**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 all 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.

**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 gastrointestinal (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 5% 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 (5′-AGAGTT TATCCTGCTGCTCAG-3′) and pH-b (5′-AAGAGGTAGTTACAGCCAGGCA- 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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**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 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 all 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.
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, lpsA-glmM, or 16S rDNA fragments were digested with HaeIII (for ureAB or 16S rDNA), HindIII (for vacA, flaA, or lpsA-glmM), HinII (for 16S rDNA), HphI (for vacA), or SmaI (for flaA, ureAB, or lpsA-glmM) for 16 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 clone of a 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 DNA activity, 0.9 ml portions of the cell suspensions were incubated with 0.1 ml of 37°C formamide 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 agarose plugs. The required volume of cell suspension was centrifuged, and the bacteria were resuspended in 10 ml of 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 cooled for 15 min, and the enzyme buffer was replaced with 100 mM Tris-HCl (pH 8.0) (0.1 ml for 2 plugs). This suspension was mixed with an equal volume of agarose plugs. The required volume of agarose plugs was centrifuged, and then 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. 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 ml of 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 enzyme buffer for 30 to 60 min at room temperature. The enzyme buffer was replaced with 100 ml of fresh enzyme buffer containing 25 U of NotI, NheI, or Smal (New England Biolabs) and incubated overnight at 37°C (25°C for Smal). The plugs were chilled on ice for 15 min, and the enzyme buffer was replaced with 10 ml of 10 mM Tris–HCl–10 mM EDTA (pH 8.0), followed by loading of the enzyme plugs into the running gel (Pulsed-field gel electrophoresis, Bio-Rad Laboratories, Hercules, Calif.). An 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 replaced with 10 mM Tris-borate-EDTA running buffer (Bio-Rad Laboratories) was used. For restriction enzyme digestion, plugs were incubated with 1 ml of the appropriate enzyme buffer 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 ml of 10 mM Tris–HCl–50 mM EDTA (pH 8.0) at 4°C for 3 months.

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-HhaI patterns. We considered isolate A1a to be a putative 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 were summarized in Fig. 2. For 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 lpsA-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 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 an allelic combination Genotypic diversity of 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 11.25 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 lpsA-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 by implementing both PCR-RFLP and PFGE analysis.

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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 \( I_{xy} \) was 0.43. The Monte Carlo procedure indicated that the \( I_{xy} \) was significantly different from zero. Moreover, calculation of \( I_{xy} \) 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

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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 *Sac*III (D), *ure*AB fragments digested with *Hae*III (E), *lspA-glnA* 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.
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. Therefore, transmission from parent (mother) to child and perhaps from child to child predominates, occurring con-


