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Typing of *Campylobacter pylori* by Bacterial DNA Restriction Endonuclease Analysis and Determination of Plasmid Profile

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***Campylobacter pylori* isolates from 37 symptomatic patients and 3 asymptomatic volunteers were examined by chromosomal DNA restriction endonuclease analysis and determination of plasmid profile. Restriction digests with *Hin*dIII, *Hae*III, *Pvu*II, and *Bgl*II produced clear and reproducible results that permitted discrimination between different strains. Only 35% of *C. pylori* isolates were found to have plasmid DNA. Isolates from different patients, including those from two pairs of siblings, had unique restriction patterns and plasmid profiles. Consecutive isolates obtained 1 year apart from each of two asymptomatic volunteers had identical restriction patterns and plasmid profiles, suggesting persistence of the same strain. A pair of isolates obtained one year apart from the third volunteer differed in plasmid DNA content but had similar chromosomal DNA restriction patterns. Plasmid profile determination and bacterial DNA restriction endonuclease analysis provide a reliable means of discriminating between different strains of *C. pylori* and may be useful for typing these organisms in epidemiologic studies.**

The discovery of the association of the organism which is currently referred to as *Campylobacter pylori* with inflammatory diseases of the stomach and duodenum has generated considerable excitement in the medical community. There is mounting evidence that this bacterium has an etiologic role in the development of gastritis, but its role in gastric and duodenal ulcers remains uncertain (1, 2, 4, 10, 14). It is known that the organism may be found in diverse human populations around the world and that its prevalence increases with age (1, 7, 8, 20). However, only limited data exist regarding the epidemiology of *C. pylori* infection. There has been one report of the isolation of *C. pylori* from a site other than the stomach or intestinal tract (9), but to date it has not been isolated from the inanimate environment. The source or natural reservoir for this organism and its route(s) of transmission remain unknown. Information about the natural history and optimal therapy of *C. pylori*-associated gastritis is also lacking. A reliable method of typing these organisms would permit detailed epidemiologic investigations and the determination of virulence factors. Neither biotyping nor serotyping classification schemes for *C. pylori* are available. Restriction endonuclease analysis of genomic DNA has recently been described as a means of typing *C. pylori* (11, 12). In this study, we describe modifications to previously reported methods and confirm that determination of plasmid profiles and bacterial DNA restriction endonuclease analysis are useful techniques for identifying epidemiologically linked *C. pylori* isolates. The use of these techniques also allowed us to make some important observations regarding the carriage of *C. pylori* in asymptomatic subjects and its epidemiology in family members.

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MATERIALS AND METHODS

Source of isolates. *C. pylori* isolates from symptomatic patients and asymptomatic volunteers who had participated in a previous study (8) (mean age, 48 years) and isolates obtained from two pairs of symptomatic siblings (ages, 11 to 16 years) with duodenal ulcers were examined. Gastric antral biopsies were obtained through an Olympus GIF Q10 fiberoptic endoscope (Carsen Medical & Scientific Co. Ltd., Markham, Ontario, Canada). The endoscope and biopsy forceps were disinfected in 2% glutaraldehyde after each procedure. Biopsy specimens were minced and inoculated onto 5% sheep blood agar and a modified Skirrow medium (Oxoid Ltd., Basingstoke, England) within 2 h of procurement. The plates were incubated at 35°C under microaerobic conditions (Gas Generating Kit for Campylobacters; Oxoid) and were examined daily for 7 days. *C. pylori* was identified by accepted criteria (1, 15), and isolates were stored at −70°C in 0.5 ml of sheep blood.

Preparation of chromosomal DNA. *C. pylori* isolates were grown on 5% horse blood agar plates. Cells (approximately 250 mg) were scraped from three to five plates and then were washed by centrifugation (8,700 × *g*, 10 min, 4°C), twice in phosphate-buffered saline (pH 7.2) and twice in TE (10 mM Tris hydrochloride, 100 mM sodium EDTA, pH 8.5). The pellet was suspended in 4 ml of TE, and lysozyme (type II; Sigma Chemical Co., St. Louis, Mo.) was added to a final concentration of 3.0 mg/ml. The suspension was incubated for 12 min at 37°C and then was lysed with sodium dodecyl sulfate (final concentration, 1%). The lysate was incubated for 60 min at 37°C in the presence of RNase A (bovine pancreas; Boehringer Mannheim Biochemicals, Indianapolis, Ind.) at a final concentration of 0.05 mg/ml. Pronase (final concentration, 0.8 mg/ml; Boehringer Mannheim) and proteinase K (final concentration, 0.5 mg/ml; Boehringer Mannheim) were added, and the lysate was incubated overnight at 37°C. DNA was extracted with an equal volume of phenol: chloroform (1:1 [vol/vol]) and precipitated overnight in 0.3 M sodium acetate and 2.1 volumes of absolute ethanol at −20°C. The DNA was pelleted by centrifugation at 12,100 × *g* for 60 min at 4°C. The ethanol was decanted, and the pellet was air dried and redissolved in sterile distilled water. A portion was taken, and the A_{260} and the A_{280} were measured in a spectrophotometer (Beckman DB-G) to ascertain purity

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and for quantitation. The procedure yielded 0.5 to 1.5 mg of DNA. The DNA preparations were suspended in sterile distilled water and stored at −20°C.

Restriction endonuclease analysis of bacterial DNA and gel electrophoresis. Approximately 20 μg of genomic DNA from each isolate was digested to completion in excess of enzyme and according to the instructions of the manufacturer. The following enzymes were used: *Hin*dIII, *Hae*III, *Pvu*II, *Bgl*II, *Cfo*I, *Sma*I, *Ava*I, *Dra*I, *Kpn*I, *Rsa*I, *Sal*I, and *Eco*RI (Boehringer Mannheim). The reaction was terminated by the addition of gel-loading buffer (40% sucrose, 0.25% bromphenol blue). The DNA fragments were separated by gel electrophoresis in a 0.8% horizontal agarose gel overnight (16 h) at 30 V in TAE buffer (40 mM Tris hydrochloride, 20 mM sodium acetate, 2 mM EDTA, pH 7.6). The gels were stained with ethidium bromide (1 μg/ml) and photographed with filtered UV illumination on Polaroid P/N type 665 film. Three or more different enzymes were used per isolate. For 21 strains, restriction endonuclease analysis was repeated at least three times with different extractions and DNA preparations each time to determine the reproducibility of the technique. Restriction patterns were compared by visual inspection, and any observed differences were interpreted as signifying different strains. Two isolates were considered to be the same strain if no band differences were noted in restriction patterns demonstrated with three different enzymes.

Plasmid profiles were demonstrated by electrophoresis of undigested DNA according to conditions described above.

Elution of chromosomal DNA. Separation of chromosomal DNA from plasmid DNA was performed by the method of Maniatis et al. (13). Briefly, a trough was cut into the electrophoresis gel in front of the leading edge of the chromosomal band. The trough was filled with TAE buffer, and the distal side blocked with dialysis tubing. Electrophoresis was resumed until the chromosomal DNA band had entered the trough. Migration of the band was monitored by UV light. The eluted DNA was extracted with phenol: chloroform (1:1) and then treated and electrophoresed as outlined above.

RESULTS

A total of 45 *C. pylori* isolates from 40 patients were examined for plasmid DNA content and by chromosomal DNA restriction endonuclease analysis. Among the strains studied were three pairs of isolates obtained 1 year apart from each of three asymptomatic volunteers (subjects 1, 2, and 3) and a pair of isolates recovered from gastric biopsies at two distinct sites during the same endoscopy from each of two siblings (siblings A1 and A2).

Among the 40 epidemiologically unrelated isolates, 14 (35%) were found to have one or more plasmids. The plasmid profiles of these isolates differed from each other. The two isolates recovered 1 year apart from subject 1 also had different plasmid profiles (Fig. 1, lanes 1 and 2). Consecutive isolates were obtained 1 year apart from subject 2 (Fig. 1, lanes 3 and 4) and subject 3 (data not shown); the profile of each of the repeat isolates was identical to that of the corresponding initial isolate. The two isolates taken from sibling A1 during the same endoscopy also had identical plasmid profiles (Fig. 1, lanes 5 and 6).

To evaluate the applicability of restriction endonuclease analysis of *C. pylori*, tests were first performed with 12 endonucleases. The best results were obtained with *Hin*dIII, *Hae*III, *Pvu*II, and *Bgl*II. These enzymes produced clear

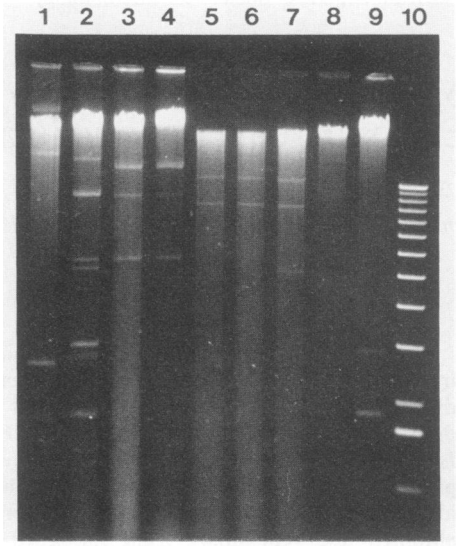

FIG. 1. Agarose gel electrophoresis of undigested DNA from *C. pylori* isolates demonstrating plasmid profiles. Lanes: 1, initial isolate from subject 1; 2, second isolate recovered from subject 1 1 year later; 3, initial isolate from subject 2; 4, second isolate recovered from subject 2 1 year later; 5, initial isolate from sibling A1; 6, second isolate recovered from different biopsy site in sibling A1; 7, isolate from sibling A2; 8, isolate from sibling B1; 9, isolate from sibling B2; 10, molecular weight marker (1-kilobase DNA ladder; Bethesda Research Laboratories, Inc., Gaithersburg, Md.) with sizes ranging from 12.216 to 0.516 kilobase pairs.

and reproducible digestion patterns that could easily be assessed for comparing different *C. pylori* isolates. *Eco*RI, *Kpn*I, and *Ava*I also digested *C. pylori* DNA, but these restriction enzymes yielded large fragments such that unique restriction patterns were not easily discernible. Studies with a limited number of strains indicated that digestion with *Dra*I resulted in extensive digestion without discrete banding. When restriction endonuclease analysis was repeated after up to six subcultures of an isolate, or with up to six different DNA preparations from a single strain, consistent and reproducible results were obtained with *Hin*dIII, *Hae*III, and *Pvu*II. Similarly, restriction endonuclease analysis of DNA preparations from several *C. pylori* colonies isolated from the same biopsy specimen yielded the same restriction patterns.

C. pylori isolates from different patients, including the isolates from two pairs of siblings, had unique restriction endonuclease analysis patterns, differing by several bands in each case (Fig. 2 and 3). *Hae*III and *Pvu*II digests of chromosomal DNA from two consecutive isolates obtained from two asymptomatic volunteers and one of the pairs of siblings are shown in Fig. 2 and 3. The restriction patterns of the second isolates were identical to those of the original isolates from subject 2 and sibling A1 (and also from subject 3 and sibling A2; data not shown). There was, however, a small difference in the restriction endonuclease patterns of the pair of isolates recovered from subject 1. In order to determine whether this was due to differences in plasmid or chromosomal DNA, chromosomal DNA was eluted and restriction endonuclease analysis was repeated for these two isolates. *Hin*dIII digests of the eluted chromosomal DNA were identical (Fig. 4). *Hae*III, *Pvu*II, and *Bgl*II digests of the eluted chromosomal DNA confirmed these results (data not shown).

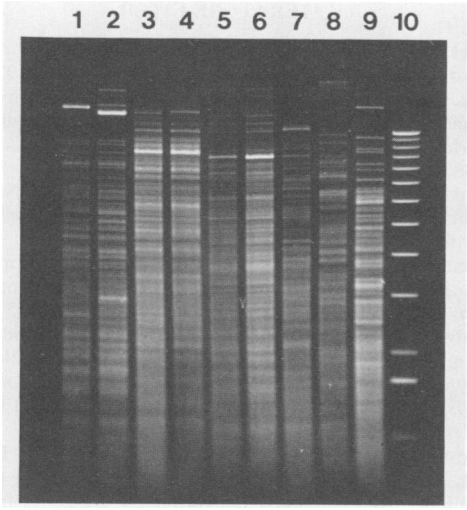

FIG. 2. Restriction endonuclease analysis of genomic DNA from *C. pylori* isolates with *Hae*III. Lanes: 1, initial isolate from subject 1; 2, second isolate recovered from subject 1 1 year later; 3, initial isolate from subject 2; 4, second isolate recovered from subject 2 1 year later; 5, initial isolate from sibling A1; 6, second isolate recovered from different biopsy site in sibling A1; 7, isolate from sibling A2; 8, isolate from sibling B1; 9, isolate from sibling B2; 10, molecular weight marker (1-kilobase DNA ladder; Bethesda Research Laboratories) with sizes ranging from 12.216 to 0.516 kilobase pairs.

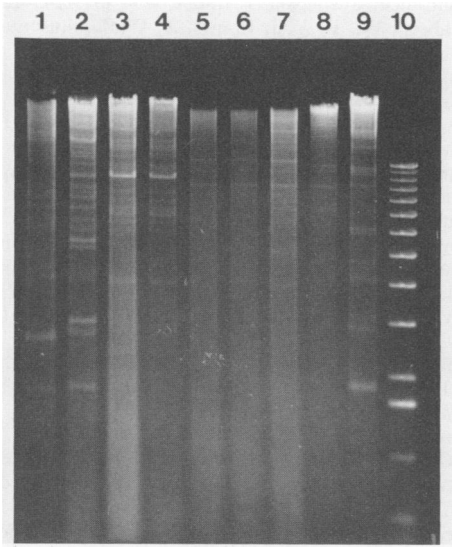

FIG. 3. Restriction endonuclease analysis of genomic DNA from *C. pylori* isolates with *Pvu*II. Lanes: 1, initial isolate from subject 1; 2, second isolate recovered from subject 1 1 year later; 3, initial isolate from subject 2; 4, second isolate recovered from subject 2 1 year later; 5, initial isolate from sibling A1; 6, second isolate recovered from different biopsy site in sibling A1; 7, isolate from sibling A2; 8, isolate from sibling B1; 9, isolate from sibling B2; 10, molecular weight marker (1-kilobase DNA ladder; Bethesda Research Laboratories) with sizes ranging from 12.216 to 0.516 kilobase pairs.

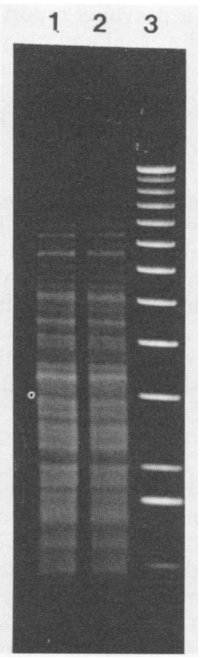

FIG. 4. *Hin*dIII digests of eluted chromosomal DNA from *C. pylori* isolates with different plasmid profiles (subject 1). Lanes: 1, initial isolate; 2, second isolate recovered 1 year later; 3, molecular weight marker (1-kilobase DNA ladder, Bethesda Research Laboratories) with sizes ranging from 12.216 to 0.516 kilobase pairs.

DISCUSSION

Epidemiologic studies to determine the ecology, means of transmission, and natural history of *C. pylori* infection have been impeded by the lack of simple typing methods such as serotyping or biotyping. The use of immunoblot fingerprinting of *C. pylori* has been described (3), but this method is not practical for routine use and its accuracy is dependent on standardized antigen and antibody preparations. Plasmid profile determination (19) and chromosomal DNA restriction endonuclease analysis (11, 12) have been shown to be sensitive methods for detecting differences in *C. pylori* isolates. In this report, we describe the application of these techniques for studying *C. pylori* infection in asymptomatic adults and in siblings with duodenal ulcers.

Plasmid profile analysis has been shown to be a useful technique for characterizing isolates from a variety of bacterial species (17). In our experience, only 14 (35%) of 40 *C. pylori* isolates were found to contain plasmids, limiting the usefulness of this technique by itself as an epidemiologic typing tool. In two previous reports (12, 19), plasmid DNA was detected in 48% of *C. pylori* isolates, most with unique plasmid profiles. These plasmids appear to have been cryptic.

Our observation of considerable genomic diversity among *C. pylori* isolates confirms results previously reported by Majewski and Goodwin (12) and by Langenberg et al. (11). Isolates obtained from different patients invariably demonstrated unique DNA restriction endonuclease patterns. Although Rauws and co-workers (E. A. J. Rauws, W. Langenberg, J. Oudbier, C. J. J. Mulder, and G. N. J. Tytgat, Gastroenterology **96**:A409, 1989) used bacterial restriction endonuclease analysis to determine that a single *C. pylori* strain infected eight members of one family, we found that isolates from siblings within the same family were

clearly different. In a previous report, we also determined that isolates from a husband and his wife were different by examining lipopolysaccharide structure and restriction endonuclease analysis of chromosomal DNA (B. Shames, S. Krajden, C. Babida, R. V. Gurgis, L. Kurjanczyk, and J. L. Penner, Abstr. 1st Meet. Eur. *Campylobacter pylori* Study Group, Bordeaux, France, 7 to 8 October 1988, abstr. no. P-124). Similarly, Majewski and Goodwin (12) recovered different strains from a brother and his sister and from a husband and his wife. Therefore, it appears that closely related family members may be infected with different strains of *C. pylori* despite the opportunity for acquisition of the organism from a common source or after prolonged intimate contact between family members.

Whereas Majewski and Goodwin (12) found that consecutive isolates of *C. pylori* from 5 of 10 patients were different, Langenberg et al. (11) reported that the first and subsequent isolates from six patients had identical restriction endonuclease patterns. We found that consecutive isolates from the same patient were identical for two of three individuals. These isolates were recovered 1 year apart from each of the untreated asymptomatic volunteers. In the other asymptomatic volunteer (subject 1), the first and second isolates had different plasmid DNA content; it appeared that the second isolate had acquired one or more plasmids. The possibility of coinfection with an unrelated strain was excluded because the restriction endonuclease analysis patterns of the plasmid-free chromosomal DNA of both strains were identical. This example illustrates the utility of combining plasmid profile determination with chromosomal restriction endonuclease DNA analysis for confirming the identities of strains. In addition, the observation that *C. pylori* may persist in the stomach for prolonged periods of time without symptoms or evidence of disease progression indicates that infection by this organism does not often resolve spontaneously. This persistence of *C. pylori* and the associated gastric inflammation have also been noted in symptomatic patients where eradication of the organism and resolution of the inflammation occurred only with appropriate antimicrobial therapy (10).

Since the first description of the isolation of *C. pylori* in culture in 1983 (J. R. Warren and B. Marshall, Letter, Lancet **i**:1273–1275, 1983), much has been learned about the association of this organism with inflammatory diseases of the upper gastrointestinal tract, the resulting systemic immune response (5, 20), and various methods of diagnosis (6, 7, 16, 18, 20). However, many questions remain about its environmental source and routes of transmission, pathogenic mechanisms, natural history, and optimal therapy. These questions will be more easily answered with the development of a suitable animal model of human disease and with a practical method of typing these organisms. As this report indicates, plasmid profile determination and bacterial DNA restriction endonuclease analysis together provide a sensitive and reliable means of discriminating between different strains of *C. pylori*. Therefore, these techniques should prove to be useful typing tools in future epidemiologic investigations.

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