Helicobacter pylori is an extremely diverse species. The characterization of strains isolated from individual patients should give insights into colonization and disease mechanisms and bacterial evolution. We studied H. pylori isolates from patients in the Japanese-Peruvian Polyclinic in Lima, Peru, by determining metronidazole susceptibility or resistance and by random amplified polymorphic DNA (RAPD) fingerprinting (a measure of overall genotype). Strains isolated from several biopsy specimens from each of 24 patients were studied. Both metronidazole-susceptible and resistant strains were isolated from 13 patients, whereas strains of more than one RAPD type were isolated from only seven patients. We propose that the homogeneity in RAPD fingerprints for strains isolated from most persons reflects selection for particular H. pylori genotypes during chronic infection in individual hosts and the human diversity in traits that are important to this pathogen. Carriage of related metronidazole-resistant and -susceptible strains could reflect frequent metronidazole use in Peru and alternating selection for resistant and susceptible phenotypes during and after metronidazole therapy.

Helicobacter pylori causes chronic infections in the human gastric mucosa that can last for years or decades despite inflammatory and immune responses and changes in gastric physiology that it elicits. It is a major cause of chronic active gastritis and peptic ulcer disease and an early risk factor for gastric cancer, even though most infections are asymptomatic [1, 2]. To better understand the specificity of H. pylori–host interactions and mechanisms of colonization and virulence, and to improve methods for preventing or curing H. pylori infection and the diseases that it causes, we have begun studying H. pylori strains isolated from persons in Peru (a country with a high prevalence of infection and associated gastroduodenal diseases) [3–7] using molecular and population genetic approaches [3–7].

The high risk of H. pylori infection in Peru and other developing countries, relative to industrialized countries, is especially apparent in childhood. About one-half of Peruvian children become infected with H. pylori during their first years of life, and most others become infected within the next one to two decades. In contrast, less than one-tenth of 5-year-old children and one-fourth of young adults in North American and Western European countries carry H. pylori [8, 9]. The high risk of H. pylori infection also provides an explanation for the poor long-term results of drug treatment in Peru, relative to industrialized countries. In one study [10], three-fourths of patients whose infections seemed to have been cured by therapy, on the basis of test results 1 month after treatment, were found to again be infected <1 year later. In contrast, for persons in industrialized countries, equivalent therapy generally results in permanent eradication of H. pylori [11, 12].

Assuming complete eradication, rather than suppression below threshold detection levels, this study [10] shows that immune responses generated during a given H. pylori infection do not protect against subsequent infection, which is in accord with the failure of these responses to clear established infections. H. pylori is thought to be transmitted by fecal-oral and oral-oral routes and via recently contaminated food and water. The high risk of infection in Peru is attributable to insufficient sanitation, (which causes frequent exposure to the pathogen [5, 13–15].

Studies using random amplified polymorphic DNA (RAPD) fingerprinting (also called arbitrarily primed PCR) or other fingerprinting methods have shown that most independent H. pylori isolates are distinguishable from one another [16–20]. In industrialized countries, studies of H. pylori isolates from multiple sites indicated that just one strain predominates in most infected persons, although two or more strains were re-
covered from a small fraction of patients (generally ~5%–10%) [21–24]. Complementary studies using the gnotobiotic piglet, a short-term infection model, showed that inoculation with a mixture of two strains led to both strains becoming established in each animal for the period during which the experiments could be run (1 to several weeks) [25]. Such observations, plus a model of individual bacteria-host specificity presented below, led us to test whether Peruvians (who are at higher risk of *H. pylori* infection than are most people studied to date) would also tend to carry just one predominant strain or multiple strains.

We report the results of a study of *H. pylori* strains isolated from patients at the Japanese-Peruvian Polyclinic in Lima, Peru. The bacteria recovered from individual patients were often mixed in terms of resistance or susceptibility to metronidazole but, remarkably, were quite uniform in terms of RAPD fingerprint and thus overall genotype.

**Materials and Methods**

This study was approved by the institutional review boards of The Johns Hopkins University School of Hygiene and Public Health (Baltimore), the Universidad Peruana Cayetano Heredia (Lima), and Washington University School of Medicine (St. Louis). Endoscopy was performed essentially as previously described [26], with informed consent on adult patients of both sexes (age range, 21–65 years), who presented with dyspeptic symptoms to the Japanese-Peruvian Polyclinic in Lima in 1993 and 1994. To prevent cross-contamination, endoscopes were carefully cleaned and sterilized between patients by using successive washes of antimicrobial solution, acid alcohol, and sterile water. During each endoscopic procedure, two-to-four biopsy specimens (one or two from the antrum and one or two from the cardia or body) were taken for culture; each specimen was placed in a tube containing 0.5 mL of tryptic soy broth (Difco Laboratories, Detroit, MI) containing 20% glycerol and frozen at ~70°C until cultured.

*H. pylori* were grown on Petri plates containing brain heart infusion (BHI) agar (Difco Laboratories) supplemented with 7% horse blood (Colorado Serum, Denver), 0.5% IsoVitaleX (BBL Becton Dickinson Microbiology Systems, Cockeysville, MD), and antibiotics (6 mg of vancomycin/L, 5 mg of trimethoprim/L, and 0.8 mg of amphotericin B/L). The plates were incubated in a microaerobic atmosphere (5% O₂, 10% CO₂, and 85% N₂) and 100% humidity generally for 3–6 days, depending on the growth rate of the strain [27].

*H. pylori* strains were identified by their distinctive slow growth rate; small colony morphology; positive tests for urease, catalase, and oxidase; and/or their spiral shape when viewed microscopically. The biochemical tests were performed on a glass slide or on a piece of filter paper; one or a few colonies were picked with a sterile loop and suspended in a drop of each test solution. The urease test solution consisted of 10% urea in PBS (pH 7.2) and 1% phenol red, the catalase test solution consisted of 30% H₂O₂, and the oxidase test solution consisted of 1% 1,3,5-trimethyl-1H-phenalenediamine dihydrochloride in water or a commercial oxidase kit (Difco Laboratories). A positive urease test was indicated by a rapid change (within 1 minute) from orange to pink, a positive catalase test was indicated by rapid bubbling of the H₂O₂ solution, and a positive oxidase test was indicated by rapid development of a dark blue color [27].

Liquid cultures were prepared by scraping confluent growth from 3- to 5-day-old plates and then inoculating this suspension into 5 mL of BHI broth containing 0.5% IsoVitaleX and 7% horse serum in a 100 × 15-mm Petri plate, which was then incubated with gentle shaking in a microaerobic incubator for several days. The possibility of contamination by other less fastidious faster growing bacteria was tested by spreading a 50-μL aliquot on L agar (Difco) and incubating it overnight at 37°C.

To determine if at least some *H. pylori* from each biopsy were resistant to metronidazole or clarithromycin, bacteria were grown in liquid medium to titers of ~2 × 10⁶ cells/mL; 100 μL of each culture was transferred into a well of a sterile ELISA microtiter plate and diluted serially in 10-fold increments in PBS to densities of ~10⁶ cells/mL. Ten-microliter aliquots of each dilution were then spotted on solid medium containing 8 μg of metronidazole/mL or 4 μg of clarithromycin/mL and on control medium that was free of metronidazole and clarithromycin. Growth on metronidazole- or clarithromycin-containing media indicated resistance. Pools of resistant bacteria from spots having at least 20 colonies were replated on the same medium and grown further for DNA extraction and DNA fingerprinting.

The method of RAPD fingerprinting employed here involves PCR amplification with oligonucleotides of arbitrarily chosen sequences; low stringency annealing conditions are used to allow these oligonucleotides to prime DNA synthesis from sites to which they are only partially and fortuitously matched [28–30]. This method generates arrays of DNA fragments that reflect the spacing of pairs of fortuitous primer binding sites in the strain in question. It samples diversity throughout genomes of interest and allows closely related strains to be identified. RAPD fingerprinting is advantageous for *H. pylori* because it requires much less genomic DNA (and effort) than traditional methods, such as genomic restriction analysis or ribotyping [18–24]. Control experiments have shown that the RAPD fingerprint of a strain remains constant during its passage in culture, in an animal model, or in a single infected person.

Although it is one of the most sensitive of current methods of fingerprinting, only a tiny fraction of the genome is sampled with any given arbitrary primer, and the RAPD fingerprint is usually not changed by just one or a few mutations (such as those underlying clarithromycin or metronidazole resistance). Strains exhibiting different RAPD fingerprints usually contain DNA sequence differences at many sites in their genomes [16, 25, 30]. *H. pylori* is so diverse as a species, however, that any
given isolate can usually be distinguished from other independent isolates with just a single primer [16].

RAPD fingerprinting was carried out essentially as previously described [16]; aliquots of genomic DNA were used that had been phenol-extracted from 3–5 mL of stationary-phase liquid cultures with BHI broth and quantitated by electrophoresis in 0.7% agarose gels. The reactions were carried out in 25-μL volumes containing 1–20 ng of H. pylori DNA; 3 mM MgCl₂; 20 pmol of primer; 1.0±1.2 U of AmpliTaq DNA polymerase (Perkin Elmer, Norwalk, CT); 250 μM dCTP, 250 μM dGTP, 250 μM dATP, and 250 μM dTTP in 10 mM Tris-HCl (pH 8.3); 50 mM KCl; and 0.001% gelatin under a drop of mineral oil.

The primers used were 1254 (5'-CCGCAGCCAA) and 1281 (5'-AACGGCACAC); the primers were synthesized by DNA-Genetics (Malvern, PA). The following cycling program was used: 40 cycles of 94°C for 1 minute, 36°C for 1 minute, and 72°C for 2 minutes and then a final cycle of 72°C for 10 minutes. After PCR amplification, 8 μL of the product was mixed with 2 μL of gel-loading buffer (0.1% bromophenol blue and 50% glycerol), and samples were loaded on a 2% agarose gel containing 0.5 mg of ethidium bromide/mL in 1 × tris acetate EDTA buffer [30]. The 1-kilobase DNA ladder (GIBCO BRL, Gaithersburg, MD) was used as a size standard, and gels were photographed under ultraviolet light after electrophoresis and ethidium bromide staining.

Results

Overview. The possibility that individual Peruvians infected with H. pylori would often carry more than one strain was studied with patients at the Japanese-Peruvian Polyclinic in Lima. About 20–100 single colony isolates of H. pylori were pooled from each of two to four biopsy specimens (one or two from the antrum and one or two from the cardia or body) from each of 24 H. pylori–infected patients. The bacterial pools were characterized by determining susceptibility or resistance to metronidazole and clarithromycin and by RAPD fingerprinting.

Our primary reliance on pools of bacteria was based on three observations suggesting that such a strategy would be informative. First, early histological studies showed that H. pylori growth tends to be patchy, with some sites in infected stomachs being highly colonized and other sites being essentially free of H. pylori [26]. Second, experimental mixed infections with two H. pylori strains in gnotobiotic piglets showed nonrandom distributions of the input strains, with one strain predominating at some biopsy sites and the other strain predominating at other sites [25]. Taken together, these results suggested a microcolonial mode for H. pylori growth with relatively little migration between different sites. Third, about one-half of Peruvian H. pylori strains are resistant to metronidazole, and about one-tenth of them are resistant to clarithromycin [31].

Accordingly, it seemed that in cases of mixed infection, (1) two (or more) strains might differ in terms of susceptibility or resistance to metronidazole or clarithromycin, (2) metronidazole-susceptible bacteria might often predominate at one or more sites, and (3) DNA diversity in these bacterial populations might be detected by comparing RAPD fingerprints of pools of H. pylori that had been selected for metronidazole resistance with those from an unselected largely metronidazole-susceptible population.

Resistance. Metronidazole-resistant H. pylori strains were isolated from 17 of the 24 infected patients studied (table 1). For 13 of these patients, most H. pylori isolates from one or more biopsy sites were metronidazole-susceptible, even though many or most of the H. pylori isolates from the other biopsy sites were metronidazole-resistant. For four of these 17 patients, the entire H. pylori population might have been metronidazole-resistant.

<table>
<thead>
<tr>
<th>Biopsy site</th>
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<th>Metronidazole-susceptible</th>
<th>RAPD fingerprint</th>
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<tr>
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<tr>
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<td>C</td>
<td>A</td>
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<td>09</td>
<td>A, A, C,* C</td>
<td>B</td>
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<td>A,* B</td>
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<tr>
<td>56</td>
<td>A, A, C</td>
<td>A, C</td>
<td>Uniform</td>
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NOTE. A = antrum; B = body; C = cardia; RAPD = random amplified polymorphic DNA.

* Different RAPD fingerprints were obtained from unselected pools and metronidazole-resistant and clarithromycin-resistant subpools of bacteria isolated from the indicated biopsy specimens. To estimate the total number of strains that were distinguishable by RAPD fingerprinting (see text), we assumed that one strain caused any uniform infection and that two strains caused each mixed infection.
resistant. This inference was based on finding the bacterial pool from each biopsy site to be metronidazole-resistant, a lack of difference in RAPD fingerprints in bacterial pools grown on metronidazole-free vs. metronidazole-containing medium, and single colony isolates from each bacterial pool being metronidazole-resistant. There was no obvious preference for colonization of the antrum vs. cardia with the metronidazole-susceptible or -resistant strains.

Resistance to clarithromycin was determined in parallel. One of the 24 patients tested was found to harbor clarithromycin-resistant *H. pylori*, but this patient’s infection was mixed (due to clarithromycin-resistant and -susceptible strains).

**DNA fingerprinting.** The overall genetic diversity of *H. pylori* strains in a given patient and in strains from all patients was assessed by RAPD fingerprinting. These tests were carried out on DNA from pools of *H. pylori* recovered from two to four biopsy sites per patient and on DNA from metronidazole-resistant or clarithromycin-resistant subpopulations, when present, by using two different primers in separate reactions (figure 1). The fingerprint obtained for any given patient (or sets of fingerprints for patients carrying more than one strain) differed from the fingerprint(s) obtained for any of the other patients (figures 1A and 1B). Thirty-two different strains, distinguishable by RAPD fingerprinting, were found in the bacterial pools and single colony isolates recovered from these 24 unrelated patients (see footnote to table 1). Thus, there is considerable genetic diversity among *H. pylori* strains from Peru, in keeping with the diversity seen among strains from other geographic regions [16]. For 17 of 24 patients (including nine of the 13 who had mixed infection with metronidazole-resistant and -susceptible

![Figure 1](https://academic.oup.com/cid/article-abstract/25/5/996/341525)

**Figure 1.** Random amplified polymorphic DNA (RAPD) fingerprints for *Helicobacter pylori* isolates from Peruvian patients. A. RAPD fingerprints of pools of *H. pylori* recovered from the antrum and cardia (left and right lanes, respectively) of eight representative patients; primer 1281 and 20 ng of template DNA per reaction were used. The 0.95-kb and 1.5-kb bands in the bacterial pool from the antrum of patient 37, which were in low yield in this reaction, were of an intensity equal to that in the bacterial pool from the cardia when less (1–10 ng) template DNA was used. This finding indicates that the seeming difference seen here was due to an inhibitor. B. RAPD fingerprints for five representative patients; primer 1254 and 20 ng of template DNA were used. C. RAPD fingerprints for *H. pylori* recovered from representative patients seeming to carry more than one *H. pylori* strain (RAPD type). Reactions were carried out in duplicate with use of 2 ng and 10 ng of template DNA (left and right lanes, respectively) and primer 1281. Ant = bacterial pool from the antrum; Car = bacterial pool from the cardia; Cla = clarithromycin-resistant fraction; Mtz = metronidazole-resistant fraction. This panel shows that the differences between the bacterial pools from the antrum and cardia of patients 33 and 55, and of their unselected and drug-resistant populations, were reproducible. D. Representative RAPD fingerprints for single colony isolates from bacterial pools from the antrum (Ant) and cardia (Car) of patient 55; 10 ng and 2 ng of template DNA (left and right lanes, respectively) and primer 1281 were used. kb = kilobase; m = molecular size marker.
strains), the same RAPD fingerprint was obtained for each bacterial pool recovered from a given patient (table 1). A single colony isolate from one of the bacterial pools was tested in eight of these 17 cases, and in each case, the fingerprints generated were found to match those for the parental pools. These results suggest that the \textit{H. pylori} population in any given patient is usually quite uniform in overall genotype. Seven of the 24 colony isolates and the parental pool from the cardia biopsy were found to match those for the parental pool. These results were expected on the basis of earlier findings that \textit{H. pylori} is an extremely diverse species [16]. However, for three-fourths of the patients, just one RAPD fingerprint was observed, even though many carried mixtures of metronidazole-susceptible and -resistant variants of this predominant strain type. Findings that metronidazole-susceptible and -resistant isolates could have similar RAPD fingerprints illustrate that this technique, like other current fingerprinting methods, samples only a minority of genomic sequences and thus only rarely detects any particular mutation [32]. The many cases in which just one RAPD type (and thus overall genotype) of \textit{H. pylori} was recovered seemed remarkable, because independent \textit{H. pylori} isolates are easily distinguished [16], Peruvians are probably exposed to \textit{H. pylori} throughout their lives [3–7, 10], and a few cases of apparently mixed infection have already been seen in persons living in other lower risk environments [21–24].

One explanation proposes that only a minority of \textit{H. pylori} genotypes grow in culture. It seems unlikely to us, in part because individual infections were so often mixed with metronidazole-susceptible and -resistant strains. A second explanation assumes that these patients have significantly less exposure to \textit{H. pylori} than most Peruvians because they are of the middle socioeconomic class and thus have better sanitation. This possibility is being tested in a study of \textit{H. pylori} isolates from residents of a shantytown. Our preferred explanation is based on humans being diverse in traits such as availability of potential receptors, immune and inflammatory responses, and other features of gastric physiology that should be important to \textit{H. pylori}. Evolutionary considerations [33] predict increasing adaptation of a given strain to the individual host who carries

Discussion

We studied the diversity of \textit{H. pylori} strains isolated from patients in the Japanese-Peruvian Polyclinic in Lima to test ideas of \textit{H. pylori}–human host specificity and to better understand forces governing \textit{H. pylori} evolution. The bacteria were typed by RAPD fingerprinting, which measures overall genetic relatedness, and their susceptibility to metronidazole or clarithromycin was determined. Bacteria from two to four biopsy specimens per patient were studied, because \textit{H. pylori} growth is patchy [26] and studies involving experimental mixed infection have shown that different strains could predominate at different sites [25].

Each person’s strains were easily distinguished from one another by RAPD fingerprinting, which was expected on the basis of earlier findings that \textit{H. pylori} is an extremely diverse species [16]. However, for three-fourths of the patients, just one RAPD fingerprint was observed, even though many carried mixtures of metronidazole-susceptible and -resistant variants of this predominant strain type. Findings that metronidazole-susceptible and -resistant isolates could have similar RAPD fingerprints illustrate that this technique, like other current fingerprinting methods, samples only a minority of genomic sequences and thus only rarely detects any particular mutation [32].

The “different” strains recovered from the same patient often seemed more closely related by fingerprint than most pairs of strains recovered from different patients, although this impression still needs further testing. To learn if the small differences in RAPD fingerprints were reproducible or artifact, the samples from each patient that seemed to have mixed populations were retested by using two levels (∼10 ng and ∼2 ng) of template DNA in duplicate reactions. This retesting was based on our observations that (1) when different DNA preparations from the same \textit{H. pylori} strain yield different RAPD fingerprints, the inconsistency usually results from a weak PCR inhibitor in one DNA preparation and (2) inhibitory effects can usually be overcome by using less of the DNA preparation (K. Srivastava, J. Lelwala-Guruge, and D. E. Berg, unpublished data). The results obtained confirmed that the differences in RAPD fingerprints seen for bacterial pools isolated from seven patients were reproducible and thus probably were a reflection of genotype (see figure 1C).

Representative results for \textit{H. pylori} strains isolated from two patients are shown in figure 1C. For patient 33, different RAPD fingerprints were obtained for isolates from the antrum and cardia. Although all bacteria recovered from the antrum were metronidazole-susceptible, many of those recovered from the cardia were metronidazole-resistant. The fingerprints for the metronidazole-resistant fraction and unselected bacterial pools from the cardia were matched. For patient 55, different RAPD fingerprints were obtained for the pools of \textit{H. pylori} from the antrum and cardia that had not been selected for metronidazole resistance. In addition, some bacteria from each biopsy site were metronidazole-resistant and/or clarithromycin-resistant. The RAPD fingerprint for the rare clarithromycin-resistant isolates from the antrum differed from that for the clarithromycin-susceptible isolates that predominated at this site but matched the fingerprint for the bacteria from the cardia. Thus, a minority population was revealed by selecting for clarithromycin resistance, although we do not know whether the two types coexisted at this site or reflected carry-over of clarithromycin-resistant isolates from another site in the same patient during endoscopy.

A few single colony isolates from some of these apparently mixed infections were tested, and the results confirmed that these \textit{H. pylori} populations were indeed heterogeneous. (Figure 1D illustrates this finding for the case of patient 55.)
it (by mechanisms of mutation, gene transfer from other strains, and growth rate selection) and thus an ability to grow better in this host than most unrelated strains that he or she also ingests.

Several observations support the idea of individual host specificity. First, there are sporadic reports of infections that (unlike most) were followed from the outset and that, in some cases, were cured spontaneously soon after they began [34, 35]. Transient infection was also seen in experiments with rhesus monkeys: significantly, a strain that infected one monkey only transiently caused persistent infection in another, and conversely, a monkey who had been only transiently infected by one strain was persistently infected by another strain [36]. Transient infection is also suggested by findings of dramatic fluctuations in gastric urease levels in individual Peruvian infants over time [6]. Each of these transient infections may reflect cases in which the genotype of the infecting strain was poorly matched to the particular individual host.

In considering why many patients might carry a mixture of metronidazole-susceptible and -resistant \textit{H. pylori}, we note that metronidazole therapy is used for many diseases in Peru (usually at doses that partially inhibit growth of metronidazole-susceptible \textit{H. pylori}) and that two important metabolic enzymes are regulated differently in metronidazole-resistant and -susceptible strains [37]. If metabolic features of metronidazole-resistant strains make them grow less well than metronidazole-susceptible strains when no drug is present, the mixed infections with metronidazole-resistant and -susceptible strains might reflect the frequent usage of metronidazole in Peru and the alternating enrichments for the two phenotypes during and after each metronidazole treatment.

One explanation for finding more than one RAPD type in individual patients assumes that there are differences among sites such as the antrum and cardia or the gastric epithelium and overlying mucin in traits that could be important to \textit{H. pylori} and that some persons become infected by strains specific to these various sites. A related explanation imagines interdependence among strains (e.g., one strain grows best on undamaged tissue but induces inflammation, and another strain grows best when there is an abundance of inflammatory exudate). A third explanation supposes a population in transition [38]. Examples would include (1) recent ingestion of a strain that is better suited to the patient than the first strain colonizing him or her, and (2) a physiological change in the patient, such as worsening gastritis, that might affect the fitness of the original strain relative to a superinfecting strain.

The model of individual host specificity that we propose has important implications for virulence in societies where the risk of \textit{H. pylori} infection is high, especially in cases of gene transfer among strains that a person might ingest (because gene exchange can often generate derivatives that are better suited to a given host much more efficiently than a spontaneous mutation clone [39]). If the extent of tissue damage and inflammation tends to be related to bacterial density, evolutionary considerations predict that the average virulence of \textit{H. pylori} isolates for their individual hosts will be highest in people who encounter new strains of this pathogen repeatedly throughout their lives.

**Acknowledgment**

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**References**