Spiral Bacteria in the Human Stomach: The Gastric Helicobacters

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During the past decade, Helicobacter pylori *has become recognized as one of the most common human pathogens, colonizing the gastric mucosa of almost all persons exposed to poor hygienic conditions from childhood. It also is often found, albeit with a lower frequency, in groups of high socioeconomic status.* H. pylori *causes chronic active gastritis and is a major factor in the pathogenesis of duodenal ulcers and, to a lesser extent, gastric ulcers. In addition, the presence of this bacterium is now recognized as a risk factor for gastric adenocarcinoma and lymphoma. Nevertheless, most infections appear without clinical consequences. In this second decade of intensive research, it is important to understand why* H. pylori *is sometimes a dangerous pathogen, and to determine how it can be eradicated in those at highest risk for severe disease.*

At the end of the 19th century, several types of spirochetes and spirilla were observed for the first time in the stomach of animals (1,2). Beginning at the turn of the 20th century, similar spiral bacteria were found in gastrectomy specimens from patients with gastric cancer and peptic ulcer disease (3,4). In addition, gastroenterologists and surgeons noted—but could not explain—the almost universal presence of antral gastritis in patients with duodenal ulcers and the frequent presence of atrophic gastritis in patients with gastric ulcer and cancer. Nevertheless, the possibility that peptic ulcer disease or gastric cancer might be caused by an infectious agent was generally discounted. The observation made in 1975 that gram-negative bacteria were present in 80% of patients with gastric ulcer (5) was largely ignored by the scientific community which, at the time, was busily developing potent antiulcer agents (6). Skepticism remained the overwhelming reaction to the 1983 reports describing the frequent association between antral gastritis and the presence of *Campylobacter*-like bacteria (7), as well as of their culture and isolation from patients with gastritis (8). A similar reaction followed the subsequent demonstration that these *Campylobacter*-like bacteria were present in almost all patients with gastric and duodenal ulcers, and were generally associated with antral gastritis (9). In the past decade, however, a number of studies have confirmed and extended these early observations. A consensus regarding the major role of this bacterium, now named *Helicobacter pylori*, in causing gastroduodenal ulceration was formally presented in 1994 (10). Furthermore, in June 1994, the International Agency for Research on Cancer Working Group stated , "*H. pylori* plays a causal role in the chain of events leading to cancer," referring to adenocarcinoma and lymphoma of the stomach as well as to the more benign mucosal-associated lymphoid tissues (MALT) (11-13).

An important consequence of the considerable interest generated by these clinical observations is that extensive bacteriologic and molecular studies have been performed on this bacterium and similar organisms. 16S rRNA gene sequence analysis has revealed important differences between *H. pylori* and the closely related *Campylobacter*, *Flexispira*, and *Wolinella* genuses. These differences have necessitated the creation of the genus *Helicobacter*, which, to date, includes eight gastric, three intestinal, and two hepatic species (14). Each of these *Helicobacter* species colonizes different, or a spectrum of, mammalian species.

This review summarizes our current knowledge of the two *Helicobacter* species that have been observed in the human stomach and reported on extensively in the literature: *H. pylori*, the type strain, and *H. heilmannii*, also known as *Gastrospirillum hominis* (15,16).

Characteristics of Gastric Helicobacters Observed in Humans

H. pylori, a gram-negative bacterium with a curved, spiral, or gull-wing shape, is 2.5 to 3.5 μm long and 0.5 to 1.0 μm in diameter and has a periodicity of 1 to 2 μm. It has smooth surfaces, and one to six polar-sheathed flagellae emerge from one of its rounded ends. Since it is morphologically similar to *C. jejuni*, it was initially named "pyloric *Campy-*

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lobacter" and subsequently *C. pyloridis* and *C. pylori* before finally being named *H. pylori*. This organism colonizes only the non-acid-secreting mucosa of the stomach and is not found where parietal cells are numerous. Thus, it may be observed in the gastric antrum and the cardia, but also in the corpus, when atrophic gastritis is present, and attached to the gastric epithelial cells found in the duodenum, when gastric metaplasia is present.

G. hominis (*H. heilmannii*) is tightly spiraled, and is 3.5 to 7.5 µm in length and 0.9 µm in diameter; it has a periodicity of 0.8 to 1 µm and up to 12 flagellae at each pole. 16S rRNA indicates that this organism belongs to the genus *Helicobacter*, and is more closely related to a *Helicobacter* sp. isolated from the stomach of cats (*H. felis*) than to *H. pylori* (17). The name *H. heilmannii* was proposed in honor of the late German pathologist Heilmann. However, the subsequent examination of the rRNA of different clinical isolates indicates that there is enough heterogeneity among isolates tentatively identified as *H. heilmannii* that it is premature to propose an official name (17) . This bacterium colonizes only the parietal cell area of the gastric mucosa and may be found within parietal cells (18,19).

Diagnosis

H. pylori infection may be diagnosed by harvesting gastric biopsy specimens during endoscopy, by culturing and isolating the bacterium under microaerobic conditions (90% N_2, 5% O_2, and 5% CO_2), and by characterizing the enzymes (urease, catalase, and oxidase) it produces. Visualization of the bacterium by light microscopy on slides stained with hematoxylin and eosin, Gram, Giemsa, Genta, or Warthin-Starry stain is also of great benefit since it allows the concurrent diagnosis of the extent of the antral chronic-active gastritis that *H. pylori* causes. However, because *H. pylori* colonization is focal, negative biopsy results do not exclude the possibility of infection in areas not sampled. Infection also may be diagnosed by determining plasma and salivary immunoglobulin (Ig) G or IgA levels with enzyme-linked immunosorbent assays (20,21). This latter technique is noninvasive, specific, and sensitive and is believed to reflect the mucosal and systemic immunity induced by *H. pylori* infection.

Two other tests, which rely on the production of urease, also can be used to identify *H. pylori*. One is the CLO (for *Campylobacter*-like organisms) test, which is performed by placing a mucosal biopsy specimen in medium containing urea and a pH-sensitive dye that changes color in the presence of OH^- ions. The second test is the noninvasive ^{14}C or ^{13}C breath test following the oral administration of ^{14}C- or ^{13}C-urea. Neither of these tests is specific for *H. pylori* since *G. hominis*, which generates urease, also gives a positive reaction. Until specific methods based on the polymerase chain reaction (PCR) amplification of 16S rRNA (17) become widely available, the diagnosis of *G. hominis* infection must rely on histologic morphologic characteristics; histologic identification must be confirmed by transmission electron microscopy since other spiral organisms, e.g., *Flexispira rappini*, also may be present in the stomach of humans (22).

Epidemiology

The seroepidemiology of *H. pylori* has been extensively studied in the United States and in other countries (23). The high frequency of seropositivity (up to 100% in some age groups in Albania) and acquisition of the infection during infancy are characteristic of disadvantaged socioeconomic groups living in crowded or poor hygienic conditions and appears to be independent of gender and ethnic origin. In adults of higher socioeconomic groups, the rate of seroconversion is estimated at 0.5% per year, although the frequency of seropositivity increases with age and may be as high as 40%. A longitudinal study has indicated that the high frequency of seropositivity in older adults might be due to a higher rate of *H. pylori* infection in Western countries in the years between the two world wars than during recent years (cohort effect) (24). Alternatively, the increase in frequency of infection in older adults might be due to years of low but cumulative risk for infection. Although the route of transmission for this infection is not known, the contamination of drinking water may play a role in certain developing countries (25). In the United States and in other regions, direct contact and/or consumption of food or water contaminated by saliva (26), gastric contents, or feces (27) may be major factors. The recent observation that *H. pylori* can be isolated from cats (28) suggests that transmission from pets to humans (or humans to pets) is also possible.

The epidemiology and route of transmission of *G. hominis* are largely unknown. The frequency of this infection appears to range from less than 1% of the population in industrialized countries (29) to 3% to 8% in developing countries (30). Although the detection of spirilla in the stomach of cats and dogs suggests possible transmission from pets, marked morphologic differences exist between these spirilla and the organism found in the stomach of humans.

Pathogenicity

H. pylori is considered a pathogen because its presence is always associated with chronic active gastritis, and eradication of the bacterium is always followed by resolution of gastritis. In addition,

nearly all patients with duodenal ulcer disease have *H. pylori* gastritis, and ulcer relapse is exceptional after *H. pylori* eradication. Thus, the presence of *H. pylori* seems necessary for the production of duodenal ulcers, with the exception of ulcers attributed to the use of nonsteroidal antiinflammatory agents or to the Zollinger-Ellison syndrome (10). The association with gastric ulcers is not as strong, although *H. pylori* infection is present in 80% of patients with gastric ulcers who do not consume nonsteroidal antiinflammatory agents (10). However, most *H. pylori*-infected persons do not report any clinical symptoms. This may be because these persons are colonized by less virulent strains or because other host or bacterial cofactors are required for overt disease.

In addition, three prospective cohort studies have demonstrated that *H. pylori*-infected persons have an increased risk of developing intestinal-type, but not undifferentiated, gastric adenocarcinoma (10). In fact, the association of *H. pylori* with either gastric ulcer or gastric cancer may be underestimated in these studies: the atrophic gastritis that follows long-term infection makes the gastric niche less hospitable for the bacterium, which may either eliminate *H. pylori* or make it difficult to detect. Nevertheless, atrophic gastritis per se is believed to be a precancerous lesion that leads to carcinogenesis without the presence of *H. pylori*.

The pathogenicity of *G. hominis* is unclear. The organism has been associated with upper gastrointestinal complaints, and its carriage is generally accompanied by gastritis, although the inflammation and gastric atrophy are less than noted with *H. pylori* (31,32). In addition, *G. hominis* was observed in gastric cancer patients (3) as well as in patients with only minimal gastritis (29). In this relatively small number of cases, the frequent concurrent infection with *H. Pylori* makes interpreting the respective pathogenic role of either bacterium difficult. It is probable that G. *hominis* will turn out to be at least somewhat pathogenic, as it makes urease and products of urease action that have been implicated in inflammation.

Colonization and Virulence Factors

H. pylori multiplies with great efficiency in the hostile environment within the stomach but survives poorly in the gastric lumen; it is mainly found where the pH ranges between 4 and 7, i.e., under the mucous layer and in close proximity, or even attached, to gastric superficial epithelial cells. The virulence and the ecologic niche of *G. hominis* are unknown, although its presence within parietal cells of patients with gastrointestinal complaints (18,19) suggests that it is even more resistant to acid than *H. pylori*.

The production of urease was the first putative colonization or virulence factor studied. The production of this enzyme is shared by the two organisms, and it may explain their extraordinary ability to survive in an environment previously considered sterile because of the presence of proteolytic enzymes, as well as the low pH of gastric contents. Because the ecologic niches of these bacteria are rich in urea, urease generates OH^- ions that neutralize gastric acid. Although the neutralization of gastric acid benefits the two bacteria, the production of hydroxide ions also is toxic to gastric epithelial cells in vivo, as indicated by in vitro experiments (33).

Two other important virulence factors shared by *H. pylori* and *G. hominis* are their spiral shape and the motility of their flagellae, which render them resistant to peristaltic flushing of the gastric contents and enable them to persist in the mucous layer. Because *G. hominis* appears to infect fewer persons than *H. pylori*, a more important role might be attributable to characteristics that are unique to *H. pylori*; these include the production of other enzymes (catalase, oxidase, protease, and phospholipase), as well as the synthesis of specific adhesin proteins that enable them to adhere to mucous and epithelial cells, both in vivo and in vitro (34-36).

The putative virulence factor of *H. pylori* that has commanded the most attention during the past few years has been its vacuolating cytotoxin (*vacA* gene product). Intragastric administration of the toxin to mice causes some (but not all) of the tissue damage seen in *H. pylori*-infected persons (37). In addition, cytotoxin production is highly correlated with the production of a high molecular weight (120 to 128 kilodaltons) major protein antigen that is called cytotoxin-associated protein (*cagA*) and is not the toxin itself (38).

Diversity of *H. pylori*

H. pylori isolates may differ with respect to each of the virulence factors described above; this diversity is likely to contribute to variation in colonization or disease. For example, urease-negative strains have been isolated, and the vacuolating cytotoxin is produced by only a subset of *H. pylori* strains (*vacA*+ or tox+ strains) (39-41). This observation is probably clinically relevant because most or all strains from duodenal ulcer patients, and many strains from gastric cancer patients, produce cytotoxin, whereas only a fraction of strains from patients with gastritis alone produce the cytotoxin (42,43). This phenotypic diversity is mirrored in great diversity on the DNA level. Thus, only cytotoxin-producing strains contain the gene for this cytotoxin-associated protein (*cagA*) (38,42), although genetic tests have shown that *cagA* protein is not needed for toxin production (44). Strains that

do not produce the 128-kDa *cagA* protein generally lack the entire *cagA* gene and additional neighboring genes. Although the function of the *cagA* region is unknown, its presence or absence is easily scored by hybridization or PCR and thus serves as an easy marker for probable cytotoxin production and possible virulence of *H. pylori* strains. Additional virulence factors are likely to be present. For example, another recently discovered region constitutes at least 21 kilobases of the *H. pylori* genome in hybridization experiments, and its presence is highly correlated with the presence of *cagA*: 39 of 40 strains lacking *cagA* also lacked this region, and 50 of 52 strains containing *cagA* contained this region. This newly discovered region is being called *cagII*, and the effort to sequence it is nearly complete (D. E. Berg, pers. comm.). Preliminary searches have identified several open reading frames with strong homologies to virulence functions from other microbes (45).

In addition to these extensively studied genes, genetic diversity of various *H. pylori* strains can be demonstrated by the use of two sensitive, efficient, and reliable PCR-based methods (46,47). This approach is particularly useful because it allows tracing of strains in epidemiologic studies.

Infection and Immune Response

One of the most puzzling aspects of gastric infection with *H. pylori* is its persistence despite intense local and systemic immune responses. These immune responses are extremely complex and vary among infected humans. The systemic response is characterized by a marked increase in plasma IgG, which remains present for months after the infection has been cured. The local response includes the production of IgA, which binds to the surface antigens of *H. pylori* in vitro and coats the bacterium in vivo. In addition, infection is consistently associated with an intense inflammatory response and the infiltration of cells into the gastric mucosa. Although polymorphonuclear cells are often present, most cells in such infiltrates are mononuclear cells. Both B and T cells are present, and recent studies have indicated that the natural killer activity of peripheral blood lymphocytes can be increased by *H. pylori*, possibly by its stimulating the production of interferon and other cytokines (48). Thus, the long-term carriage of the infection may be related to the ability of the bacterium to influence the T-cell response. Fragmentary evidence also suggests that this infection can be abortive and cure spontaneously without the use of antibiotics (A. Dubois and D. E. Berg, unpublished).

On the other hand, the mucosal response may promote colonization, as indicated by the observation that patients with acquired immunodeficiency syndrome (AIDS) tend to have a lower rate of infection than aged-matched subjects who are negative for human immunodeficiency virus (49,50). The latter study (50) also demonstrated that AIDS patients had a different pattern of gastritis, characterized by greater mononuclear cell responses, fewer lymphoid follicles, and a greater prevalence of intestinal metaplasia. The immune response may also prevent the invasiveness of *H. pylori*, as suggested by the anecdotal but puzzling observation of invasive *H. pylori* infection in a patient with AIDS (51).

Treatment

Although *H. pylori* is sensitive to many antimicrobial drugs in vitro, it is difficult to eradicate from the stomach. This may be ascribed to antibiotic breakdown by gastric acid, clearance by gastric emptying, and the difficult-to-penetrate mucous layer in which the bacterium resides. Resistance of *H. pylori* to specific antibiotics, especially metronidazole, is also frequent. Therefore, it is generally accepted that a combination of at least two, and possibly three, antimicrobial agents should be given for a minimum of 1 week. The regimen found to be most effective is the administration of amoxicillin (or tetracycline) plus metronidazole and bismuth subsalicylate 2 to 4 times a day for 2 to 3 weeks (52). The use of one antibiotic associated with an antisecretory agent, such as a histamine H_2 receptor antagonist, has given disappointing results. In contrast, the combination of a proton pump inhibitor (H^+-K^+ ATPase antagonist) with amoxicillin or acid-stable macrolides (clarythromycin or roxithromycin) appears more promising; a number of studies are being conducted to determine the optimal dose, duration, concomitant therapy, and cost-effectiveness of these compounds (53,54). Recently, it was shown that at least a 7-day course of any of these regimens is required to obtain a high (90%) cure rate, but that continuing treatment for more than 10 days does not significantly improve its efficacy. Finally, topical therapy for 1 h was recently tried with excellent results, albeit in only one center at this time (55). This treatment involves a 2-day administration of a mucolytic agent to dissolve the mucous layer and of a proton pump inhibitor. On the third day, a balloon is introduced into the second portion of the duodenum under fluoroscopic control, and a solution of pronase, amoxicillin, metronidazole, and bismuth subsalicylate is injected into the stomach, where it is left for 1 h. The presence of the duodenal balloon appears to prevent emptying of the antibiotics and the mucolytic agent, thus ensuring maximum efficacy of the therapy.

Future Research

The past 12 years have seen extensive progress in research on *H. pylori* as a cause of chronic active gastritis, duodenal ulcer disease, and gastric cancer. This has been largely due to an unusual collaboration among gastroenterologists, pathologists, molecular geneticists, bacteriologists, and immunologists. However, our understanding of how *H. pylori* colonizes and causes diseases is far from complete, and it will benefit from studies performed in animal models that can be experimentally infected with *H. pylori* (56-59). In addition, no easily administered treatment leading to eradication of this bacterium in all patients is yet available, although a better knowledge of its physiology may lead to the development of such a "silver bullet." Studies in animals that are not naturally infected with *H. pylori* suggest possibilities for vaccines (56,57), and ongoing trials in nonhuman primates are exploring the possibility of immunizing hosts that can be naturally infected with this organism. Although the elimination of peptic ulcer disease and of certain forms of gastric cancer will require extensive and coordinated efforts from public health authorities, this goal now appears to be within the reach of the scientific and medical community.

Acknowledgments

The author thanks Drs. P. Baker and D.E. Berg for their helpful comments and suggestions during the preparation of this review.

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HIV-1 Patients May Harbor Viruses of Different Phylogenetic Subtypes: Implications for the Evolution of the HIV/AIDS Pandemic

The virus variants isolated from HIV-infected persons worldwide share remarkable diversity, especially in the envelope glycoprotein, gp120. Phylogenetic studies have clustered HIV-1 isolates into eight subtypes (A-H). Nevertheless, even within a single infected person, HIV is present as a "quasispecies," or a swarm of closely related variants. This genetic diversity, which in the case of HIV-1 accumulates at a rate of approximately one nucleotide substitution per genome per replication cycle, gives the virus an enormous flexibility to respond to a wide array of in vivo selection pressures. As a consequence, drug-resistant and immunologic escape mutants are rapidly generated in infected persons through all stages of infection. On a global scale, the HIV pandemic is recognized as consisting of many separate epidemics, each with characteristic geography, affected populations, and predominant viral strain type. With an estimated 15 million infected persons, the geographic distribution of viral subtypes is becoming more dispersed, and these demarcations are further confounded by growing evidence of mixed infections.

The epidemic emergence of mixed heterotypic infections with HIV-1 and HIV-2 variants has been recognized for some time in the geographic areas where both types of viruses are present. We reported these infections in Côte d'Ivoire and Brazil (1, 2); they have also been reported from India (3). In contrast, homotypic mixed infections of distinct HIV-1 variants have only recently been suggested by the presence of broadly reactive sera and evidence of HIV recombinants from geographic regions in which multiple HIV-1 subtypes are circulating. Dual HIV-1 infection in two patients from Thailand has been demonstrated by viral DNA sequence analysis (4).

As the HIV-1 pandemic has grown, the simultaneous presence of multiple subtypes in a region has become common. As a consequence, an increased frequency of HIV-1 mixed infections could be expected. Thus, there is a need to estimate the prevalence and geographic distribution of this type of infection. Sequence analysis of HIV proviral DNA has been the method of choice to characterize HIV genetic diversity. However, because even relatively limited sequence determinations of small polymerase chain reaction (PCR) fragments are time consuming and very labor-intensive, this method is not particularly practical for large-scale molecular epidemiologic studies. To address this problem, we have developed a genetic method based on restriction site polymorphism to screen for homotypic HIV-1 mixed infections within infected populations. The concept of this assay is based on the observed correlation between the restriction maps of HIV-1 isolates with their phylogenetic classification, which is based on the sequence data. Thus, certain restriction enzymes may be used to predict the phylogroup of HIV-1 infected samples. The differences in electrophoretic mobility of endonuclease digestion products result from restriction site polymorphisms in the selected region of the HIV-1 genome and allow for quick recognition of the distinct phylogenetic subtypes. A 297 bp pol fragment spanning the entire viral protease gene is used for our analysis. The viral gene is amplified by nested PCR using DNA templates from uncultured peripheral blood mononuclear cells (PBMC) or virus culture. Preliminary classification of HIV-1 strains to well defined subtypes A, B, C, D, and F is done by sequential endonuclease restriction analysis. AluI restriction polymorphism in a PCR-amplified protease gene segregates viral strains into two groups: subtypes B and D belong to one group, and subtypes A, C, and F to another (Figure 1A). Further differentiation of HIV-1 subtypes within those two groups is accomplished by analysis of HinfI, BclI, MaeI, SpeI, and ScaI restriction enzyme digestion patterns of the protease gene (Pieniazek et al., manuscript in preparation). The electrophoretic migration patterns visualized by ethidium bromide staining or by radiolabeled probes are then determined on a 10% acrylamide gel. In single infections, a single restriction pattern is detected, whereas in multiple infections involving HIV-1 strains of distinct subtypes, complex digestion patterns are observed in infected persons. As an example, in Figure 1A, we present three distinct AluI restriction patterns of the protease gene that are characteristic for single infections by viruses of subtypes A, C, and F (pattern #1) and by subtypes B and D (patterns #2 and #3). In Figure 1B, we show a typical combination of two distinct AluI restriction patterns (#1 and #2) found in a patient infected with two viral strains of subtypes F and B. Basing our analysis on the conserved protease gene region, we should detect most HIV-1 strains; however, some highly divergent isolates could escape PCR amplification as a result of primer mismatches. Moreover, since a single nucleotide substitution could either generate or destroy a restriction site, sequence analysis remains the ultimate tool in identifying variants of multiple infections. Nevertheless,

this assay can be conveniently applied to screen a large number of samples.

By using this method, we have screened HIV-1 proviral DNA from 208 specimens collected from countries in South America, Africa, and Asia where HIV-1 strains of distinct subtypes are found. We observed the simultaneous presence of two distinct digestion patterns in PCR amplified protease gene (Figure 1B) in 31 samples; our observation suggests superinfection with HIV-1 strains of distinct origin. To eliminate the potential for laboratory cross-contamination, we analyzed the restriction patterns of the protease gene from multiple aliquots of the patient's PBMC. In addition, the analysis was repeated on DNA from a second collected blood sample from each of the patients. The analyses for the first five of 31 patients were completed, and data are summarized here (details are in Janini et al., manuscript in preparation). Sequence and phylogenetic analysis of the viral protease gene (Figure 2) in PBMC from those five patients confirmed dual infections caused by HIV-1 strains of subtypes B and F in one person (Br5), subtypes F and D in another patient (Br22), and subtypes C and D in a married couple (Br19 and 20). Moreover, in the child (Br30) of this couple, two distinct AluI digestion patterns were also found; the major HIV-1 strain clustered among subtype C viruses of the parents. The minor strain of this child is likely to represent subtype D, but there was not sufficient material for cloning and further sequencing of this strain.

Detection of naturally occurring heterotypic and homotypic multiple infections may have important implications for immunotherapies because infection with one HIV subtype may not fully protect against subsequent superinfections with distinct HIV strains. However, we do not know if the acquisition of viruses in the dually infected adult patients was sequential or simultaneous. Nevertheless, the consequences of mixed infections may profoundly affect the ability of the virus to change and may modify the direction of the pandemic through altered patterns of viral pathogenesis, increased genetic variation through recombination, and the generation of

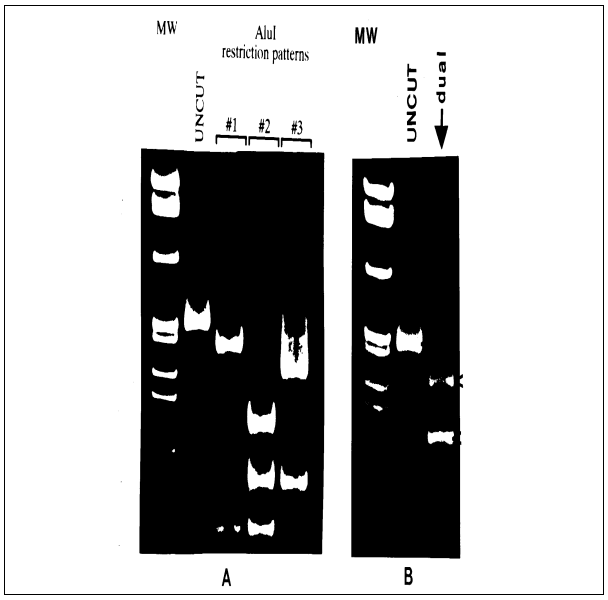

Figure 1.
A. Three distinct AluI digestion patterns of PCR amplified protease gene representing single HIV-1 infections by viral strains of subtypes A, C, and F (pattern #1), and subtypes B and D (patterns #2 and #3).
B. The presence of two distinct AluI digestion patterns (#1 and #2) of the protease gene in PBMC of the patient dually infected by viral strains of subtypes F and B (lane 3). Arrows indicate diagnostic fragments detected by hybridization with the radioactive probe (2). MW represents molecular weight markers—ΦX174 RF DNA, HaeIII digest.

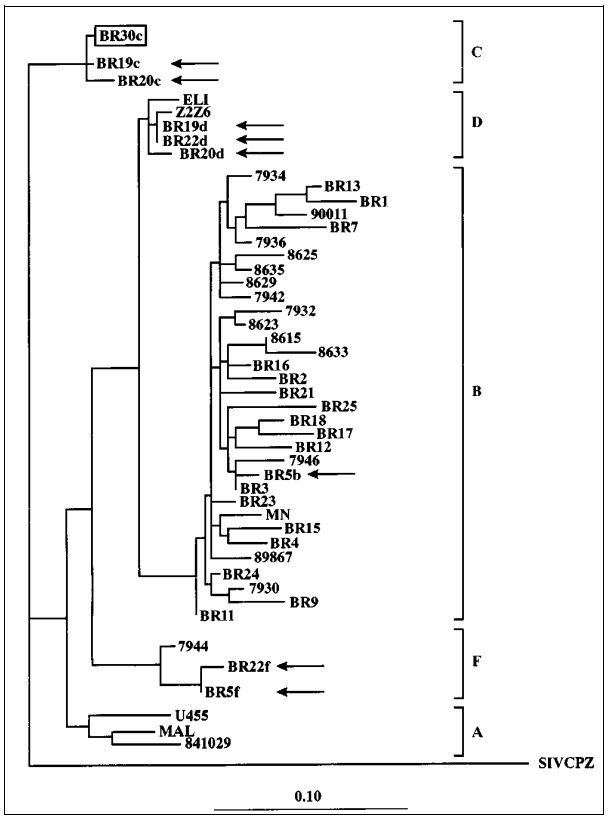

Figure 2.
Phylogenetic classification of HIV-1 strains in dually infected patients. HIV-1 sequences from dual infections (Br5, 19, 20 and 22) are indicated by arrows, and the major strain in the infected child (Br30) is boxed. The tree was constructed on the basis of the DNA sequences of the protease gene by using the maximum likelihood method with the fastDNAml program (6). SIV-cpz protease sequence was used as an outgroup. The distinct HIV-1 subtypes are delineated. The scale bar shows the ratio of nucleotide substitutions for given horizontal branch length. Vertical distances are for clarity only.

pseudotype virions, including phenotypically mixed virus particles. It is to be anticipated that such events would ultimately broaden the cellular tropism for HIV and mandate the designed polyvalent immunotherapies. Finally, our data together with recently published genetic analysis for HIV-1 and HIV-2 (5) suggest that multiple homotypic infections with divergent HIV strains may be more common than previously thought. The screening assay described here will be useful in estimating incidences of such HIV-1 infections. We believe that this information is crucial for both evaluating the pandemic and developing intervention strategies.

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