Helicobacter pylori: Clonal Population Structure and Restricted Transmission within Families Revealed by Molecular Typing

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***Helicobacter pylori* infects up to 50% of the human population worldwide. The infection occurs predominantly in childhood and persists for decades or a lifetime. *H. pylori* is believed to be transmitted from person to person. However, tremendous genetic diversity has been reported for these bacteria. In order to gain insight into the epidemiological basis of this phenomenon, we performed molecular typing of *H. pylori* isolates from different families. Fifty-nine *H. pylori* isolates from 27 members of nine families were characterized by using restriction fragment length polymorphism analysis of five PCR-amplified genes, by pulsed-field gel electrophoresis (PFGE) of chromosomal DNA, and by *vacA* and *cagA* genotyping. The 16S rRNA gene exhibited little allelic variation, as expected for a unique bacterial species. In contrast, the *vacA*, *flaA*, *ureAB*, and *lspA-glmM* genes were highly polymorphic, with a mean genetic diversity of 0.83, which exceeds the levels recorded for all other bacterial species. In conjunction with PFGE, 59 *H. pylori* isolates could be differentiated into 21 clonal types. Each individual harbored only one clone, occasionally with a clonal variant. Identical strains were always found either between siblings or between a mother and her children. Statistical analysis revealed clonality of population structure in all isolates. The results of this study suggest the possible coexistence of a large array of clonal lineages that are evolving in each individual in isolation from one another. Transmission appears to occur primarily from mother to child and perhaps between siblings.**

Helicobacter pylori-associated gastritis is today recognized as the major cause of duodenal and gastric ulcers, gastric adenocarcinoma and mucosa-associated lymphoid tissue lymphoma (9). *H. pylori* infects up to 50% of the human population worldwide. The infection occurs predominantly in childhood. Once the stomach is colonized, the organism persists for decades, if not for a lifetime. Many lines of evidence suggest person-to-person transmission of *H. pylori*, possibly by an oral-oral, fecal-oral, or gastric-oral route (4, 8, 9, 32–35, 44, 48). However, tremendous genetic diversity has been reported for these bacteria (23, 26). The molecular and epidemiological bases for this phenomenon are not understood.

Limited data suggest that the *H. pylori* population exhibits a panmictic or recombinational structure (1, 19, 24, 37, 39), and horizontal gene transfer followed by frequent DNA recombination has been considered to be an important source of the immense genetic diversity. However, clonal groupings of *H. pylori* have also been detected from different ethnic groups in New Zealand, China, The Netherlands, and other countries (1, 7, 25, 43, 45, 46). Moreoveor, comparison of the complete genomic sequences of two unrelated *H. pylori* isolates (2, 42) reveals that the overall genomic organizations, gene orders, and predicted proteomes are quite similar, with the majority of nucleotide changes representing synonymous substitutions. Based on these observations, Wang et al. (47) pointed out that mutation must be of major importance in generating the genetic variation of *H. pylori*.

In the present study, 59 *H. pylori* isolates from 27 different family members of nine families were characterized in order to define the *H. pylori* population structure. The results are most compatible with the concept that a large array of clonal lineages coexist and are evolving in isolation from one another. The evidence also indicates that transmission of *H. pylori* occurs predominantly and perhaps even exclusively within families. If this is correct, *H. pylori* would represent the first human pathogen recognized to display this remarkably restricted mode of transmission.

MATERIALS AND METHODS

Subjects. Nine index children from different families, designated families A through I, were referred to the Department of Pediatrics, Johannes Gutenberg University, Mainz, Germany, due to recurrent abdominal pain. One or more gastric (antral and corpus) and duodenal biopsy specimens were obtained from each of these patients and from at least one additional family member (see Fig. 2). In total, 27 members from nine families were investigated, represented by 2 to 6 members in each family. These patients were 23 children (11 boys and 12 girls with an age range of 2 to 18 years) and 4 adults from three families (2 women and 2 men from 29 to 44 years of age). The other parents were not sampled. They lived either within or in close proximity to Mainz.

Culture and PCR. Biopsy specimens were cultured on Columbia agar with 7% human erythrocytes and an *H. pylori*-selective supplement at 37°C under microaerobic conditions. *H. pylori* was identified and bacterial genomic DNA was prepared as previously described (23). PCR amplification of the *vacA*, *flaA*, *ureAB*, *lspA-glmM* (formerly *ureCD*), or *cagA* gene fragments was performed using the primer pairs described previously (23). The *cagA* gene status was determined by the presence or absence of *cagA* amplicons. The *vacA* genotypes, including signal(s) sequences and midregion (m) types, were characterized by a one-step PCR method (22). For ribotyping, a 1.5-kb fragment was amplified with broad-specificity 16S ribosomal DNA (rDNA) primer pairs pA-f (5′-AGAGTT TGATCCTGGCTCAG-3′) and pH-b (5′-AAGGAGGTGATCCAGCCGCA-3′) (15), using an initial denaturation step at 94°C for 2 min, followed by 35 cycles of denaturation at 94°C, annealing at 55°C, and extension at 72°C (each for 1 min), and a final extension at 72°C for 10 min. In order to eliminate contaminating DNA within PCR reagents, UV light and 8-methoxypsoralen (Sigma,

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Munich, Germany) were used (31). Genomic DNA was added after irradiation of the PCR mixture with UV light (366 nm) at room temperature for 20 min.

PCR-RFLP analysis. PCR-amplified *vacA*, *flaA*, *ureAB*, *lspA-glmM*, or 16S rDNA fragments were digested with *Hae*III (for *ureAB* or 16S rDNA), *Hha*I (for *vacA*, *flaA*, or *lspA-glmM*), *Hin*fI (for 16S rDNA), *Hph*I (for *vacA*), or *Sau*3AI (for *flaA*, *ureAB*, or *lspA-glmM*) for 3 h at 37°C in the appropriate buffer recommended by the supplier (New England Biolabs, Schwalbach/Taunus, Germany), and the DNA digests were analyzed on ethidium bromide-stained 2% agarose gels. Each isolate was thus characterized by 10 individual gene-enzyme patterns. A combination of these 10 patterns was designated a PCR-based locus-specific restriction fragment length polymorphism (PCR-RFLP) type, which defined the clonal type of an *H. pylori* isolate. Additionally, a combination of two restriction patterns of 16S rDNA was defined as a ribotype. The *cagA* gene fragments were not analyzed by PCR-RFLP, since the *cagA* gene could be detected in only 34 of 59 *H. pylori* isolates.

PFGE. The methods previously described by Taylor et al. (41) were modified. Fresh 2-day cultures of *H. pylori* were harvested and washed three times using normal saline. Alternatively, in order to inhibit DNase activity, 0.9-ml portions of the cell suspensions were incubated with 0.1 ml of 37% formaldehyde solution for 1 h at room temperature, and then washed (18). Approximately 1 ml of cell suspension with an optical density at 600 nm of 0.9 was needed for the preparation of two agarose plugs. The required volume of cell suspension was centrifuged, and bacteria were resuspended in 10 mM Tris-HCl–10 mM EDTA (pH 8.0) (0.1 ml for 2 plugs). This suspension was mixed with an equal volume of 1.2% agarose equilibrated to 56°C to prepare 0.6% agarose plugs. The solidified plugs were incubated overnight in ESP lysis buffer (0.25 M EDTA [pH 9.0], 1% lauroyl sarcosine, 1 mg of proteinase K per ml) (Sigma, St. Louis, Mo.) at 56°C. The plugs were washed two times in 15 ml of the Tris-EDTA buffer described above for 2 h each at 4°C. For the subsequent enzyme reactions, the plugs were washed once in 15 ml of 10 mM Tris-HCl–0.5 mM EDTA (pH 8.0) for 30 min at 4°C. The plugs could be stored in 10 mM Tris-HCl–50 mM EDTA (pH 8.0) at 4°C for 3 months.

For restriction enzyme digestion, plugs were incubated with 1 ml of the appropriate 1× enzyme buffer for 30 to 60 min at room temperature. The enzyme buffer was replaced with 100 μl of fresh enzyme buffer containing 25 U of *Not*I, *Nru*I, or *Sma*I (New England Biolabs) and incubated overnight at 37°C (25°C for *Sma*I). The plugs were chilled on ice for 15 min, and the enzyme buffer was replaced with 10 mM Tris-HCl–10 mM EDTA (pH 8.0), followed by loading of the plugs into the 1% running gel (Pulsed field certified agarose; Bio-Rad Laboratories, Hercules, Calif.). A lambda ladder PFG marker (New England Biolabs) served as a size standard. For pulsed-field gel electrophoresis (PFGE), CHEF-DR III PFGE systems (Bio-Rad Laboratories) were used. An agarose gel prepared in 0.5× Tris-borate-EDTA running buffer (Bio-Rad Laboratories) was subjected to electrophoresis for 20.3 h at 200 V and a 0.5- to 60.4-s switch time at a constant temperature of 14°C. Agarose gels were stained with ethidium bromide and photographed. A combination of three restriction patterns in an isolate was designated a PFGE type.

Statistical analysis. For the RFLP data, different patterns of each of the analyzed genes were treated as different alleles. A distinct combination of 10 individual patterns in an *H. pylori* isolate was designated an RFLP type (clonal type) and equated with an allelic combination. Genetic diversity (h) for a locus j is calculated as $h_j = 1 - \Sigma \chi_i^2[n/(n - 1)]$, where χ_i is the frequency of the ith allele at the locus, n is the number of isolates in the sample, and $n/(n - 1)$ is a correction for bias in small samples (38). Genotypic diversity was calculated by the same formula, in which χ_i is the frequency of the ith RFLP type and n is the number of RFLP types. Genetic diversity and genotypic diversity have a range of between 0 and 1. The mean diversity per locus is the arithmetic average of h over all loci examined. The genetic distance between pairs of isolates was calculated as the proportion of loci at which dissimilar alleles occur, i.e., the proportion of mismatches (38). The dendrogram was constructed from PCR-RFLP typing data by the unweighted pair group cluster method with arithmetic means in the computer program MEGA (28).

The index of association between loci (I_A) was calculated as described previously (30). The I_A value is a measure of the degree of association between loci and has an expected value of zero for a large random-mating (panmictic) bacterial population. In contrast, for bacterial species that are clonal at all levels as a result of geographic isolation or infrequent genetic recombination, the expected value of I_A should deviate significantly from zero. To determine whether I_A is significantly greater than zero, a Monte Carlo procedure can be performed. Computer programs written by J. G. Lorén (17) were used to calculate locus and genotypic diversity, to calculate I_A, and to perform the Monte Carlo simulations.

RESULTS

Figure 1 illustrates the RFLP and PFGE patterns obtained with nine isolates from the mother, the father, and two children of family A. These results are representative. Whenever possible, analyses were performed on isolates obtained from the antrum (a), corpus (c), and duodenum (d). Most patients harbored only a single *H. pylori* isolate. If a second isolate was detected, it differed only in a single restriction pattern, except for one isolate (H4a). An example is the isolate from the antrum of the mother (A1a), which varied from the corpus isolate A1c exclusively in the *flaA*-*Hha*I patterns. We considered isolate A1a to be a mutational variant of the parent isolate A1c.

Conspicuously, apparently identical isolates were very frequently found within families, where they were identified between siblings or between mother and child. The collective results of molecular typing are summarized in Fig. 2. For 59 *H. pylori* isolates, analysis of the 16S rRNA gene demonstrated an average of 2.5 RFLP patterns, a mean genetic diversity of 0.08, and a mean genotypic diversity of 0.14. The sparse allelic variation in the 16S rRNA gene was as expected for a unique bacterial species (14). In contrast, the *vacA*, *flaA*, *ureAB*, and *lspA-glmM* genes were highly polymorphic, with an average of 11.25 RFLP patterns per gene, a mean genetic diversity of 0.83, and a mean genotypic diversity of 0.91 for all of 59 *H. pylori* isolates. These values exceed the level of diversity recorded for all other bacterial species (19). The results of PFGE typing were concordant with the PCR-RFLP analysis in 46 of 52 cases. Three variants (A1a, D2a, and I2c) were distinct from their parent strains by PCR-RFLP analysis but showed PFGE patterns identical to those of the parent strains. Vice versa, three variants (B1c, H4a, and H4c) were distinguishable from their parent strains by PFGE but not by PCR-RFLP analysis. Hence, detection of strain variants is sometimes possible only by implementing both PCR-RFLP and PFGE analysis.

The genetic relationship among the 59 *H. pylori* isolates exhibited a tree-like phylogenetic structure with a deep but limited branching pattern (Fig. 2). Twenty-one clonal types clustered into 10 divisions (designated I to X). Isolates from one family tended to cluster into the same division. Isolates from different families were sometimes also found to be related, as evident in divisions II and IV.

Siblings tended to harbor identical isolates (families A, C, D, G, and H), but distinct *H. pylori* isolates could also be found between siblings (families D, E, F, and I). We were able to analyze *H. pylori* isolates from only one pair of parents (family A). The mother harbored the same isolate as her children, whereas the father harbored an unrelated strain. Intriguingly, the mother in family B also had the same isolate as her son. In contrast, the *H. pylori* strain of the father in family F was distinct from the isolates recovered in either of his children. These results indicate that *H. pylori* can be transmitted within families and possibly from parent (mother) to child or between siblings. However, a common source of exposure for *H. pylori* infection could not be completely ruled out.

The complete set of *H. pylori* isolates and subsets of the population were analyzed for multilocus linkage disequilibrium (Table 1). The 59 isolates differed on average at 6.80 of the 10 loci examined, and the I_A was 0.43. The Monte Carlo procedure indicated that the I_A was significantly different from zero. Moreover, calculation of I_A for the 21 PCR-RFLP types resulted in a value of 1.42, which was significantly different from zero ($P < 0.001$) based on the Monte Carlo procedure. These results, analyzed at the level of all isolates or RFLP types, demonstrated a significant degree of linkage disequilibrium among the *H. pylori* isolates from different families, consistent with the clonal structure described by Maynard Smith et al. (30).

DISCUSSION

Previous studies have suggested that *H. pylori* is panmictic and that the immense genetic diversity among *H. pylori* strains
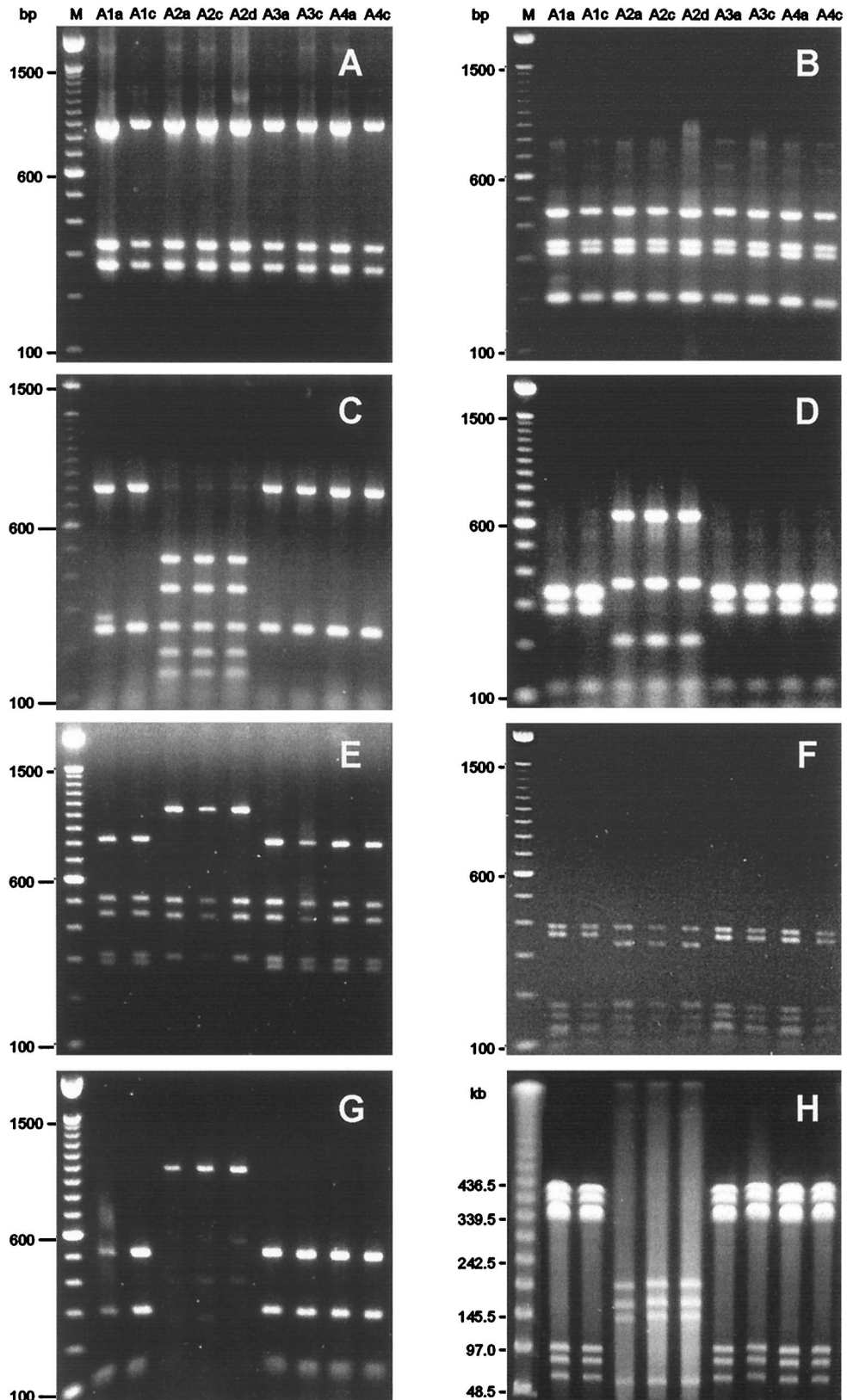
FIG. 1. Representative molecular typing results for *H. pylori* isolates from family A. PCR-RFLP analyses of 16S rDNA fragments digested with *Hae*III (A), 16S rDNA fragments digested with *Hin*fI (B), *flaA* fragments digested with *Hha*I (C), *flaA* fragments digested with *Sau*3AI (D), *ureAB* fragments digested with *Hae*III (E), *lspA-glmA* fragments digested with *Hha*I (F), and *vacA* fragments digested with *Hph*I (G) and PFGE of genomic DNA digested with *Not*I (H) are shown. Lanes: M, size markers; A1a to A4c, *H. pylori* isolates, designated according to the origins of the isolates (A, family A; 1 to 4, family members; a, c, and d, antrum, corpus, and duodenum, respectively).
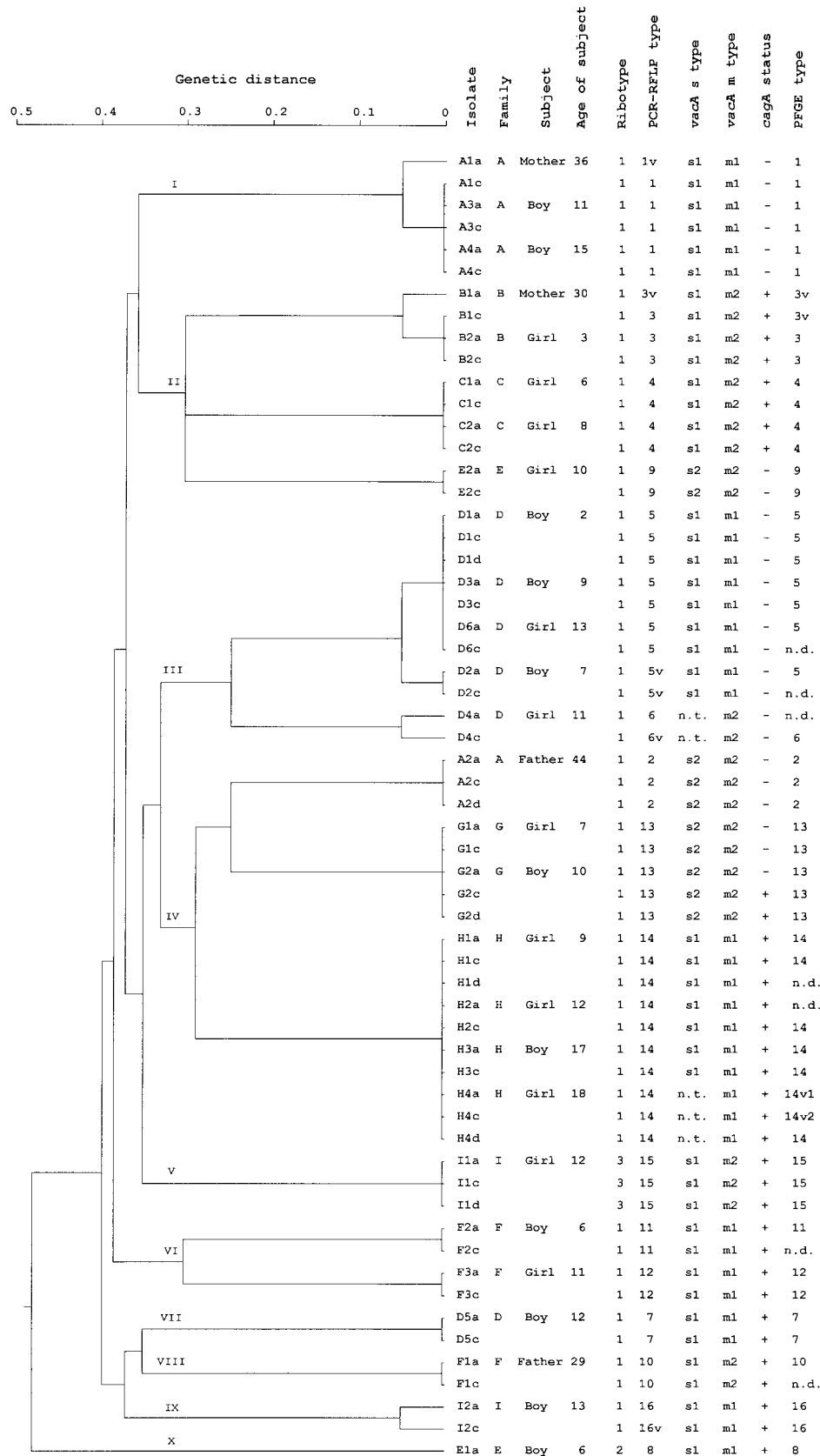
FIG. 2. Genetic relationships among 59 *H. pylori* isolates from nine different families. The dendrogram was constructed from PCR-RFLP typing data by the unweighted pair group cluster method with arithmetic means. Each distinct combination of 10 individual gene and enzyme patterns was designated a PCR-RFLP type, corresponding to a clonal type. Ten major lineages, termed I through X, separating at a genetic distance of 0.3 are indicated to the left. The columns to the right indicate the designation of *H. pylori* isolates, the origin of the isolates, and the molecular typing results. n.d., not determined; n.t., not typeable.

TABLE 1. Analysis of association between loci in 59 *H. pylori* isolates from family members[a]

Isolate group	Isolates				PCR-RFLP types only			
	No.	X	I_A	P	No.	X	I_A	P
vacA s1 type	44	6.83	0.61	<0.001	16	7.67	1.64	<0.001
vacA s2 type	10	4.02	0.78	>0.05	3	6.00	0	>0.05
vacA m1 type	34	6.05	0.99	<0.001	11	7.58	2.52	<0.001
vacA m2 type	25	6.51	0.24	>0.05	10	7.00	1.36	0.001
cagA positive	34	6.78	0.36	>0.05	13	7.89	1.66	<0.001
cagA negative	25	5.88	1.59	<0.001	9	6.64	2.92	<0.001
From antrum	27	7.13	0.74	<0.001	19	7.60	1.14	<0.001
From corpus	26	6.90	0.22	>0.05	16	7.44	0.50	>0.05
All	59	6.80	0.43	<0.01	21	7.58	1.42	<0.001

[a] X, mean number of allelic mismatches between pairs of *H. pylori* isolates or between PCR-RFLP types; I_A, index of association between loci; P, probability based on the Monte Carlo procedure. Values of I_A that differ significantly from zero indicate a clonal structure in bacterial populations, with rare or absent recombination. In contrast, values of I_A that do not differ significantly from zero indicate a panmictic structure, with frequent recombination.

arises by frequent horizontal DNA transfer and recombination (1, 19, 24, 37, 39). Indeed, direct evidence for recombination between different *H. pylori* strains has been observed in humans (27) and in mice (10). However, several lines of evidence indicate that mutations may also play an important role in generating the observed diversity in *H. pylori* (47). We show in this report that the bacterium *H. pylori* does in fact exhibit a clonal structure. According to these data and previous studies (23), each individual will harbor very few *H. pylori* strains (usually only one). This hypothesis was substantiated by our data (Fig. 2) that a large array of clonal lineages coexist, which evolve in isolation, i.e., in each individual. This would account for the finding that separate lineages of *H. pylori* have been detected from different ethnic groups in New Zealand (Polynesians and Europeans), in China, in The Netherlands, and in other countries (1, 7, 25, 43, 45, 46). Identical *flaA*, *flaB*, or *vacA* alleles were also found in unrelated *H. pylori* strains from a population in Capetown, South Africa (39). These results are consistent with the existence of divergent *H. pylori* clonal lineages at the level of different ethnic groups or different geographic regions.

The results presented in this report strongly suggest that mutations which occur within the individual host are capable of generating variant *H. pylori* strains. Recombination, although possible, will be a rather rare event in the absence of multiple infections. In fact, infection with multiple *H. pylori* strains appears to be infrequent in the developed world. The "different" strains recovered from the same patient often seemed more closely related by fingerprinting than other strains recovered from different patients. In experimentally infected rhesus monkeys, a mixed infection tends to resolve quickly, with just one strain generally predominating within a year (8). If the working hypothesis is correct, the clonal population structure of *H. pylori* would only slowly be eroded by recombination. The fact that the mode of *H. pylori* transmission is probably very restricted gains particular importance in this context. Although the number of subjects included here was still relatively small, all of the results would be in line with the contention that *H. pylori* transmission occurs mainly, if not exclusively, within families. Thereby, transmission from parent (mother) to child and perhaps from child to child predominates, occurring conceivably via the gastric-oral, oral-oral, or fecal-oral route. This contention would be consistent with many studies that have utilized epidemic (5, 11–13, 16, 35) or molecular typing (4, 8, 32, 34, 44, 48) methods. A small number of epidemic studies have concluded otherwise (3, 6, 20, 21), but this may have been due to factors affecting *H. pylori* transmission, e.g., the general prevalence of *H. pylori* in different age groups, the timing of acquisition, and the socioeconomy and ethnic composition of the studied population. An investigation by Goodman and Correa (21) with rural Colombian children appeared to reveal transmission of *H. pylori* from older to younger siblings. However, Rowland (36) has commented on the problems surrounding the interpretation of those data. The hypothesis that *H. pylori* transmission occurs predominantly from parents to child, and not vice versa, is supported by the observations that (i) *H. pylori* acquisition occurs predominantly in childhood and (ii) the prevalence of *H. pylori* infection in parents is much higher than that in children. Additionally, interspousal transmission of *H. pylori* occurs rarely (29, 40). Our molecular analysis of *H. pylori* suggests the vertical spread from parent (mother) to child, perhaps combined with the horizontal transmission between siblings, as the pathway for *H. pylori* transmission. If this proves to be correct, *H. pylori* would represent the first human pathogen recognized to display this remarkably restricted mode of transmission.

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