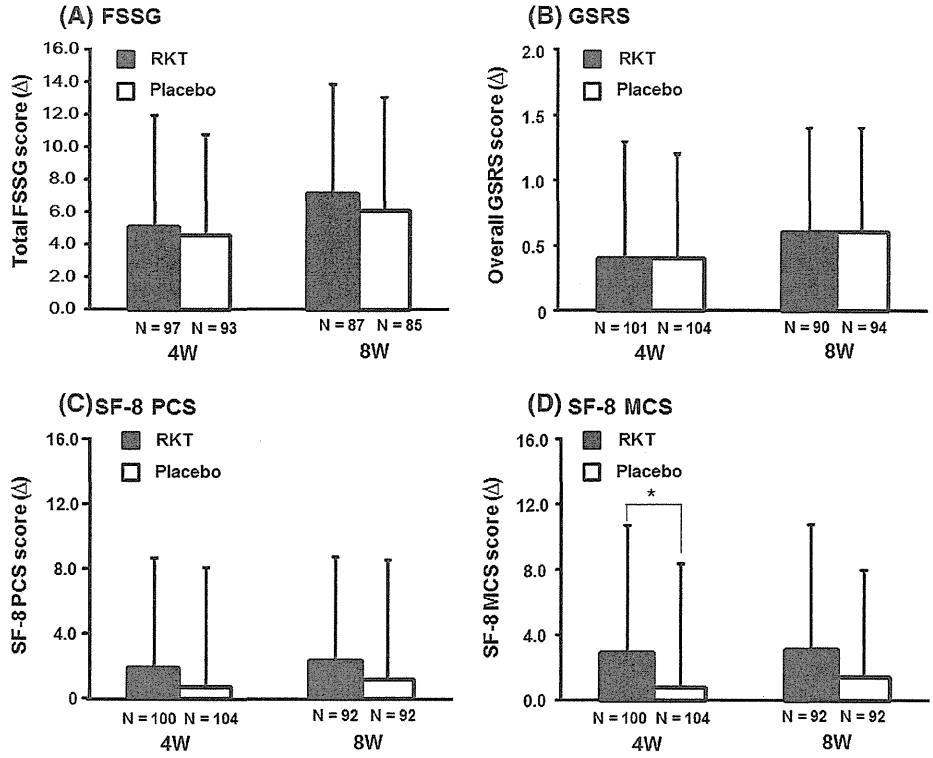

Fig. 2 Improvement degrees of total FSSG, total GSRS, and SF-8 scores after 4- and 8-week treatments in the RKT and placebo groups. *FSSG* frequency scale for the symptoms of GERD, *GSRS* Gastrointestinal Symptom Rating Scale, *SF-8* Short-Form Health Survey-8, *PCS* physical component summary, *MCS* mental health component summary. Values are expressed as mean ± SD. $*P < 0.05$ (Wilcoxon rank-sum test)

to confirm the definitive efficacy of RKT as the possible candidate for PPI-refractory NERD patients. After confirming the significant efficacy of RKT during 4- and 8-week treatments for GERD symptoms and QOL, we showed the efficacy of RKT for mental QOL scores (MCS scores of SF-8) after the 4-week treatment among the primary endpoints in patients with PPI-refractory NERD, compared to that for placebo treatment.

Patients suffering from GERD symptoms generally show decreased health-related QOL compared with that of the general population [30, 31]. In addition, low QOL levels in patients with GERD may be related to their long-lasting severe symptoms, as well as to their potential psychological factors [32]. RKT significantly suppressed increases in human plasma levels of adrenocorticotropic hormone and cortisol under stress conditions compared with placebo [33], which may suggest its efficacy for improving mental QOL scores. It has been reported that RKT reverses an increase in plasma levels of neuropeptide Y, a representative neurotransmitter of the sympathetic nervous system of the autonomic nervous system (ANS), to control those levels [34]. RKT also decreased afferent vagal nerve activity but increased the efferent activities of the gastric branches of the vagal nerve [35]. A clinical study using the gastric barostat method demonstrated that RKT may also improve stress-induced gastric hypersensitivity and/or changes in gastric wall tone [36]. These findings suggested that RKT has the potential to induce recovery effects for mental stress-associated ANS imbalances. On the other hand, there were no differences in GERD symptoms between the groups. Several factors may be responsible for these unexpected results. One factor is an excessively high placebo response rate observed in the present study. A meta-analysis of 24 GERD clinical trials showed an overall placebo response rate of approximately 19 % [37]. However, the placebo response rate in this study (42 %) was greater than that in the clinical trials reported so far. Several studies have shown high overlap among NERD, FD, and IBS [30, 38]. Because patients with FD or IBS responded highly to placebo treatment (about 40 %) in the clinical trials [37], we assume that overlapping patients with FD and/or IBS unfortunately might have been relatively highly included in our cohort than the other studies since preventing overlapping is very difficult in multicenter clinical trials. Another factor is that differences in duration of prior PPI mono-treatment should be considered. In addition, we have fully recognized the necessity for physiological testing using multichannel intraluminal impedance-pH monitoring to identify the respective pathophysiological types (considering a possibility of functional heartburn, etc.) related to persistent GERD symptoms after the standard PPI treatment. However, the difficulty of such physiological testing in each clinical institution made it challenging to achieve the second step

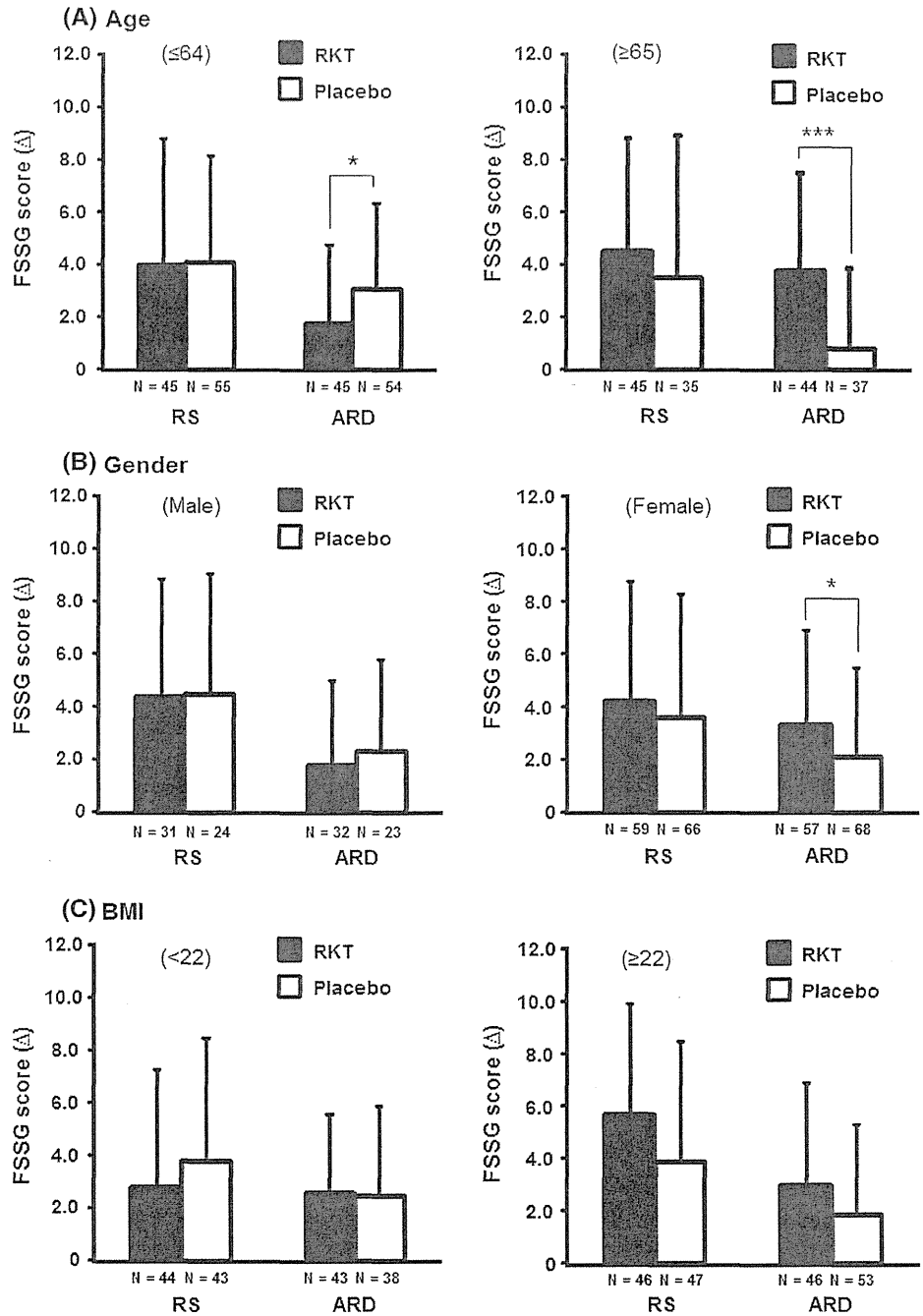

Fig. 3 Improvement degree in FSSG scores after 8-week treatment in the RKT and placebo groups. A–C show age (≤64 or ≥65 years), gender (male or female), and body mass index (BMI; ≥22 or <22) subgroup analyses. The FSSG questionnaire comprises 12 items: two domains of reflux symptom (RS) and acid-related dysmotility symptom (ARD). Values were expressed as mean ± SD. $^{*}P < 0.05$, $^{***}P < 0.01$ significant difference between each paired group (Wilcoxon rank-sum test)

of the pharmacological treatment rationale for NERD patients after the first step treatment using PPI. These points may be limitations of the present study as well as other similar clinical trials.

Subgroup analysis showed that the improvement degree of dyspeptic symptoms in the RKT group was significantly greater than that seen in the placebo group, especially for elderly and female patients. The pathogenesis of PPI-refractory NERD is characterized by multiple factors such as delayed gastric emptying [39], the presence of hiatal hernia [40], TLESR dysfunction [41], prolonged esophageal contraction [42], gas or bile acid reflux [43], visceral hypersensitivity [44], and psychological comorbidities [45] rather than acid reflux. Recent basic [16–18] and clinical [46] studies demonstrated pharmacological action of RKT similar to that of prokinetic agents. On the other hand, RKT improves acid regurgitation-associated circumstances in the lower esophagus by preventing decrease in tight

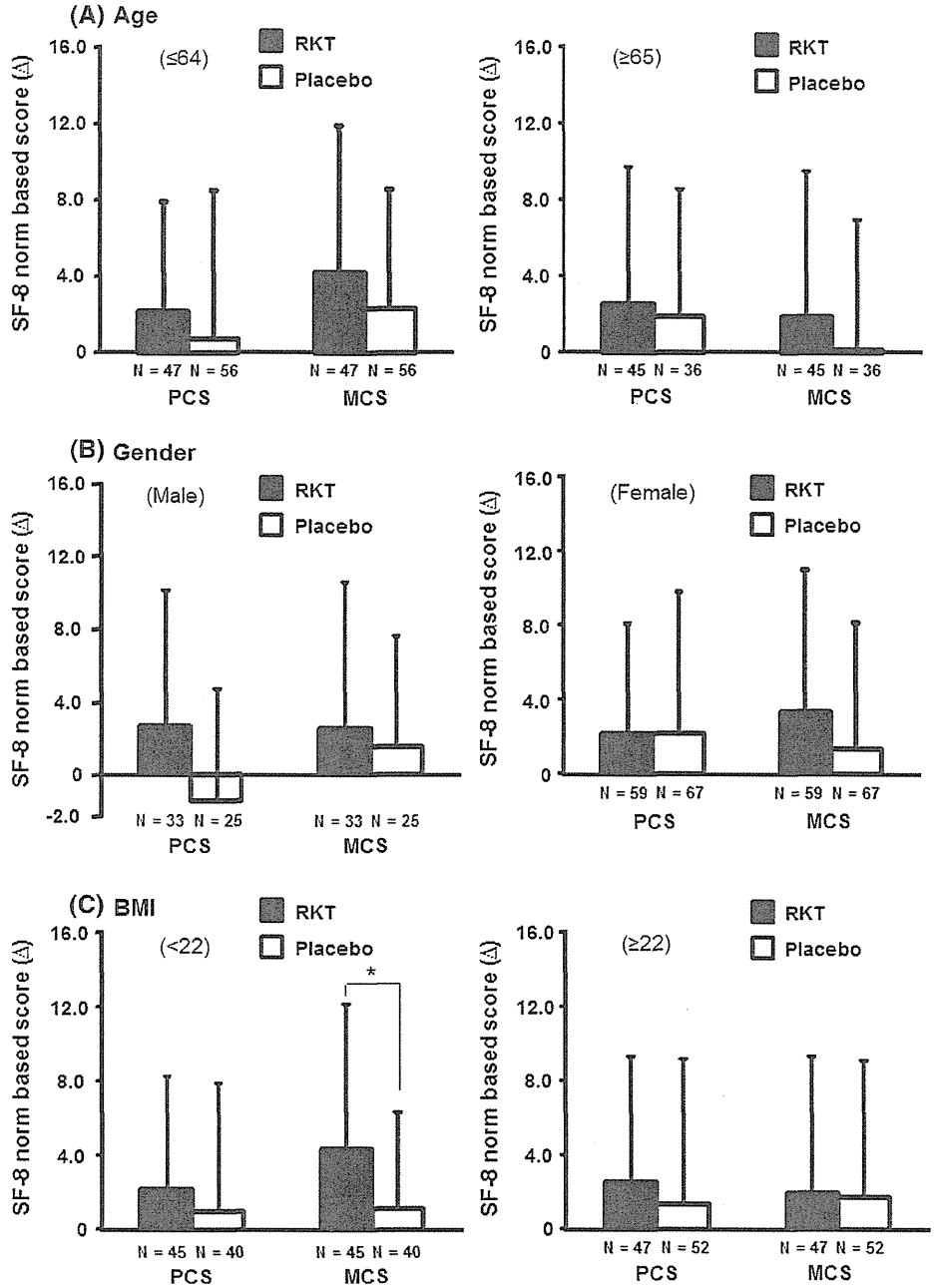

Fig. 4 Improvement degree in SF-8 scores after 8-week treatment in the RKT and placebo groups. A–C show age (≤64 or ≥65 years), gender (male or female), and body mass index (BMI; ≥22 or <22) subgroup analyses. *SF-8* Short-Form Health Survey-8, *PCS* physical component summary, *MCS* mental health component summary. Values were expressed as mean ± SD. $^*P < 0.05$ significant difference between each paired group (Wilcoxon rank-sum test)

junction proteins and an increase in the intercellular spaces in the epithelial mucosa [47]. RKT has a potent and differential absorption for bile salts [48]. Interestingly, RKT enhances the secretion of ghrelin, an appetite-stimulating peptide, as well as the reactivity of its receptor [44, 49], and restores GI motility by improving the ghrelin response in rat GERD models [50]. RKT also ameliorated age-associated anorexia by improving the ghrelin response in aged mice [51]. These reports may suggest the characteristic effects of RKT on ghrelin resistance and aging-associated symptoms. Thus, all of these findings suggest that RKT may relieve the dyspeptic symptoms in patients with PPI-refractory NERD via these mechanisms. However, it remains unknown why its efficacy was limited in these characteristic populations. Therefore, further investigations are required.

In conclusion, the present G-PRIDE study showed that RKT may be useful for improving mental QOL in patients with PPI-refractory NERD, especially for female and elderly patients.

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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.,

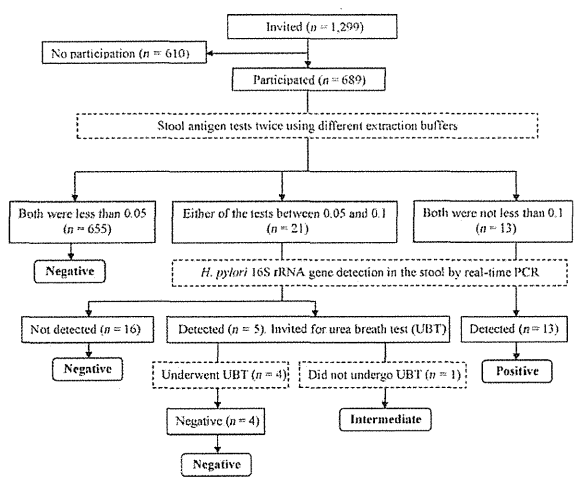

Figure 1 Among 1299 children invited to the study in 2010, 689 gave stool samples. The figure shows who underwent the further tests, which were measurement of *H. pylori* DNA in the stool samples and urea breath test. In consequence, 675 were *H. pylori* negative, 13 were positive and one was intermediate.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

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 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

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.

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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Evaluation of a stool antigen test using a mAb for native catalase for diagnosis of *Helicobacter pylori* infection in children and adults

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Non-invasive diagnosis of *Helicobacter pylori* infection is important not only for screening of infection but also for epidemiological studies. Stool antigen tests are non-invasive and are convenient to identify *H. pylori* infection, particularly in children. We evaluated the stool antigen test, which uses a mAb for native catalase of *H. pylori* developed in Japan. A total of 151 stool samples were collected from participants (52 children and 99 adults) of the Sasayama Cohort Study and stored between −30 and −80 °C. The stool antigen test used was Testmate pylori antigen (TPAg), and was performed according to the manufacturer's instructions. Furthermore, we conducted a quantitative real-time PCR test and compared the PCR results with those of the TPAg test. When compared with the results in real-time PCR, the sensitivity of TPAg was 89.5 % overall, 82.7 % for children and 92.4 % for adults, and the specificity was 100 %. The accuracy was 93.4 % overall, 90.4 % for children and 94.9 % for adults, and there was no significant difference in the accuracy of TPAg between children and adults. Five of 28 children (18 %) and five of 38 adults (13 %) were PCR positive with negative TPAg results. Four of five children with positive PCR and negative TPAg results were given a ^{13}C-urea breath test and all four children tested negative. No significant correlation was observed between the TPAg results and DNA numbers of *H. pylori* in faeces among children or adults. A stool antigen test (TPAg) using a mAb for native catalase is useful for diagnosis of *H. pylori* in children and adults. Additionally, this test has particularly high specificity.

INTRODUCTION

Since the discovery of *Helicobacter pylori* (Marshall & Warren, 1984), many studies have revealed that this bacterium causes gastritis, peptic ulcer diseases, gastric cancer and extragastric diseases such as idiopathic thrombocytopenic purpura or iron-deficiency anaemia (Choe *et al.*, 1999; Emilia *et al.*, 2001; Uemura *et al.*, 2001). *H. pylori* has spread throughout the world as a pathogen, and it was estimated in 2002 that about 60 million Japanese individuals are infected with this pathogen (Asaka, 2002). Recently, the infection rate in children and young adults has been decreasing in Japan. It is not practical to perform an upper gastrointestinal endoscopy for diagnosis of *H. pylori* infection for asymptomatic children or for epidemiological analysis. In addition, serum antibody tests are not recommended for younger children because of their low sensitivity (Leal *et al.*, 2008). A ^{13}C-urea breath test (UBT) can be performed on older children but not on young children (Leal *et al.*, 2011b). The stool antigen test is non-invasive and convenient to assess *H. pylori* infection, particularly for children. The Testmate pylori antigen (TPAg) enzyme immunoassay (EIA) utilizes a mAb to check for native *H. pylori* catalase (Wakamoto Pharmaceutical) with high specificity (Suzuki *et al.*, 2002a, b). In addition, an EIA is used for diagnosis of *H. pylori* and is more useful to determine the results of eradication therapy than a UBT (Shimoyama *et al.*, 2010).

The aim of this study was to examine in children whether a mAb-based stool antigen test is applicable to determine the presence of *H. pylori*. Furthermore we performed quantitative real-time PCR with 16S rRNA gene primers of *H. pylori* using faecal samples from children and adults.

Abbreviations: EIA, enzyme immunoassay; TPAg, Testmate pylori antigen; UBT, ^{13}C-urea breath test

METHODS

Stool samples. We conducted a retrospective study using stored samples from participants of the Sasayama Cohort Study in two consecutive years: 2010 and 2011. In brief, subjects of the study were recruited from seven elementary schools, six kindergartens and three nursery schools in Sasayama city, Hyogo, Japan, over the course of the two years. In 2010, 1299 children aged 0–9 years were asked to give stool samples and 689 participated in the study. In 2011, 835 of the 1909 children asked, aged 0–12 years, participated. Furthermore, we collected 109 stool samples from the staff of the elementary schools, kindergartens and nursery schools, and 103 stool samples were collected from the family members (adults) of the selected children during the course of the study. In this study, faecal specimens excluding diarrhoeal stool samples were collected. None of the adults recruited in this study had previously undergone eradication therapy for *H. pylori*. A faecal sample (~2–10 g) was collected from each child or adult and stored at between −30 and −80 °C until used. In this study, we chose the TPAg-positive and -negative stool samples from both children and adults.

Informed consent was obtained from all participants or parents. The research protocol was reviewed and approved by the Ethics Committee of the institution.

Stool antigen test. The TPAg EIA (Wakamoto Co.) was used for detection of stool antigen according to the manufacturer's instructions. Briefly, 30 mg faecal specimen was diluted with 1 ml diluent. Faecal solution (50 µl) of was added to each well and mixed with the reagent. After 30 min incubation at 25 °C, each well was washed five times with washing buffer. Substrate solution (100 µl of 3,3′,5,5′-tetramethylbenzidine with H_2O_2) was added to each well and mixed for 1 min. The reaction was terminated with 50 µl 0.5 M H_2SO_4. Absorbance at 450 nm/630 nm was measured by a spectrophotometer and the cut-off value of the test was taken as 0.100.

Bacterial strain and culture conditions. *H. pylori* TK1402 strain was used as standard strain for quantitative real-time PCR (Osaki *et al.*, 2006). *H. pylori* TK1402 was cultured under microaerobic conditions (AnaeroPack MicroAero A-28; Mitsubishi Gas Chemical Co.) at 37 °C on a *Brucella* plate containing 1.5 % agar and 7 % horse serum for 48 h and inoculated in *Brucella* broth containing 7 % horse serum. After an 18 h incubation under microaerobic conditions, 1 ml culture (OD_{600}=1.0) was collected.

DNA extraction and quantitative real-time PCR. *H. pylori* DNA was extracted from a cultured strain using a Wizard Genomic DNA Purification kit (Promega). One millilitre of an 18 h bacterial broth culture containing 1×10^8 c.f.u. *H. pylori*, quantified by microaerophilic cultivation, was centrifuged. The pellet was resuspended in 480 µl 50 mM EDTA, and 120 µl 400 µg lysozyme ml^{-1} was added followed by incubation of the mixture at 37 °C for 60 min. Subsequent steps were performed according to the manufacturer's instructions. Finally, 100 µl nuclease-free water was used for elution of the purified DNA.

DNA from stool samples was isolated using a QIAamp Stool kit (Qiagen) according to manufacturer's instructions, with some modifications. Briefly, 180–200 mg frozen faeces was suspended into 450 µl buffer ASL with 0.3 g glass beads (GB-01, diameter 0.1 mm; TOMY), and the suspension was mixed vigorously for 30 s using a Multi-beads Shocker (MB755U; Yasui Kikai) at 1500 r.p.m. three times at 30 s intervals. After a 5 min incubation at 75 °C, the suspension was mixed again in the same manner. After centrifugation at 14 000 *g* for 5 min, subsequent steps were performed according to the manufacturer's instructions. Two hundred microlitres of the DNA solution in buffer AE was eluted at the final step.

Quantitative real-time PCR of confirmed *H. pylori* antigen-positive faecal samples was done using *H. pylori* 16S rRNA gene-specific primers (16S2-F: 5′-CGCTAAGAGATCAGCCTATGTCC-3′; 16SB2-R: 5′-CCGTGTCTCAGTTCCAGTGTGT-3′) (Osaki *et al.*, 2012). The PCR was performed in duplicate or triplicate. First, two reaction wells were used for each sample. If the two wells showed positive or negative reactions, the PCR result was determined as positive or negative, respectively. If the two wells showed opposite results (positive and negative), the PCR was performed again using one more well. In this case, a positive result was determined according to at least two positive reactions for the sample in the PCR. Quantitative data were calculated from a standard curve generated by amplifying serial dilutions of a known quantity of amplicon. To confirm the specificity of the PCR product, a melting-curve analysis was done after amplification to distinguish the target PCR product from the non-target PCR product. The melting curves of PCR products were obtained by slow heating at temperatures in dissociation steps. The melting temperature (T_m) value of control strains was used to confirm PCR specificity. It was shown that the data with standard $T_m \pm 1$ °C were the same as the control product.

UBT. Children were fasted at least 4 h before the UBT and breath samples were collected before and 20 min after ingestion of ^{13}C-urea. The dosage of ^{13}C-urea was 100 mg for all ages. An infrared spectrometer (UBiT- IR300; Otsuka Electronics Co.) was used in this study and an increase of more than 3.5 ‰ was considered positive (Kato *et al.*, 2002).

Statistical analysis. Analyses were carried out separately among children and adults, and combined when necessary. Agreement between positive or negative results of the TPAg and PCR was evaluated. TPAg results were classified into three levels, and the mean DNA number of *H. pylori* in faeces was calculated for each level. Correlation between results of the TPAg and PCR was evaluated by calculating Spearman's rank correlation coefficient.

RESULTS

In the 2010 group, 13 and 676 children were identified as TPAg positive and negative, respectively. In the 2011 group, 15 and 820 children were identified as TPAg positive and negative, respectively. We chose 24 random samples (from children aged 2–12 years, mean 5.9 ± 2.0) of 28 positives and 28 random samples (from children aged 2–8 years, mean 5.0 ± 1.7) of 1496 negatives, and performed quantitative real-time PCR using 16S rRNA gene primers of *H. pylori*.

Of the stool samples obtained from the staff, 29 of 109 samples were positive by the TPAg. Concerning family members, 42 were positive and 61 were negative in the TPAg. Sixty-one of 71 positive stool samples and 38 of 141 negative stool samples obtained from adults (staff and family members) were chosen and used for quantitative real-time PCR using 16S rRNA gene primers of *H. pylori*.

The correlation between the results from the TPAg and PCR tests is presented in Table 1. All TPAg-positive samples from both children and adults were also shown to be positive by PCR. Five of 28 children (18 %) and five of 38 adults (13 %) revealed PCR-positive results with negative TPAg results. When compared with the results in real-time PCR, the sensitivity of TPAg was 89.5 %

Table 1. Correlation of the results of TPAg and PCR tests

	Children		Adults		
	PCR (+)	PCR (−)	PCR (+)	PCR (−)	Total
TPAg (+)	24	0	61	0	85
TPAg (−)	5	23	5	33	66
Total	29	23	66	33	151

overall, 82.7 % for children and 92.4 % for adults, and the specificity was 100 %. The accuracy was 93.4 % overall, 90.4 % for children and 94.9 % for adults, and there was no significant difference in the accuracy of the TPAg between children and adults. Among the subjects, four children with positive PCR and TPAg results were given a UBT and were found to be positive (Table 2). Four of five children with positive PCR and negative TPAg results were given a UBT and all four were negative (Table 2).

The relationship between TPAg values and the numbers of the *H. pylori* quantified by quantitative real-time PCR results and the DNA numbers of *H. pylori* in faeces is presented in Fig. 1. When samples were limited to the PCR-positive results, no significant correlation was observed between TPAg values and the numbers of *H. pylori* in faeces among children or adults.

DISCUSSION

In the present study, the accuracy of the TPAg for diagnosis of *H. pylori* infection in children was investigated and compared with the PCR results using *H. pylori* 16S rRNA gene-specific primers. All TPAg-positive stool samples agreed with the results of the PCR. However, in some cases, there were discrepancies in the test results between the TPAg and PCR results. In these cases, a UBT was performed for some of the children with discrepant results, and the results of the UBT agreed with those of the TPAg. Using PCR as a standard, it was demonstrated that the sensitivity and specificity of the TPAg were 89.5 % (85/95) and 100 % (56/56), respectively. However among PCR-positive children or adults, a significant correlation was not observed between the TPAg values and the numbers of *H. pylori* in faeces.

Table 2. Comparison of the results of TPAg, PCR and UBT tests for eight children

Case no.	Age (years)	TPAg (OD)	*H. pylori* DNA (g^{-1})	UBT (‰)
519	5	0.438	7037	32.7
509	5	0.790	3696	27.3
714	7	1.153	92686	25.4
X01	12	0.402	19023	33.9
325	3	0.019	7149	1.1
504	5	0.016	19811	1.5
631	6	0.021	5552	1.9
651	6	0.013	5574	0.0

The stool antigen test is non-invasive and convenient because of the ease of obtaining samples. It is used not only for diagnosis but also for epidemiological studies of *H. pylori* in children. Several types of stool antigen test have been used for the diagnosis of *H. pylori* infection, and in these tests EIA or immunochromatography is used with polyclonal or mAbs. However, the guidelines of the European Helicobacter Study Group state that the diagnostic accuracy of the stool antigen test is equivalent to the UBT if a validated laboratory-based mAb test is used (Malfertheiner *et al.*, 2012). In addition, the Japanese guidelines for the management of *H. pylori* infection also recommend the use of mAb stool antigen tests for confirming infection (Asaka *Leal.*, 2010).

Native catalase was identified as an antigen produced by *H. pylori* (Suzuki *et al.*, 2002a, b) and the TPAg uses a mAb against catalase. The TPAg does not react with bacterial antigens of other *Helicobacter* species or intestinal bacteria, whereas it reacts with antigens from most *H. pylori* clinical isolates (Sato *et al.*, 2012). The TPAg has been prepared for two types of testing: rapid TPAg (immunochromatography) and TPAg EIA. The rapid TPAg is reported to have a high accuracy for the diagnosis of *H. pylori* infection in asymptomatic children (Cardenas *et al.*, 2008), but a meta-analysis to evaluate the performance of stool antigen tests showed a low accuracy of one-step mAb tests (Leal *et al.*, 2011a). There are no reports concerning the TPAg EIA using a mAb to catalase for children. We used the stool antigen test for epidemiological study of *H. pylori* in Japanese children (Okuda *et al.*, 2001). In Japan, the prevalence of *H. pylori* infection has become particularly low (~5 %) (Okuda et al., 2001) and therefore a good diagnostic method to reduce incidences of false positives is needed. It is well known that quantitative real-time PCR is a very sensitive test to detect low numbers of *H. pylori*, regardless of viability. According to the results by the UBT for four children with TPAg-negative and PCR-positive results, it was clarified that the PCR-positive results were false positives. In contrast, the TPAg is not as sensitive as PCR in terms of detection of small numbers of *H. pylori*. The exact reason why 10 specimens from five children and five adults with TPAg-negative results were positive in the PCR test is not known, but it is possible that the PCR detected very low numbers of *H. pylori* or dead *H. pylori*, or transit passage of *H. pylori* without persistent infection in gastric mucosa.

In this study, we evaluated a TPAg comparing PCR using *H. pylori* 16S rRNA gene-specific primers. All 85 TPAg-positive stool samples obtained from 24 children and 61 adults agreed with the results of the PCR with 100 % specificity. This showed that the TPAg is an excellent method for epidemiological studies in low-prevalence area. However, 10 (five children and five adults) of the 66 TPAg-negative stool

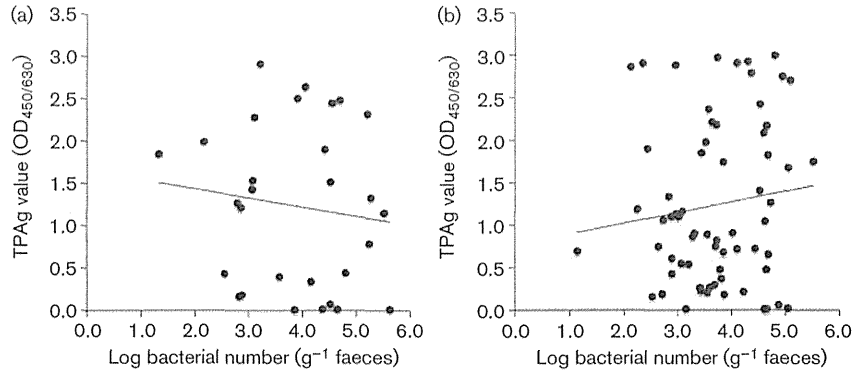

Fig. 1. Relationship between TPAg value and the number of *H. pylori* estimated by quantitative real-time PCR in faecal samples of 29 children (a) and 66 adults (b). Pearson's product moment correlation (shown by lines) was −0.122 (P=0.53) and 0.116 (P=0.35) among samples from children and adults, respectively.

samples were positive by PCR. This may show a low sensitivity of the TPAg, but we conducted a UBT for four of the six children with discrepant results from the TPAg-negative stool samples. The results of the UBT agreed with the results of the TPAg. As PCR can detect very low quantities of *H. pylori* DNA, this discrepancy may be due to transit passage of *H. pylori* in the intestinal tract. Ou *et al.* (2013) also reported that gastric mucosa fluorescence quantitative PCR (fqPCR) is more sensitive than routine histology, a rapid urease test, UBT alone or UBT in combination to detect *H. pylori* infection in children. One hundred and thirty-eight patients who underwent gastroscopy and a UBT were included in the study. Using the gastric mucosa fqPCR method of testing, 38 '*H. pylori* positive patients' tested positive, and additionally, eight (8 %) of the 100 'gold standard negative' children also tested positive (Ou *et al.*, 2013).

We have previously reported that intra-familiar infection of *H. pylori* is possible by mother-to-child or father-to-child transmission in three families by multilocus sequencing type analysis using faecal specimens (Osaki *et al.*, 2013). Furthermore, by using DNA specimens extracted from the *H. pylori* isolates, it was shown that the mother or father might be the main origin of intra-familiar infection of *H. pylori* (data not shown).

In this study, the correlation between TPAg values and the number of *H. pylori* evaluated by quantitative real-time PCR was examined, but no correlation was observed. It is likely that this result was based on the higher sensitivity of the quantitative real-time PCR assay for detection of *H. pylori*. It is also possible that the non-correlation between the results in the TPAg and real-time PCR might be due to the difference in the target molecules in these detection methods. In four representative cases with TPAg-positive/PCR-positive and four cases with TPAg-negative/PCR-positive results, the TPAg matched the UBT results. The TPAg is a clinically useful test in terms of its ease to perform.

Conclusion

In the present study, a stool antigen test (TPAg) using a mAb for native catalase was evaluated for diagnosis of *H. pylori* in children and adults, and the results were compared with those from a quantitative real-time PCR assay. It was demonstrated that the mAb-based TPAg had high sensitivity and 100 % specificity, indicating that TPAg is a useful method to detect *H. pylori* not only in adults but also in children.

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Analysis of intra-familial transmission of *Helicobacter pylori* in Japanese families

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Intra-familial infection is considered to be one of the main routes of transmission for *Helicobacter pylori* in Japan. We assessed the genomic profiles of *H. pylori* isolates from family members by multi-locus sequence typing (MLST) and identified the original strain infecting the index child. A total of 19 isolates from five families were analysed by MLST using seven housekeeping genes and by random amplification of polymorphic DNA (RAPD)-PCR. Phylogenetic analysis was performed using nucleotide sequences of the seven loci. Two or more different types of *H. pylori* strains were indicated in three (K-1, K-2 and K-5) out of five families. Independent genotypes of *H. pylori* strains were detected from all members of the other two families suggesting that these strains (K26-28 and K29-33) may be dominant. Mother-to-child transmission of *H. pylori* was demonstrated in four out of five families, whilst transmission from father-to-child and sibling-to-sibling were demonstrated in two families and one family, respectively.

INTRODUCTION

Helicobacter pylori is a curved Gram-negative bacterium that has been implicated in chronic gastritis, peptic ulcers, gastric adenocarcinoma and mucosal-associated lymphoid tissue lymphoma (Ernst & Gold, 2000; Marshall *et al.*, 1985; Uemura *et al.*, 2001). It was first discovered in the stomachs of gastritis patients by Marshall & Warren (1984). The infection prevalence of *H. pylori* has decreased in the industrialized world (Genta, 2002; Blaser & Atherton, 2004). Improved hygiene, housing conditions and the elimination of *H. pylori* from the population have resulted in a lower prevalence in children (Kosunen *et al.*, 1997; Rehnberg-Laiho *et al.*, 2001; Roosendaal *et al.*, 1997). It is considered that *H. pylori* will have been transmitted to an individual before the age of 5 years in many countries including developed (Weyermann *et al.*, 2009) and developing countries with high infection prevalence (Fiedorek *et al.*, 1991). However, in industrialized countries, the prevalence of *H. pylori* infection is low early in childhood and slowly rises with increasing age (Kuipers *et al.*, 1993). New infections are thought to occur as a consequence of direct human-to-human transmission by the oral–oral or faecal–oral route or both (Allaker *et al.*, 2002; Ferguson *et al.*, 1999; Leung *et al.*, 1999; Parsonnet *et al.*, 1999). In a recent study in Dutch children, relatively high *H. pylori* colonization rates in children of non-Dutch ethnicity who were born and raised in a western city were demonstrated. However, decreased colonization rates were also found in all ethnic groups in the study, implying the importance of environmental factors in *H. pylori* transmission in modern cities (den Hollander *et al.*, 2014).

In 1993, it was estimated that approximately 0.4 % of the 60 million Japanese who were infected with *H. pylori* have been diagnosed with gastric cancer (Asaka *et al.*, 1993). It has been reported that *H. pylori* infection rates gradually increased with age (Asaka *et al.*, 1992). Although this increase with age was similarly reported in more recent work (Asaka, 2002; Shiota *et al.*, 2013), the total prevalence of *H. pylori* infection is decreasing continuously in Japan (Shiota *et al.*, 2013). It can be implied that the detection rate of the pathogen originating from the environment has recently declined due to improving water provision and sewer services, and therefore one of the main transmission routes for this pathogen is unlikely to be a major factor in Japan. Several epidemiological studies reported that *H. pylori*-infected family member(s) are the risk factor for paediatric infection with *H. pylori* (Goodman & Correa, 2000; Vincent *et al.*, 1994). *H. pylori*-infected parents, particularly mothers, are suspected as the infectious sources. Although it was proven that other family members could transmit *H. pylori* infection, more precise analysis is needed to clarify the origin of the pathogen in each case. Multi-locus sequence typing (MLST) analysis of *H. pylori*

Abbreviations: MLST, multi-locus sequence typing; RAPD, random amplification of polymorphic DNA; SNP, single nucleotide polymorphism.

Two supplementary figures and two supplementary tables are available with the online Supplementary Material.

by comparing seven housekeeping genes (*atpA*, *efp*, *trpC*, *ppa*, *mutY*, *yphC* and *urel*) has been reported (Falush *et al.*, 2003; Kennemann *et al.*, 2011; Yamaoka, 2009), and we have used this method while investigating intra-familial transmission of *H. pylori* using faecal samples (Osaki *et al.*, 2013). In this study, to further investigate intra-familial transmission of *H. pylori*, the strains isolated from several family members including index children were cultured and analysed by MLST.

METHODS

Study design and definitions. This study was undertaken with approval from the ethics committees of Kyorin University, Tokyo and Sapporo Kosei General Hospital. Children attending clinic with gastric disorders and/or iron deficiency anaemia during the period April 2011 to December 2012 at Sapporo Kosei General Hospital were recruited for the study. Gastric biopsy specimens or gastric juice samples from all participants were obtained before eradication of *H. pylori* infection. Family members recruited to participate in this study were then tested for status of *H. pylori* infection by the presence of *H. pylori* IgG in their serum (HM-CAP; Enteric Products) or presence of antigen in their stool (Premier Platinum HpSA; Meridian Diagnostics) using commercially prepared kits and according to manufacturers' instructions. Gastric biopsy specimens were then collected only from *H. pylori*-positive family members.

Isolation of *H. pylori* from gastric specimens. The biopsies or gastric juice samples from patients or their family members were inoculated onto *H. pylori* selective agar media (Nissui Pharmaceutical) and cultured for 5 days at 37 °C under microaerobic conditions (AnaeroPack; Mitsubishi Gas Chemical Company) as described previously (Konno *et al.*, 2005; Osaki *et al.*, 2008). Single colonies were picked from the plates and sub-cultured on *Brucella* medium (Becton, Dickinson and Company) supplemented with 1.5 % (w/v) agar and 7 % horse serum (BHS medium). The isolates were identified by colony morphological analysis and urease-positive characterization. Each isolate was suspended in brain heart infusion broth (Nissui Pharmaceuticals) and stocked at −80 °C until use.

DNA extraction from *H. pylori* isolates. *H. pylori* strains were inoculated onto BHS medium and cultured for 48 h at 37 °C under microaerobic conditions. The DNA was extracted using the Wizard Genomic DNA purification kit (Promega) according to manufacturer's instructions.

MLST analysis. The extracted DNA (10 ng) was used as template for the amplification of seven housekeeping genes (*atpA*, *efp*, *trpC*, *ppa*, *mutY*, *yphC* and *urel*) using targeted primer pairs (Table S1; available in the online Supplementary Material). PCR amplicons were purified using the Wizard SV Gel and PCR Clean-Up System (Promega) according to the manufacturer's instructions for sequence studies.

The purified PCR amplicons were sequenced in a Bio-Rad DNA Engine Dyad PTC-220 Peltier Thermal Cycler using ABI BigDye Terminator v3.1 Cycle Sequencing kits with AmpliTaq DNA polymerase (FS enzyme, Applied Biosystems), according to the manufacturer's instructions. Single-pass sequencing was performed on each template using a second primer (forward or reverse; Table S1). The fluorescently labelled fragments were purified from the unincorporated terminators with an ethanol precipitation protocol. The samples were resuspended in distilled water and subjected to electrophoresis in an ABI 3730xl sequencer (ABI). The DNA sequence of each gene locus was registered on the MLST website (http://pubmlst.org/helicobacter/) (Jolley & Maiden, 2010). The allele number corresponds to the exact matching sequence for each gene to the strain listed in the database. In the case that the sequence had one or more base differences a new allele number(s) was listed on the MLST website.

Phylogenetic analysis. Phylogenetic analysis was carried out to compare nucleotide arrangement. The gene sequences for the seven loci were combined into one linear arrangement. The sequences were aligned and the maximum-likelihood tree was obtained by using MEGA5.1 (Arizona State University software) (Tamura *et al.*, 2011). The evolutionary history was inferred using the unweighted pair group method with arithmetic mean (UPGMA) method. The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. As a control strain, the genome sequence of Japanese *H. pylori* strain F32 was obtained from Pubmed (http://www.ncbi.nlm.nih.gov/genome) and used for comparison.

Random amplification of polymorphic DNA (RAPD)-PCR. RAPD-PCR was carried out as described previously (Akopyanz *et al.*, 1992; Konno *et al.*, 2005). The extracted genomic DNA of *H. pylori* isolates was assessed by RAPD-PCR using the D1254 primer (Akopyanz *et al.*, 1992).

RESULTS

The five index children aged 6–10 years, their siblings aged 5–17 years, and their parents aged 28–51 years participated in this study (Fig. 1, Table S2). A total of five families were recruited for the detection of intra-familial transmission

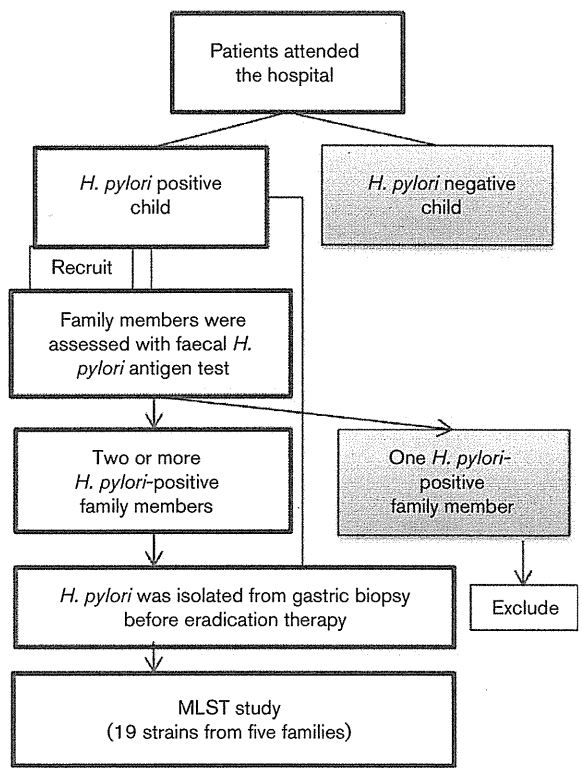

Fig. 1. Flow chart of this study.

and for determination of the family member harbouring the original strain.

In family K-1, three *H. pylori* isolates from the father (K16), mother (K17) and index child (K15) were compared. The alleles of seven loci (*atpA*, *efp*, *mutY*, *ppa*, *trpC* and *ureI*) in the isolates matched between K15 and K17 (Table 1). In family K-2, the alleles in the isolate (K37) from the index child were exactly the same as those from the mother (K35) and the sibling (K36), but not the same as those of the father (K34) (Table 1). The phylogenetic analysis of both families is shown in Fig. 2. There is large sequence diversity between the father's *H. pylori* strain and the index child's strain or mother's strain in both families. The genotype of *H. pylori* (K36) isolated from the sibling in family K-2 also matched that of the isolates from the mother and the index child, but not the isolate from the father (Fig. 2b). We could not find any nucleotide arrangement differences between K15 and K17 for family K-1 and between K37 and K36 for family K-2. These results indicate that these infections in families K-1 and K-2 may have occurred relatively recently.

The MLST results from families K-3 and K-4 are shown in Table 1. The alleles of all seven loci in *H. pylori* isolates matched almost those of all family members including the father and the sibling in each family. In addition, in the phylogenetic analysis the nucleotide arrangement was markedly similar, implying that only one original strain colonized all members in each family (Fig. 3). However, since the mother's isolates (K27 and K30) were located upstream from the other two isolates on the phylogeny tree (K26 and K28 in K-3, K29 and K32 in K-4, except K33), this suggested that the mother was infected with *H. pylori* first and then other family members were infected with the mother's *H. pylori* strain. The K33 (index child) strain was located at the same position as the mother's isolate (K30) on the phylogenetic tree, suggesting that the infection between the mother and the index child may have occurred relatively recently. The order of intra-familial infections between parents and child (sibling) cannot be determined.

For family K-5, the same sequence type strains were detected by MLST in all three children, but different types were detected in their mother and father (Table 1, Fig. 4). In total, three different types of *H. pylori* strains were detected in family K-5. This implied that the *H. pylori* strain was therefore transmitted between the index child (K24) and the two siblings (K23 and K25) and that the strains isolated from either mother (K22) or father (K21) were not the source of intra-familial transmission. The phylogenetic study supports this implication, as the genotype of the index child's isolate (K24) was divided from the nodes of its father's isolate and mother's isolate (K21 and K22) (Fig. 4). Sibling-to-sibling transmission(s) of *H. pylori* was clearly illustrated in family K-5.

Table 1. MLST analysis of *H. pylori* strains isolated from family members

Family	Member	Strain	Allele type number							
			atpA	*efp*	*mutY*	*ppa*	*trpC*	*ureI*	*yphC*	ST
K-1	Father	K16	2305	2129	2342	2153	2418	2390	2307	2747
	Mother	K17	2309	2170	2347	1125	2419	2391	2345	2748
	Index child	K15	2309	2170	2347	1125	2419	2391	2345	2748
K-2	Father	K34	2313	2199	2359	942	458	1968	2401	2749
	Mother	K35	2324	2202	2360	2235	2433	2406	2404	2750
	Sibling	K36	2324	2202	2360	2235	2433	2406	2404	2750
	Index child	K37	2324	2202	2360	2235	2433	2406	2404	2750
K-3	Father	K26	1760	2185	2354	936*	457	2393*	457	2753
	Mother	K27	1760	2185	2354	2232*	457	2393*	457	2754
	Index child	K28	1760	2185	2354	2232*	457	2400*	457	2755
K-4	Father	K29	2319	2186†	2355†	945	954	36	957	2756
	Mother	K30	2319	2191†	2355†	945	954	36	957	2757
	Sibling	K32	2319	2191	2358†	945	954	36	957	2758
	Index child	K33	2319	2191	2355†	945	954	36	957	2757
K-5	Father	K21	2313	2182	459	2155	2420	1968	2393	2760
	Mother	K22	2315	2184	2351	445	2426	1968	2394	2761
	Sibling no. 1	K23	951	909	950	2230	2427	2392	2400	2762
	Index child	K24	951	909	950	2230	2427	2392	2400	2762
	Sibling no. 2	K25	951	909	950	2230	2427	2392	2400	2762

ST, Sequencing type.

Allele numbers over 2000 were given for this study from the MLST website (http://pubmlst.org/helicobacter/)

*One SNP difference between *ppa*936 and *ppa* 2232, *ureI*2393 and *ureI*2400 in family K-3.

†One SNP difference between *efp*2186 and *efp*2191, *mutY*2355 and *mutY* 2358 in family K-4.