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* isolates from family members may provide further evidence of intra-familial transmission of *H. pylori* if the same strain infects multiple family members.
by comparing seven housekeeping genes (atpA, efp, trpC, ppa, mutY, yphC and ureI) 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 \textit{H. pylori} using faecal samples (Osaki et al., 2013). In this study, to further investigate intra-familial transmission of \textit{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 \textit{H. pylori} infection. Family members recruited to participate in this study were then tested for status of \textit{H. pylori} infection by the presence of \textit{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 \textit{H. pylori}-positive family members.

**Isolation of \textit{H. pylori} from gastric specimens.** The biopsies or gastric juice samples from patients or their family members were inoculated onto \textit{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 \textit{H. pylori} isolates.** \textit{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 ureI) 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 \textit{H. pylori} strain F52 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 \textit{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.
and for determination of the family member harbouring the original strain.

In family K-1, three \textit{H. pylori} isolates from the father (K16), mother (K17) and index child (K15) were compared. The alleles of seven loci (\textit{atpA}, \textit{efp}, \textit{mutY}, \textit{ppa}, \textit{trpC} and \textit{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 \textit{H. pylori} strain and the index child's strain or mother's strain in both families. The genotype of \textit{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 \textit{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 \textit{H. pylori} first and then other family members were infected with the mother's \textit{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 \textit{H. pylori} strains were detected in family K-5. This implied that the \textit{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 \textit{H. pylori} was clearly illustrated in family K-5.

<table>
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<td>2313</td>
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ST, Sequencing type.  
Allele numbers over 2000 were given for this study from the MLST website (http://pubmlst.org/helicobacter/)  
*One SNP difference between \textit{ppa}936 and \textit{ppa}2232, \textit{ureI}2393 and \textit{ureI}2400 in family K-3.  
†One SNP difference between \textit{efp}2186 and \textit{efp}2191, \textit{mutY}2355 and \textit{mutY}2358 in family K-4.
The MLST results were confirmed by RAPD fingerprinting (Fig. S1). There was no discrepancy between the results from MLST and RAPD-PCR.

**DISCUSSION**

MLST is a standard method of molecular typing for pathogenic and non-pathogenic bacteria. We have conducted this study using MLST to clarify the mode of intra-familial transmission among several *H. pylori*-positive family members with an index child.

In this study, four cases (families K-1, K-2, K-3 and K-4) of mother-to-child transmission were demonstrated in five families. The alleles of all loci in *H. pylori* isolates matched almost those of all family members including the father and the sibling in family K-3 and K-4, therefore the possibility of paternal or sibling infection cannot be excluded in two of the cases. There have been several studies concerning the intra-familial transmission of *H. pylori* evaluated by molecular analysis (Georgopoulos et al., 1996; Han et al., 2000; Kivi et al., 2003; Konno et al., 2005, 2008; Nahar et al., 2009; Nwokolo et al., 1992). The major causative mode of infection was suggested to be mother-to-child transmission in these studies and our previous study (Osaki et al., 2013). Father-to-child transmission was not detected clearly, as the father and the mother had similar genotypes of *H. pylori* in families K-3 and K-4. It has been previously reported that maternal, paternal and sibling infection are all strongly and significantly related to infection of the child with *H. pylori* in bivariate analyses (Weyermann et al., 2009). Phylogenetic analysis revealed *H. pylori* isolated from the father of the K-3 (K26) and K-4 (K29) family were located upstream from the each node compared to *H. pylori* strains from other family members (family K-3, K27 and K28; family K-4, K30 and K33), implying that the father was infected with the original *H. pylori* strain.

In two families (K-3 and K-4), the original *H. pylori* strain was not only related to infection in the children but also inter-spousally. A previous study reported this type of transmission previously by using *H. pylori* 16S rRNA ribotyping, showing that eight of 18 couples were colonized with a single *H. pylori* strain (Georgopoulos et al., 1996). An additional study reported six cases of inter-spousal infection using RAPD-fingerprinting analysis (Konno et al., 2008). It is difficult to clarify the time when inter-spousal infection occurred, but it is unlikely that the couples were...
Sibling-to-sibling(s) transmission of *H. pylori* was found in family K-5, but parent-to-child transmission was not. In this case, it is suggested that the isolates from the children originated from outside the family. There are several hypotheses as to the sources, including a study in which *H. pylori* DNA was detected in drinking water (Fujimura *et al.*, 2008). In this family, the origin of infection and the reason for the lack of parent-to-child transmission were not clear.

In Japan, oral transmission through saliva and gastric juice is thought to be the main cause of *H. pylori* infection. Japanese families have customarily fed their children pre-chewed food. It has been reported that use of soothers or bottle teats is closely related with *H. pylori* transmission in Canadian children (Sinha *et al.*, 2004). In contrast, it was reported that feeding infants food first chewed by a parent did not affect *H. pylori* status (Kurosawa *et al.*, 2000). On the other hand, children vomit more frequently and *H. pylori* strains from a young child with vomiting can be transmitted to other family members. Siblings may therefore play an important role in *H. pylori* transmission among children (Fialho *et al.*, 2010). Faecal–oral transmission is another route of infection related to sanitary conditions; water supply and sewage are considered important factors for *H. pylori* infection (Goh *et al.*, 2011). After the Second World War, sanitary conditions improved in Japan and the prevalence of *H. pylori* infection decreased (Shiota *et al.*, 2010).

In this study, sibling-to-sibling transmission of *H. pylori* without maternal or paternal infection was detected. The type of transmission of *H. pylori* has been reported in several articles (Garg *et al.*, 2006; Miehlke *et al.*, 1999). However, mother-to-child transmission was also found in these cases, and it was therefore difficult to clarify the transmission route of *H. pylori* in these families.

Households with many children have been shown to be one of the risk factors for *H. pylori* infection (Fiedorek *et al.*, 1991). In Japan, the average number of children per household is 1.70 in 2010, and the number of children in family K-5 was higher than the average. Although *H. pylori* infection from the elder-to-younger sibling was most likely, the origin of the strain isolated from the index child was unclear.

Several SNPs (single nucleotide polymorphisms) were found by comparing all MLST gene sequences. Deletion and transformation of other sequences was not detected. It was shown that the SNPs were derived from the original strain during a long infection period after transmission of *H. pylori* (Raymond *et al.*, 2004). According to Graphical review of Japanese households-from comprehensive survey of living conditions, 2010, ed. Japanese Ministry of Health, Labour and Welfare (http://www.mhlw.go.jp/tokei/list/dl/20-21-01.pdf).

It is well known that clinical strains of *H. pylori* have numeric diversity. Although the genotype of intra-familial transmitted strains were closely related each other, it was shown that K16 (K-1 family), K34 (K-2 family), K21 and K22 (K-5 family) had different allele types in the seven loci tested, compared to the isolates from other family members. These results showed that similar molecular type strains were detected in the cases of intra-familial transmission than in cases with other infection routes. In the phylogenetic study, we found similar strains were present in each family (Figs 2, 3, 4 and S2). These results support the notion that the same original strain was transmitted to family members.

Another possibility is that family members were infected with two or more strains at the same time. There have been several reports of multiple infections (Hirschl *et al.*, 1994; Fiedorek *et al.*, 1991; Miehlke *et al.*, 1999; Raymond *et al.*, 2004) with different types of *H. pylori* strains in a single individual. On study reported the examination of clonal diversity by RAPD fingerprinting method (Toita *et al.*, 2013); the isolates obtained from several patients at 5 to 9 year intervals showed identical or very similar RAPD patterns. It was concluded that each Japanese individual of an urban population is predominantly infected with a
single *H. pylori* clone (Toita et al., 2013). As we used single colony isolates of *H. pylori* from each family member and MLST analysis was performed for this isolate, mixed infection of *H. pylori* could not be detected. Two different strains of *H. pylori* were isolated from the same individuals at different sampling times, but all isolates derived from a single individual showed the same MLST in this study (data not shown). The dominant population of *H. pylori* may be determined by microbiota environmental factors. In our previous study, the composition of gastric indigenous microbiota in Mongolian gerbils may be disturbed by long-term infection with *H. pylori*, and that these changes may in fact inhibit *H. pylori* infection (Osaki, et al., 2012). Further study is necessary to clarify the mechanism of intra-familial infection.

In conclusion, person-to-person transmission between family members was detected frequently in this study, with mother-to-child, parent-to-child, intra-spousal and sibling(s)-to-sibling transmissions being demonstrated. This may be the predominant mode of *H. pylori* transmission in Japan.

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