics and *H. pylori* antibiotic resistance mutations are more frequently generated in biofilms than in planktonic cells. These observations indicated that *H. pylori* biofilm formation may play an important role in preventing and controlling *H. pylori* infections. Therefore, investigation of *H. pylori* biofilm formation could be effective in elucidating the detailed mechanisms of infection and colonization by this microorganism.

1. Introduction

Helicobacter pylori is a spiral, microaerophilic, noninvasive, gram-negative bacterium that colonizes the human gastrointestinal tract, primarily the stomach [1]. *H. pylori* is one of the most common causes of human infection, especially in developing countries, where the incidence can be up to 90% of the population [2]. *H. pylori* infection often persists throughout life. This organism has been identified as an etiological agent of chronic active gastritis, peptic ulcer disease [3, 4], gastric adenocarcinoma [5], and mucosa-associated lymphoid tissue (MALT) lymphoma [6]. In addition, a working group of the World Health Organization International Agency for Research on Cancer concluded in 1994 that *H. pylori* is a group I definite carcinogen in humans [7]. Even though most individuals infected with *H. pylori* are asymptomatic, infected individuals form a high-risk population for the above-mentioned diseases. A number of factors such as the vacuolating cytotoxin, the *cagA* and *cag* pathogenicity island (*cag*PAI), motility, adhesins, and the urease enzyme are known to be involved in the virulence of this organism [8]. *H. pylori* exists in two morphological forms [9]. One is a spiral form and the other is a nonculturable but viable coccoid form. The spiral form is the most common form involved in colonization of the human stomach. It has been reported that, for survival under unsuitable conditions, this microorganism has the ability to convert its spiral form to the coccoid form [9–13].

Recently, some studies have alluded to the ability of *H. pylori* to form biofilms *in vitro* [14–16]. In addition, *H. pylori* can form biofilms on the human gastric mucosa [17–19]. Moreover, *H. pylori* could be embedded in drinking water biofilms on the surfaces of water distribution systems in developed and developing countries [20]. Therefore, a more thorough understanding of *H. pylori* biofilm should provide useful information for the characterization of this microorganism. In this review, several scientific observations including our research data on *H. pylori* biofilm formation will be described. In addition, a novel eradication strategy for *H. pylori* biofilm will be suggested.

2. Bacterial Biofilm Formation

Most bacteria live under severe nutrient-limited conditions. To protect themselves from hostile environmental influences, bacteria often form surface attached communities described as "bacterial biofilms." Biofilms are ubiquitous in natural, industrial, and clinical environments and have been shown to play a critical role in many chronic infections [21]. Biofilms are usually composed of multiple bacterial species. For example, dental biofilms (i.e., dental plaque) contain more than 500 different bacterial species [22]. Biofilms consist of viable microbial cells along with dead cells and a wide range of self-generated extracellular polymeric substances (EPS) including polysaccharides, nucleic acids (extracellular DNA from bacteria), and proteins [23]. The EPS matrix can constitute up to 90% of the biofilm biomass. The initial attachment is driven by hydrophobic or electrostatic interactions as well as specific bacterial surface molecules. The next step is multiplication of the bacteria and formation of microcolonies with EPS surrounding the microcolonies. In the third step (maturation step), the biofilm forms thick and mushroom-like or tower-like structures with increasing numbers of bacteria. Subsequently, the enlarged biofilm shows focal dissolution and liberates planktonic bacterial cells which can spread to other locations.

Biofilm bacteria exhibit distinct properties which differ from those of planktonic cells [24, 25]. One of these is an increased resistance to antimicrobial agents [26]. The susceptibility of biofilm cells to antimicrobial agents has been shown to differ from that of planktonic cultures [24] and this is a major contributor to the etiology of infectious diseases. In addition, another distinctive property is that biofilm cells exhibited different pattern of gene expression including the expression of virulence factor genes [27]. This property can involve a cell-to-cell communication system called quorum sensing (QS) [28]. The signaling molecules are known as autoinducers (AIs). When these molecules reach a critical threshold concentration, a signal transduction cascade is triggered. Signaling by AIs in the QS system forms the basis for alterations in various gene expressions including virulence factors, secretion system, motility, sporulation, and biofilm formation [29]. Three QS molecules were well characterized (oligopeptides, AI-1, and AI-2). Oligopeptides are produced by gram-positive bacteria and their action is species-specific. Many gram-negative bacteria utilize N-acyl-L-homoserine lactone (N-AHL) molecules as AI-1 signaling molecules [30], and these activities are also species-specific. A wide range of gram-positive and gram-negative bacterial species utilize AI-2 signaling molecules which are furanosyl borate diesters, and the enzyme responsible for their synthesis is encoded by the *luxS* gene [31, 32]. These AI systems play important roles in bacterial biofilm formation.

3. The Properties of *H. pylori* Biofilms

In an initial investigation on biofilm formation by *H. pylori* two studies characterized biofilm formation by this organism [14, 15]. As the first demonstration of the *in vitro* ability to form biofilms by *H. pylori*, Stark et al. reported that a water insoluble polysaccharide-containing biofilm has been observed at the air-liquid interface when *H. pylori* strain NCTC 11637 was continuously grown in a glass fermenter [14]. Subsequently, Cole et al. reported that all of the *H. pylori* strains used in their study, including clinical isolates, laboratory strains, and a mouse-adapted strain, were able to form biofilms on glass surfaces [15]. They also reported that *H. pylori* could form a biofilm only at the air-liquid interface, which is most likely indicative of its microaerobicity. However, at present, biofilm formation by *H. pylori* has not been extensively characterized. Therefore, we analyzed the ability of *H. pylori* strains to form biofilms and characterized the underlying mechanisms involved. Initially, we established a feasible and stable model for biofilm formation by this microorganism. Briefly, sterilized glass coverslips were placed into 12-well microtiter plates. Each well was filled with 2 mL of *Brucella* broth supplemented with 7% fetal calf serum (FCS) to allow adherence of *H. pylori* at the air-liquid interface. The formation of biofilms was initiated by inoculating approximately 5×10^5 cells into each well. The cultures were incubated under microaerobic conditions at 37°C for 3 to 5 days with shaking. Using this model, the biofilm forming ability of eight *H. pylori* strains including standard SS1, ATCC 43579, ATCC 43579, and NCTC11638 strains and clinical isolates from Japanese patients was analyzed. Under these conditions, all of the strains formed biofilms at the liquid-gas interface of the cultures. Specifically, strain TK1402, which was isolated from a Japanese patient with duodenal and gastric ulcers, showed significantly higher levels of biofilm formation relative to the other strains (Figure 1) [33]. The strong biofilm forming ability of TK1402 was reflected in the relative thickness of the biofilms. To clarify the architectural characteristics of *H. pylori* biofilms, we compared TK1402 and SS1 biofilms

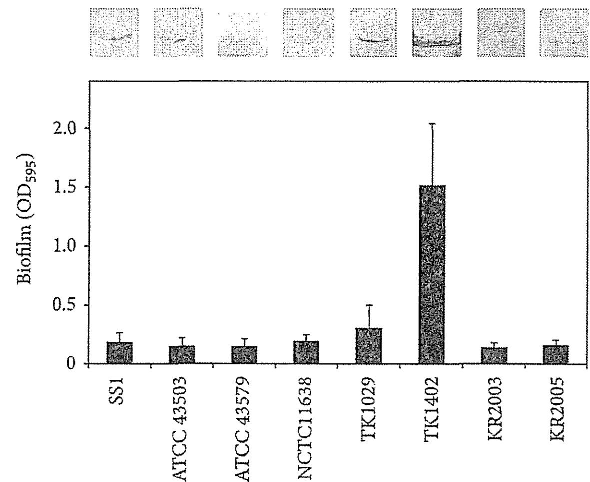

Figure 1: Biofilm formation by *H. pylori* strains. The graph shows quantification of biofilms formed after 3 days following culture in *Brucella* broth supplemented with 7% FCS. The upper photographs show typical biofilms on glass coverslips.