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Concordance of *Helicobacter pylori* Strains within Families

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Helicobacter pylori **infection is typically acquired in early childhood, and a predominantly intrafamilial transmission has been postulated. To what extent family members share the same strains is poorly documented. Our aim was to explore patterns of shared strains within families by using molecular typing. Family members of *H. pylori*-infected 10- to 12-year-old index children identified in a school survey were invited to undergo gastroscopy. Bacterial isolates were typed with random amplified polymorphic DNA and PCR-restriction fragment length polymorphism of the genes *ureA-B*, *glmM*, or *flaA*. The presence or absence of the *cag* pathogenicity island, a bacterial virulence factor, was determined by PCR. GelCompar II software, supplemented with visual inspection, was used in the cluster analysis. In 39 families, 104 individuals contributed 208 bacterial isolates from the antrum and corpus. A large proportion, 29 of 36 (81%) of the offspring in a sibship, harbored the same strain as at least one sibling. Mother-offspring strain concordance was detected in 10 of 18 (56%) of the families. Of 17 investigated father-offspring relations in eight families, none were strain concordant. Spouses were infected with the same strains in 5 of 23 (22%) of the couples. Different strains in the antrum and corpus were found in 8 of 104 (8%) of the subjects. Our family-based fingerprinting study demonstrates a high proportion of shared strains among siblings. Transmission between spouses seems to be appreciable. The data support mother-child and sib-sib transmission as the primary transmission pathways of *H. pylori*.**

Helicobacter pylori colonizes half of the world's population, and the infection is associated with chronic gastritis, peptic ulcer, gastric cancer, and gastric lymphoma. In-depth knowledge of the transmission patterns may constitute important information for future intervention strategies.

In the absence of consistent and verified environmental reservoirs, a predominantly person-to-person transmission has been postulated. *H. pylori* infection is associated with poor living conditions, and possible transmission routes are fecal-oral, oral-oral, or gastro-oral, but firm evidence is lacking (36). The infection clusters in families and is usually acquired in early childhood. A child's risk of being infected is largely determined by the presence or absence of infected family members. Having an infected mother has been suggested to be a more prominent risk factor than having an infected father (30, 35). Indications of sib-sib transmission have been reported (14). Transmission between spouses occurs but its significance is unclear (5, 9, 19).

Molecular typing of pathogens can corroborate and further characterize the transmission pathways suggested by epidemiologic data based on infection status. Shared strains among individuals indicate person-to-person transmission or acquisition from a common source. Unrelated individuals harbor distinct *H. pylori* isolates (1), while clonal lineages can be discerned within families (2, 7, 11, 16, 20, 22, 24, 29, 33, 38, 39). These observations are in line with familial transmission and an epidemic clonal population structure (37). However, available data are sparse and often based on small samples, reflecting the difficulties in obtaining gastric biopsies from children and asymptomatic individuals. The aim of the present study was to shed light on familial transmission patterns through the exploration of strain concordance by using molecular typing.

MATERIALS AND METHODS

Subjects. We identified study families via *H. pylori*-infected index children, 10 to 12 years of age, who participated in a previous serological survey in 11 schools in the Stockholm area (35). Family members residing in the same household at the time of investigation were considered. Information about family characteristics was obtained by a questionnaire completed by the parents. Blood was drawn to determine serologically the *H. pylori* infection status with an in-house enzyme-linked immunosorbent assay and immunoblot (Helico Blot 2.0, Genelabs Diagnostics, Singapore) (34). *H. pylori*-seropositive individuals were invited to undergo gastroscopy with culture of biopsy samples from the corpus and antrum. Families contributing biopsy samples from more than one individual were included in the present analysis. The ethics committees at Huddinge University Hospital and Karolinska Institutet approved the study, and participants and/or the parents gave informed consent.

Fingerprinting. Bacteria were revived from frozen stocks of primary cultures or obtained directly from primary cultures and grown on plates under standard conditions. For each biopsy, genomic DNA was prepared (QIAamp DNA Mini kit; QIAGEN, Venlo, The Netherlands) from a pool of all bacterial colonies and from a single colony after one to three additional passages.

The isolates were typed with random amplified polymorphic DNA (RAPD) and PCR-restriction fragment length polymorphism (RFLP) protocols that have been evaluated previously (6). In order to determine if a single colony was representative of the total bacterial population in a biopsy sample, we typed the single-colony DNA and the colony pool DNA with RAPD. Primer 1281 was used

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TABLE 1. Performance of the typing methods used

Typing method (restriction enzyme)	% Typeability[a]	DI (95% CI)[b]	No. of isolates in DI estimation[c]
ureAB PCR-RFLP (*Hae*III)	96 (199/208)	0.95 (0.92–0.98)	37
glmM PCR-RFLP (*Sau*3AI)	86 (179/208)	0.83 (0.75–0.91)	36
flaA PCR-RFLP (*Alu*I)	100 (54/54)	0.71 (0.52–0.90)	10
flaA PCR-RFLP (*Sau*3AI)	100 (28/28)	1.00 (NA)[d]	4
RAPD	100 (208/208)	1.00 (0.99–1.00)	37

[a] The proportion of successfully typed isolates of all those tested.
[b] DI, discriminatory index; CI, confidence interval. The DI can be interpreted as the probability of differentiating two unrelated isolates. The DIs were calculated based on 100% similarity with a collection of unrelated isolates obtained by randomly selecting one typing reaction from each family.
[c] The number of isolates used in the DI estimation does not equal the total number of families included in the study ($n = 39$). The two *flaA* reactions were added as supplements when other products could not be amplified. Hence, not all four PCR-RFLP methods were performed for all families. Moreover, two families were excluded from the DI calculations because of familial connections to other families in the study.
[d] NA, not applicable. The four isolates segregated into four clusters, resulting in a zero variance.

with the following: 20 ng of template, 0.8 μM primer, 200 μM (each) dNTP, 1× buffer, 3 mM $MgCl_2$, and 1 U of AmpliTaq Gold (Applied Biosystems, Foster City, Calif.). The cycling program was as follows: 95°C for 5 min; 40 cycles of 95°C for 1 min, 36°C for 1 min, and 72°C for 2 min; and 72°C for 10 min. The pattern was visualized by 2% agarose gel electrophoresis (100 V, 3.5 h) of 5 μl of reaction mixture and staining with ethidium bromide. For normalization purposes, the outer lanes contained DNA Molecular Weight Marker VI (Roche Applied Sciences, Basel, Switzerland).

The RAPD patterns of the single-colony samples and the pooled-colony samples were similar. The single colonies were further typed with PCR-RFLP for clustering analyses. A 2.4-kb amplicon from the *ureAB* operon and a 1.1-kb amplicon from the *glmM* (also known as *ureC*) gene were amplified and digested with *Hae*III and *Sau*3AI, respectively (6). The presence of a single major band was determined by 1% agarose gel electrophoresis. PCR products could not be obtained from all strains (Table 1), presumably due to variations of the target sequences. To compensate for the nonamplified products, we added typing reactions of a 1.5-kb *flaA* gene fragment digested with *Alu*I or *Sau*3AI (8). Two PCR-RFLPs were successfully performed for each family, and the same PCR-RFLP methods were used for all members in a family. PCR was performed with 5 ng of template, 0.2 μM forward primer, 0.2 μM reverse primer, 200 μM (each) dNTP, 1× buffer, and 2.5 U of Titanium *Taq* (BD Biosciences, San Jose, Calif.). The cycling program was as follows: 95°C for 1 min; 25 cycles of 95°C for 30 s, 55°C for 30 s, and 68°C for 2 min; and 68°C for 5 min. The PCR product was ethanol precipitated and dissolved in 12.5 μl of water. Digestion took place overnight at 37°C with 10 U of restriction enzyme. Gel electrophoresis was performed as for RAPD.

***cag* PAI PCR.** Two PCRs were used to determine the presence or absence of the *cag* pathogenicity island (PAI). The reactions were performed with the colony pool DNA sample. Two primers flanking the PAI yielded a 562-bp product when the PAI was absent (4). The other PCR (with CagAsbra primers) amplified an estimated 823-bp fragment within the PAI-encoded cag*A* gene (23a). PCR was performed with 10 ng of template, 0.4 μM forward primer, 0.4 μM reverse primer, 200 μM (each) dNTP, 1× buffer, 3 mM $MgCl_2$, and 0.75 U of AmpliTaq Gold. The cycling program was 95°C for 5 min; 30 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min; and 72°C for 10 min. Agarose gel electrophoresis was performed on 2 μl of the reaction mixture.

Fingerprint analysis. The digitized images were imported into GelCompar II software (Applied Maths, Kortrijk, Belgium) and normalized. The bands were assigned by manual editing of the software-identified bands. The position tolerance and optimization were set to 2%. The discriminatory index (DI), also designated index of diversity, of a typing technique estimates the probability that two unrelated isolates are differentiated by the technique (17). To calculate discriminatory indices for each typing method, subsets of unrelated isolates were created by randomly selecting one typing reaction from each family. Two families were excluded from the DI calculations due to family relations between members of the two families. DI 95% confidence intervals were calculated according to the method introduced by Grundmann et al. (15). In the cluster analysis, five Dice similarity matrices were calculated from the RAPD and the four PCR-RFLP experiments. The five matrices were averaged, and a dendrogram was constructed from the average matrix with the unweighted pair group method using arithmetic averages. Only major RAPD bands were considered, and the RAPD matrix was given half the weight of the more stable PCR-RFLPs. Other researchers have recommended visual inspection of the software output (10, 28). Final decisions were made after visual inspection of each family separately, and two strains were considered to be the same if a majority of the typing reactions were identical. Here also, major RAPD bands were primarily considered.

TABLE 2. Characteristics of participating, noninfected, and nonparticipating household members in the 39 families with *H. pylori* cultures from two or more family members

Relationship	No. of participants[a]	No. seronegative[b]	Nonparticipants		Total no. of household members
			No. seropositive[b]	No. with unknown serostatus[c]	
Mother	35	1	4	0	40
Father	25	2	3	3	33
Offspring	44	29	47	8	128
Total	104	32	54	11	201

[a] *H. pylori*-seropositive individuals who contributed bacterial isolates.
[b] *H. pylori* serostatus was determined with an in-house enzyme-linked immunosorbent assay and immunoblot.
[c] No blood samples were available.

RESULTS

The inclusion criterion of at least two participating family members was fulfilled for 39 families. These families had a total of 201 household members, of whom 32 were disregarded due to *H. pylori* seronegativity. Of 158 seropositive individuals, 104 underwent gastroscopy, contributing 208 bacterial isolates. Eleven subjects did not donate blood for testing (Table 2). The household members were of mixed ethnicity, and 80 of 104 (77%) participants and 36 of 65 (55%) nonparticipants were born outside western Europe and North America. The median age for participating offspring was 13 years, and the range was 3 to 22 years. For nonparticipating offspring, the median age was 13 years and the range was 1 to 23 years. The family constellations of the participants are described in Table 3.

The typeability and discriminatory indices of the techniques used are given in Table 1. In the computerized analysis, clustering of strains from different families was not anticipated. With this analysis as a guideline, a similarity cutoff at 95% was

TABLE 3. Number of families with different constellations of participating family members, excluding individuals who did not contribute isolates

Number of participating offspring[a]	Number of families with indicated participating parents[a]				Total no. of families
	Mother and father	Mother	Father	Neither	
0	17				17
1	2	5	1		8
2	2	5	1	1	9
3	1	2			3
4				1	1
5	1				1
Total	23	12	2	2	39

[a] Participants are individuals who contributed bacterial isolates.

TABLE 4. *H. pylori* strain concordance patterns of the participants

Constellation of shared strains	% Strain concordance (*n*)
Sib-sib[a]	81 (29/36)
Sib-sib, mother different strain[a]	82 (14/17)
Mother-offspring[b]	56 (10/18)
Father-offspring[b]	0 (0/8)
Mother-father	22 (5/23)

[a] The sib-sib measures are based on offspring that are part of a sibship with at least two participants. Sib-sib strain concordance is also presented after restriction to six families in which the mother harbored a unique strain.

[b] Mother-offspring and father-offspring strain concordances are based on families and indicate how often the parent harbored the same strain as one or more of his or her offspring.

chosen. The 95% similarity cutoff yielded 103 clusters, and 5 of these contained members from more than one family. Visual inspection of each family separately resulted in 80 clusters. The difference in number of clusters was largely due to antrum and corpus isolates being classified as different more frequently by the software (31 of 104 [30%]) than by visual inspection (8 of 104 [8%]). Different strains in the corpus and antrum were equally common in parents (software, 18 of 60; visual inspection, 4 of 60) and offspring (software, 13 of 44; visual inspection, 4 of 44). In the computerized analysis, 19 antrum and 18 corpus isolates diverged from a cluster into which they were assigned in the visual approach. The discrepancies between the computerized and visual analyses had limited impact on the strain concordance patterns, shown in Table 4, where the results of the two approaches coincided.

Shared strains among family members were most apparent in sibships and between mothers and offspring (Table 4). In sibships with at least two participants, 29 of 36 (81%) of the offspring were infected with the same strain as at least one sibling. In families where isolates were available from the mother and one or more offspring, shared strains between the mother and at least one offspring were detected in 10 of 18 (56%) families. In terms of mother-offspring relations, 14 of 35 (40%) were strain concordant. Clustering of siblings' strains was also observed in six families where the mother harbored her own unique strain. In these families, 14 of 17 (82%) offspring clustered with at least one sibling. We detected no father-offspring strain concordance in eight families with biopsy samples from the father and at least one offspring, corresponding to 17 investigated father-offspring relations. Spouses harbored the same strains in 5 of 23 (22%) couples. For the eight individuals who had different strains in the corpus and antrum, five corpus isolates and one antrum isolate clustered with strains of other family members. A more detailed description of the household members in each family, containing age, origin, infection status, and shared strains, is provided in an appendix to this article (http://www.mtc.ki.se/groups/granstrom/index.htm).

Strains from the same cluster generally exhibited the same *cag* PAI status, as determined by the two PCRs with the colony pool DNA samples. The cag PAI was present in 115 (64%) of 180 bacterial isolates. Twenty-eight isolates from individuals with CagA-positive immunoblots exhibited ambiguous *cag* PAI PCR results. Of these, 24 samples (from 15 individuals) gave bands in both PCRs. An explanation for this could be that the individuals harbored bacterial clones both with and without the *cag* PAI, reflecting its variability (31). Four isolates (from two individuals) yielded no products in either reaction, using both colony pool and single-colony DNA samples, possibly due to mutated *cagA* target sequences.

DISCUSSION

In the present study, conducted among high-prevalence substrata in the generally low-prevalence Swedish population (3, 35), molecular typing of *H. pylori* strains demonstrated that siblings commonly shared strains (Table 4). Moreover, strain concordance was frequently noted between mothers and offspring but not between fathers and offspring. Sibling strain clustering was also present in families where the mother harbored a unique strain. This finding may imply that acquisition of strains occurs from outside the household and that sib-sib transmission occurs in addition to mother-offspring transmission. Indirect evidence of transmission within sibships has been reported from a low-income country by using concordance in urea breath tests (14). In addition, clustering of strains in sibships has been demonstrated before (16, 20, 22, 38, 39).

The observation that mothers and offspring frequently harbor the same *H. pylori* strains is in line with epidemiologic evidence (30, 35). It has previously been suggested that children are colonized by the mother's strain more often than the father's (16). However, both father-offspring and mother-offspring strain concordances have been reported repeatedly by studies using molecular typing (2, 7, 11, 16, 20, 22, 24, 33, 38, 39). Epidemiologic data also indicate that the father has some role in children's acquisition of *H. pylori* (30, 35). Although we were unable to confirm any shared strains between fathers and offspring, our study could investigate only 17 father-offspring relations, and the father should not be disregarded as a potential infection source.

There is epidemiologic and molecular evidence in favor of transmission between spouses (5, 9, 19). The strain concordance in couples found here underlines the multifaceted nature of *H. pylori* acquisition. In four of the five cases, both parents were born in high-prevalence regions with the reported prevalence often exceeding 50% at approximately 10 years of age (36). It is possible that the spouses were infected in childhood and initially harbored different strains, one of which has subsequently been replaced by the spouse's strain. Children possibly serve as mediators of transmission. The number of children in the household has been proposed as a risk factor for infection in parents (21), and in childless couples transmission between spouses was not supported (27). Other rather small studies have found no association between the number of children and the parents' infection status (12, 32).

The direction of transmission and the sequence of events could not be determined in our study. Furthermore, the reasons behind any familial clustering patterns of *H. pylori* are still to be clarified. *H. pylori* has been cultured from vomitus, cathartic stools, and saliva, demonstrating that the bacterium is potentially transmissible by these routes (25). Close contacts within families plausibly facilitate exposure to bacteria in these ways, which are in agreement with familial transmission.

Of the total of 201 household members in the 39 families, 32

were seronegative, and 104 of 158 seropositive individuals contributed isolates (Table 2). Neither serum nor a culture was obtained from 11 subjects. Nonparticipants were mainly children, and some missing isolates would probably have clustered with isolates from other family members. Any bias introduced by nonparticipation is influenced by the proportion of familial relations examined and whether the likelihood of strain concordance differs between participants and nonparticipants.

The computerized analysis assigned corpus and antrum isolates from the same individual to different clusters more frequently (31 of 104) than the visual approach (8 of 104). This finding should be interpreted in the context of *H. pylori* being one of the most variable bacterial species (13, 18). This plasticity poses a challenge to molecular typing because strains may mutate beyond recognition of clonality. Infections with more than one strain can often be interpreted as infections with strain variants (23, 38), as suggested in the visual inspection approach. The diversification of *H. pylori* might have caused us to underestimate the extent of shared strains in families. It can be hypothesized that the harsher gastric environment of adults compared to that of children preferentially drives mutation of the parents' strains. Speaking against this possibility is the finding that the occurrence of different strains in the antrum and corpus did not differ between parents and offspring.

Further, it is possible to underestimate familial clustering by not investigating all strains that may theoretically infect an individual (40). In the presence of both shared and unique isolates, the number of shared strains will be underestimated if only the unique ones are examined. Different strains may be present either in the same biopsy or in different gastric locations. First, the possibility of multiple strains in each biopsy sample has been addressed by comparing the RAPD patterns of the single-colony samples with the respective colony pool samples. The differences were limited, and extra bands also occurred in the single-colony samples. This finding indicates that the discrepancies are a result of poor reproducibility of RAPD or infection with strain variants as opposed to infections with multiple strains acquired from different sources. Second, there is a risk of undersampling of bacteria in gastric sites not covered by the biopsies. We decreased this risk by including biopsy samples from both the corpus and antrum, but a vast area of the gastric mucosa remains unsampled. Because *H. pylori* infection appears to be comprised of colonization by mainly one strain and strain variants, the potential bias introduced by multiple-strain infections is likely to be limited.

Different strains infecting the antrum and corpus were found in eight individuals, and clustering was more common with the corpus strain (five cases) than with the antrum strain (one case). Speculatively, this observation might reflect that the corpus strain is more readily transmitted than the antrum strain. An alternate explanation may be that antrum strains evolve more rapidly than corpus strains, thereby obscuring clonal relations. Speaking against this possibility is that in the computerized analysis, antrum isolates did not tend to diverge from visually determined clusters more frequently than did corpus isolates.

Subjects with gastrointestinal symptoms were probably more likely to volunteer for endoscopy than were symptomless ones, but clinical symptoms were not monitored systematically. It is not known whether symptoms or bacterial virulence influence transmissibility. The bacterial virulence factor *cag* PAI has been associated with an increased risk of peptic ulcer and cancer. Recent Finnish data suggest that CagA-positive strains disappear more rapidly in a population than CagA-negative strains (26). We found that strains in the same cluster typically exhibit the same *cag* PAI status. Our data, which are limited in this aspect, thus do not support the notion that strains containing the *cag* PAI would be less transmissible.

The certainty of the proportions of shared strains has to be evaluated indirectly because the dependence of the observations rules out standard statistical approaches as well as bootstrap. The validity of our results relies on the performance of the molecular typing, i.e., its ability to discriminate between unrelated and clustered isolates. We have determined the discriminatory power of the techniques used and found it to be satisfactory and consistent with that previously described (6). Five of 103 clusters created by GelCompar II contained members from more than one family. Three of these instances could be attributed to missing typing reactions, and two reflect failures of the techniques' discriminatory abilities. The strain concordance patterns obtained by GelCompar II and visual inspection were essentially the same. The computerized analysis assigned corpus and antrum isolates from the same individual to different clusters more frequently than did the visual approach. The stability of the concordance patterns despite this discrepancy can be attributed to the inclusion of two isolates per individual. If one isolate is classified as unique, the individual can remain in a cluster with the other isolate.

In conclusion, our study demonstrates strong familial clustering of *H. pylori* strains. Transmission between spouses seems to be appreciable, at least in couples with children, as in our study. The concordance patterns support mother-child and sib-sib transmission as the primary transmission pathways of *H. pylori*. The transmission patterns are likely to mirror the intimacy of personal contacts, but the mechanisms remain to be explored.

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