10-11-1992

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Publication Info

Published in *Nucleic Acids Research*, Volume 20, Issue 19, 1992, pages 5137-5142.

Nucleic Acids Research, Vol. 20, No. 19 5137–5142

DNA diversity among clinical isolates of *Helicobacter pylori* detected by PCR-based RAPD fingerprinting

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Received June 16, 1992; Revised and Accepted September 1, 1992

ABSTRACT

The RAPD (or AP-PCR) DNA fingerprinting method was used to distinguish among clinical isolates of *Helicobacter pylori*, a bacterium whose long term carriage is associated with gastritis, peptic ulcers and gastric carcinomas. This method uses arbitrarily chosen oligonucleotides to prime DNA synthesis from genomic sites to which they are fortuitously matched, or almost matched. Most 10-nt primers with ≥ 60% G+C yielded strain-specific arrays of up to 15 prominent fragments, as did most longer (≥ 17-nt) primers, whereas most 10-nt primers with 50% G+C did not. Each of 64 independent *H.pylori* isolates, 60 of which were from patients in the same hospital, was distinguishable with a single RAPD primer, which suggests a high level of DNA sequence diversity within this species. In contrast, isolates from initial and followup biopsies were indistinguishable in each of three cases tested.

INTRODUCTION

The bacterium *Helicobacter pylori* colonizes the upper gastrointestinal tract in the majority of people at some time during their lives, and its long term carriage is implicated in the pathogenesis of chronic active gastritis, peptic ulcers and gastric carcinomas (1,2). Efficient, sensitive and reproducible methods are needed to distinguish among clinical isolates of this, and indeed any, pathogen, in order to: (i) assess roles of bacterial genotype vs. other factors (e.g., host genotype, nutrition, length of residence) in cancer and other disease states; (ii) trace patterns of infection in families and larger population groups; and (iii) elucidate mechanisms of long term colonization and recurrent disease. A variety of DNA-based methods have been used to identify individual isolates of *H.pylori*, including plasmid size, chromosomal restriction patterns, Southern blotting, and restriction of a specific PCR fragment (3–6). Independent clinical isolates are often not distinguished from one another in these tests. This could reflect a clonal population structure, as in several other bacterial species (7,8), or limitations in the methods used for strain identification.

We report here the use of a PCR-based DNA fingerprinting method termed 'RAPD' (for 'random amplified polymorphic DNA') (9) or AP- (for arbitrary primer) PCR (10,11) to detect DNA sequence diversity among *H.pylori* isolates. This method uses an oligonucleotide of arbitrarily chosen sequence to prime DNA synthesis from pairs of sites to which it is matched or partially matched, and results in strain-specific arrays of DNA products. Using the RAPD method, we found that each of 64 independent *H.pylori* isolates was distinguished with just a single primer, whereas isolates from initial and followup biopsies were not distinguishable in each of the three cases tested.

MATERIAL AND METHODS

H.pylori strains

NCTC11637 and NCTC11638 are from Australia (12), P466 is from Peru (kindly provided by Dr. R. H. Gilman, ref. 13) and MO19 is from Missouri (14). These five strains differ in the specificity of adherence to rat and human stomach biopsy tissue (15). The 64 strains given a WV designation were collected from patients at the Veterans Administration Medical Center, Huntington, West Virginia by one of us (T.U.W.; 16,17). Forty eight of the 53 patients for whom data were available had been diagnosed with chronic active gastritis during biopsy; two had gastric ulcers; and one had intestinal metaplasia. Forty of the 49 for whom data were available were at least 50 years old and seven were in their 40s at the time of biopsy (1987, 1988). No two patients had the same surname, implying that none were siblings and that most or all were living in separate households. The *H.pylori* isolates were given the following WV designations: 8, 9, 19, 22, 23, 36, 40, 46, 51, 54, 56, 68, 69, 76, 78, 88, 91, 99, 102, 103, 105, 107, 108, 114, 126, 130, 134, 135, 138,

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140, 141, 146, 153, 155, 156, 160, 163, 170, 173, 176, 182, 183, 185, 186, 188, 199, 202, 203, 210, 214, 217, 221, 226, 228, 229, 230, 231, 239, 244, 245, 257, 258, 263, 275. Of the WV strains, seven were isolated from sequential biopsies from three patients that had been treated with bismuth subsalicylate (Pepto-Bismol®) and clindamycin (in a clinical trial to test whether this treatment would eradicate the organism; 16): WV 146, 182 and 229 from patient 'A' (~8 weeks between initial and each followup biopsy); 188 and 231 were from patient 'B' (~14 weeks between initial and followup biopsies); and 170 and 214 from patient 'C' (~16 weeks between initial and followup biopsies).

Bacterial growth, DNA preparation, and Southern analyses

Bacteria were grown with gentle shaking under microaerophilic conditions (5% O_2 10%CO_2, 85% N_2) at 37°C in Brucella broth containing 10% human serum and 1% IsoVitaleX before DNA extraction (18). *H.pylori* DNA to be used for RAPD PCR was prepared as follows: 3 ml stationary phase (5 days) liquid cultures were collected by centrifugation, and resuspended in 200 µl GTEL buffer (50 mM glucose, 50 mM Tris HCl, pH 8.0, 50 mM EDTA, 10 mg/ml lysozyme), and incubated for one hour at 37°C. Then 500 µl of TESK lysis buffer (50 mM Tris HCl, pH 8.0, 50 mM EDTA, 1% SDS, 50 mg/ml proteinase K) was added and the solution was incubated for two hours at 55°C. RNAse was added to a final concentration of 20 µg/ml, and the mixture was incubated for 10 min at 55°C. The solution was extracted sequentially with equal volumes of phenol, phenol/chloroform/isoamyl alcohol (25:24:1) and chloroform isoamyl alchohol (24:1). 1/10 volume of 3 M sodium acetate was added. The DNA was precipitated with 2 vol ethanol, washed in 70% ethanol, and dissolved in 100 µl of distilled water. Generally 1–2 µg of DNA was obtained from 3 ml of culture. The larger amounts of DNA needed for Southern blots were prepared by the same method, but scaled up for 200 ml cultures. Standard protocols were used for electrophoresis, Southern blotting and autoradiography (19).

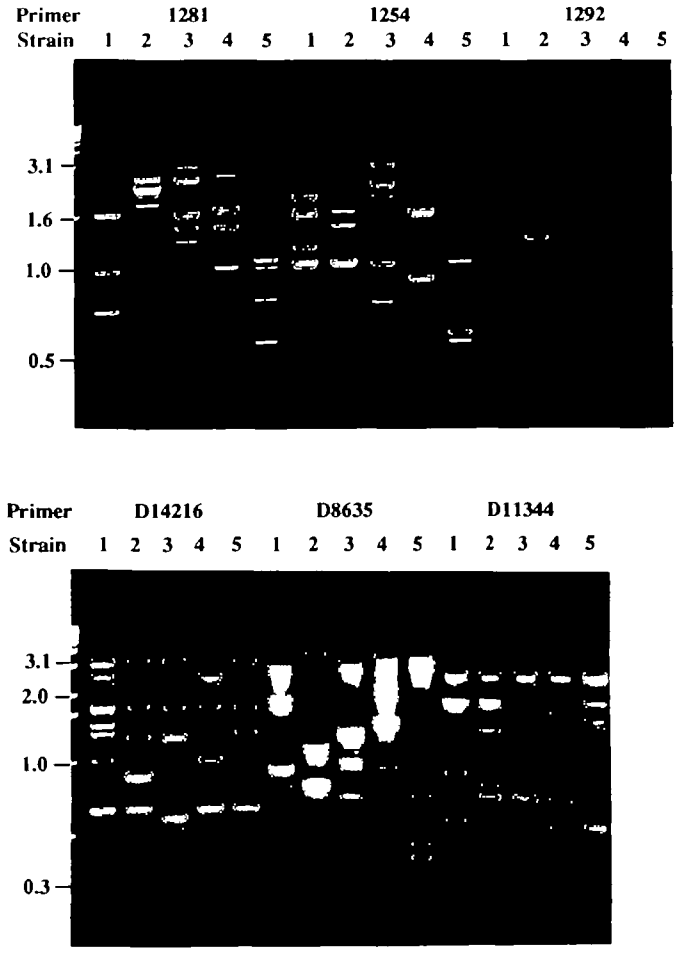

Figure 1. Representative results of tests to identify useful primers for RAPD analysis. A. Profiles with 10 nt primers. B. Profiles with longer primers. The strains used were: 1, NCTC11638; 2, WV229; 3, NCTC11637; 4, P466; and 5, MO19. The sequences of the primers used are given in Table 1A. In part A, only 5 pmoles of primer was used per reaction, rather than the 20 pmoles found subsequently to be optimal (e.g. in Figs. 2, 4, below). Sizes of relevant maker DNA fragments (M) are (in kb): 5.1, 4.1, 3.1, 2.0, 1.6, 1.0, 0.52, 0.51, 0.396, 0.344, 0.298

RAPD Fingerprinting

PCR was carried out in 25 µl containing 20 ng of *H.pylori* genomic DNA, 3 mM $MgCl_2$, 20 pmoles of primer, 1 U of AmpliTaq DNA polymerase (Perkin-Elmer Cetus), 250 µM each of dCTP, dGTP, dATP and dTTP (Boehringer) in 10 mM Tris-HCl, pH 8.3, 50 mM KCl and 0.001% gelatin, under a drop of mineral oil. A Perkin-Elmer TC480 thermal cycler was used for amplification. The cycling program when using 10-nt primers (Table 1A) was 4 cycles of [94°C, 5 min; 36°C, 5 min; and 72°C, 5 min], 30 cycles of [94°C, 1 min; 36°C, 1 min; and 72°C, 2 min], and then 72°C, 10 min (ref 9). The cycling program when using longer (≥ 17 nt) primers (Table 1B) was four cycles of [94°C, 5 min; 40°C, 5 min; and 72°C, 5 min; low stringency amplification], 30 cycles of [94°C, 1 min; 55°C, 1 min; and 72°C, 2 min; high stringency amplification], and a final incubation at 72°C for 10 min (ref 10). After PCR, 20 µl aliquots of products were electrophoresed in 2% agarose gels containing 0.5 µg/ml ethidium-bromide in the gel and 1×Tris acetate running buffer, and photographed under UV light. The 1 kb DNA ladder (Gibco, BRL) was used as a size marker (M) in all gels.

Southern blot analysis of RAPD products

DNA samples were electrophoresed in 2% agarose gels, alkali denaturated, and transfered to nylon membranes (Gibco BRL) by the capillary transfer method, and fixed to the membrane by UV irradiation. Radioactive DNA probes were made by eluting fragments from gel slices, and ^{32}P labelling using the random primer method (19).

RESULTS

Identification of informative primers

To identify primers that generate informative arrays of PCR products, we tested DNA from five clinical isolates of *H.pylori* that were obtained from four geographical sites, and that also differed in the specificity of adherence to rat and human stomach biopsy tissue (Fig. 1). Twenty oligonucleotides that were 10-nt long and had been used extensively in chromosome mapping and analysis of diversity in higher plants (9, 20, 21) were tested. Six of the eight with a 60% or 70% G+C content resulted in strain-specific arrays of up to about 15 bands in the 0.5 kb to 3.5 kb

size range, whereas only two of the 12 primers with a 50% G+C content resulted in such useful arrays under the same conditions (see Fig. 1A, and Table 1A). Nine of eleven oligonucleotides that were longer ($\geq$ 17 nts) also resulted in strain-specific arrays of fragments (Fig. 1B, Table 1B).

Concommittant tests to optimize reaction conditions were carried out with two primers (D14216 and D11344; Table 1B). The most informative patterns were obtained using 20 pmoles of primer per 25 μl reaction and 3 mM $MgCl_2$; less primer or $MgCl_2$ resulted in fewer bands, and more primer or $MgCl_2$ resulted in higher background, without additional prominent bands. The patterns were not significantly affected by changes in template DNA levels between 5 ng and 100 ng per 25 μl reaction, but levels of 1 ng or less resulted in significantly different patterns (data not shown). Consequently, 20 ng of genomic DNA was used for all further RAPD tests.

Table 1. Primers tested for RAPD analysis of *H.pylori*

A. 10 nt primers

Primer	Sequence	%G+C
Excellent patterns		
1254	CCGCAGCCAA	70
1283	GCGATCCCCA	70
1247	AAGAGCCCGT	60
1281	AACGCGCAAC	60
1290	GTGGATGCGA	60
Intermediate patterns		
1253	GTTTCCGCCC	70
1252	GCGGAAATAG	50
1280	GAGGACAAAG	50
Little if any amplification		
1288	GGGGTTGACC	70
1292	CCCGTCAGCA	70
1284	GTCAACGAAG	50
1285	AGCCAGTTTC	50
1287	CGCATAGGTT	50
1289	ACTTGCATCC	50
1248	TGCCGAATTC	50
1249	CGAACTAGAC	50
1250	GGCTTAACAC	50
1251	AAGACTGTCC	50
1255	CCGATCTAGA	50
1282	GACGACTATC	50

B. $\geq$ 17 nt primers

Primer	Sequence	Length (nts)	%G+C
Excellent patterns			
D14216	NNNAACAGCTATGACCATG	19	~44
D11344	AGTGAATTCGCGGTGAGATGCCA	23	52
D8635	GAGCGGCCAAAGGGAGCAGAC	21	67
D9355	CCGGATCCGTGATGCGGTGCG	21	71
D14307	GGTTGGGTGAGAATTGCACG	20	55
Intermediate patterns			
D14215	NNNGTAAAACGACGACCAGT	17	~47
D14217	NNNCCATGGTATTCTCCGTACCC	20	~55
D11561	TAGGATCCCGAGATCTGATC	20	50
D10730	GGCCATAGAGTCTTGCAGACAAACTGC	27	52
Little if any amplification			
D10727	GCCTAACGATCATATACATGGTTCTCTCC	29	45
D11562	TAGGATCCCCTACTTGTGTA	20	45

The 10 nt primers used were gifts of J.Williams, and have been used extensively for RAPD analyses of higher plant genomes (see ref. 20). The longer primers were from our laboratory collection (N indicates equimolar mixture of A,T,G and C). The suitability of these primers for RAPD analysis of *H.pylori* was tested using five 'standard' strains (Fig. 1). Patterns typical of those considered 'excellent' are shown under primers 1281, 1254, D14216, D8635 and D11344 in Fig. 1. Primers listed under 'intermediate' patterns resulted in just a few bands with each of the five strains tested. Primers listed under 'little if any amplification' failed to give prominent bands with at least some of the five standard strains, as illustrated with primer 1282 in Fig. 1.

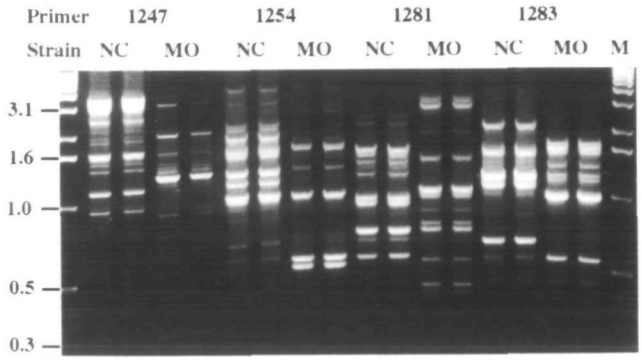

Figure 2. Representative tests of reproducibility of RAPD profiles with 10 nt primers. With each primer tested, the pairs of profiles came from DNA samples prepared on different days from separate cultures. NC designates strain NC-TC11638 and MO designates strain MO19. Marker DNA fragments (M) are as in Fig. 1.

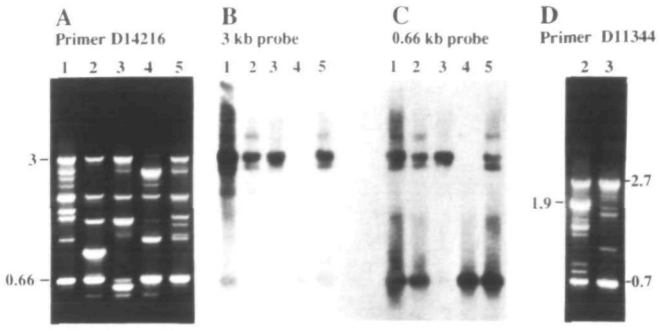

Figure 3. Representative Southern blot tests of homology with RAPD PCR products. Panel A, array of fragments generated from the five standard strains (Fig. 1) and used for Southern blotting (panel B, C). The 3 kb and 0.66 kb fragments used as probes and generated from the DNA of strain NCTC11638 are indicated. Panel B, results of probing the array shown in panel A with the 3 kb fragment from this array. Panel C, results of probing the same array with the 0.66 kb fragment. Panel D, array of fragments generated with primer D11344 and strains WV229 and NCTC11637 (from Fig. 1B), and showing the 0.7 kb, 1.9 kb and 2.5 kb fragments used as probes for additional Southern blots that are described in the text. Marker DNA fragments (M) are as in Fig. 1.

Reproducibility of RAPD patterns

The reproducibility of RAPD patterns was tested using eight 10-nt primers, two different strains, and DNA preparations made from separate cultures of each strain on different days. Identical strain-specific arrays of DNA fragments were obtained from the paired DNA preparations with each primer tested. Equivalent high reproducibility was seen using several longer primers (see Fig. 2 for representative data). This implies that differences in arrays of fragments obtained from clinical isolates (below) reflect DNA sequence diversity among *H.pylori* genomes.

The RAPD profiles reported here were obtained using Amplitaq polymerase (Cetus). A few experiments carried out with thermostable polymerases from other suppliers resulted in somewhat different, although again reproducible, arrays of RAPD products. Although polymerase effects have not been investigated in detail, it seems that using a thermostable polymerase from just one source can contribute to reproducibility.

Homologies among RAPD products

To test for homology among amplification products, five representative bands (indicated in Fig. 3A and D) were purified from agarose gels, labeled with ^{32}P, and used as probes in Southern blots to arrays of RAPD products. Three conclusions emerged from these tests, as illustrated in Fig. 3 B and C (the three probes indicated in Fig. 3D gave equivalent results). First, co-migrating fragments generated with a single primer from different strains were homologous. Second, the lack of a fragment of a particular size was not associated with a strongly hybridizing band of different size. Thus, the diversity of patterns does not usually seem to be due to small insertions or deletions that affect fragment mobility. Third, the 0.66 kb probe hybridized strongly to the 3 kb band, and the 3 kb probe hybridized weakly to the 0.66 kb band, suggesting the presence of a repetitive sequence in both the 0.66 kb and 3 kb DNA segments (Fig. 3 A–C).

Diversity among strains from a single location

The divergence of the five strains used to develop the RAPD method for *H.pylori* was consistent with their coming from different geographical locations (see above). To better assess the diversity of *H.pylori* strains, we screened isolates from 60 patients at one location, the Veterans Administration Hospital in Huntington, West Virginia. Fig. 4 shows tests of 12 representative strains with four primers. Each primer resulted in a unique pattern for each strain. The other 48 strains were tested with one of these primers (1281), and each strain resulted in a distinct fingerprint, different from all others. Twenty-two of these strains were also tested with two other primers (D14216 and D8635), and exhibited a unique fingerprint with each primer (data not shown).

Strains obtained by sequential biopsy of the same patient

Isolates were obtained in sequential biopsies two to four months apart from three patients undergoing a clinical trial to assess the effectiveness of treatment with clindamycin and Pepto-Bismol® (16). The arrays of fragments from *H.pylori* isolates from the initial and followup biopsies were identical (Fig. 5). This result again illustrated the reproducibility of the RAPD method, and provided further evidence supporting the conclusion (16) that this treatment did not eradicate the organism.

DISCUSSION

We adapted the RAPD PCR-based DNA fingerprinting method to the gastric pathogen *H.pylori* to: (i) test whether certain bacterial genotypes are associated with specific disease states; (ii) trace patterns of transmission in human populations; and (iii) elucidate mechanisms of long term residence and recurrent disease. Informative arrays of up to about 15 prominent DNA fragments were obtained with many different primers. However, most 10-nt primers with a 50%, rather than 60% or 70%, G+C content were not informative even though *H.pylori* DNA is about 65% A+T (22). Since all of these 10 nt primers generated useful arrays of fragments with higher plant DNAs (9, 20, 21), their unsuitability for *H.pylori* may reflect the rarity of pairs of linked sites in small genomes (1.7 mb for *H.pylori*) that match any particular 10 nt primer, and the weak binding of 10 mers containing only five G+C to partially matched sites. In applying the RAPD method, we distinguished each of 64 independent

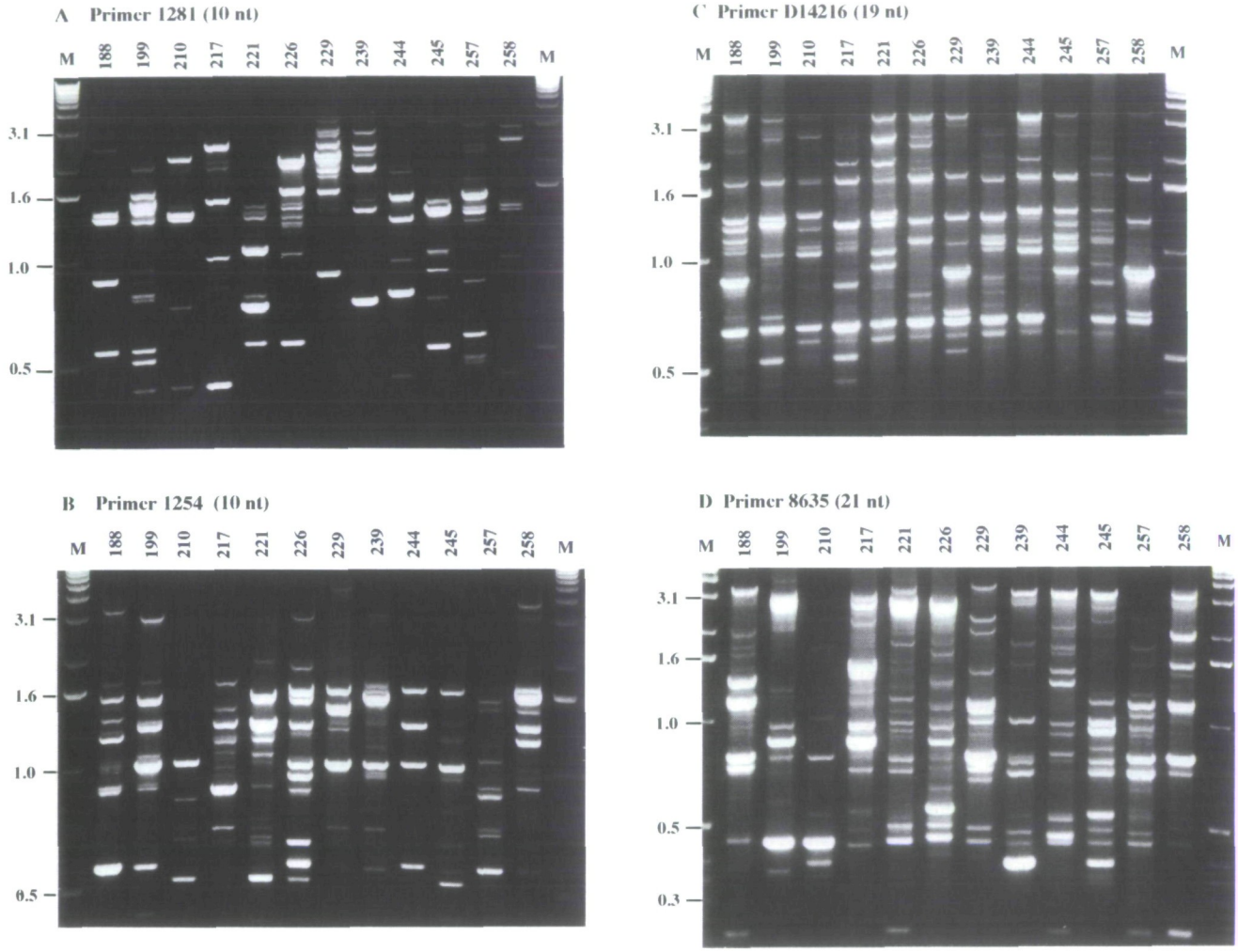

Figure 4. Representative RAPD analysis of *H.pylori* strains from patients at one location. The numbers refer to the WV strains described in Materials and Methods. Marker DNA fragments (M) are as in Fig. 1.

isolates using just a single 10 nt primer, and found that strains recovered from followup biopsies matched those obtained in the initial biopsy in each of three cases.

More diversity among *H.pylori* isolates was found in the present RAPD analysis than in recent studies of restriction site polymorphisms (RFLPs) in *H.pylori* [10 HaeIII RFLPs in a PCR-amplified urease gene segment from 22 strains (6), and 77 HaeIII or HindIII Southern blot 'ribotypes' among 146 other strains (23)]. Although different sets of clinical isolates were used in each study, the diversity of fingerprints in our experiments probably reflects a greater sensitivity of the RAPD method. This sensitivity is attributable to: (i) the sampling of the entire genome, essentially at random, when using apropriate arbitrary primers; and (ii) the existence of numerous potentially amplifiable segments, only a subset of which (the most easily copied) contribute significantly to the final array of products.

The RAPD method also appears to be more efficient than the popular ribotyping method of strain identification because only about 1% as much DNA is needed per assay ($\leq$ 20 ng rather than 2 μg per assay). Thus a few hundred μl of culture would

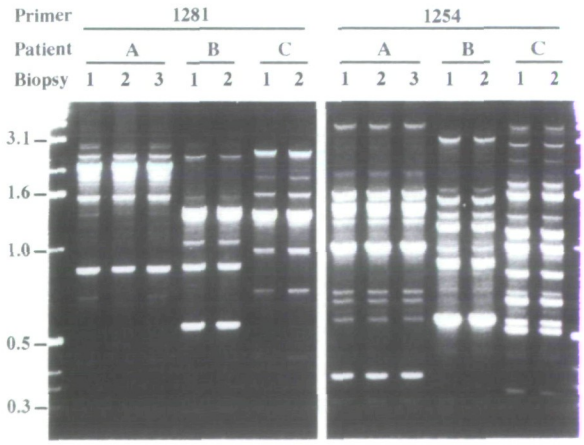

Figure 5. RAPD analyses of isolates from initial and followup biopsies of three patients. Marker DNA fragments (M) are as in Fig. 1.

provide enough DNA for multiple RAPD tests of each isolate. Economy of media, reagents and incubator space, and the ease of handling small samples all facilitate large scale screening, especially of organisms like *H.pylori* that are microaerophilic, fastidious and slow growing. These features will also be important in the development of automated protocols for strain identification.

Most studies of bacterial population structure to date have focused on organisms with growth habits very different from those of *H.pylori* (e.g. *Haemophilus*, *Legioniella*, *Listeria*, *Streptococcus*, *Shigella/E. coli* and *Salmonella*) and have been based on strain-specific differences in electrophoretic mobility of metabolic enzymes (7, 8), and also on sequencing of representative genome segments (24). These studies indicated very strong clonality, often with many clones detectable in the environment at large, and with just one or a few clones predominating in disease episodes. Extrapolating from the protein electrophoretic and RAPD analysis data, biopsy specimens of *H.pylori* seem to be more diverse than the clinical isolates of these other well-studied bacterial groups. Several factors might contribute to this diversity. In particular, *H.pylori* seems to not grow well outside of its human host, and may not benefit from a significant environmental reservoir of potentially infectious organisms (1). Although infection by *H.pylori* is quite infrequent in the US and Western Europe (frequency of 0.5%−2% per year; 25), relative to regions with poorer sanitation (13, 26), the bacteria, once acquired, can be carried for decades. Since the strains used here came from (former) members of the US Armed Forces over age of 40, they may have colonized their hosts years before biopsy, perhaps in locales far from the small city of Huntington, WV. The observed diversity of strains in this patient population might thus reflect three factors: (i) the diversity of the species world wide; (ii) the rarity of person-to-person spread, and thus relatively little of the competition among clones that would select those that are most fit; and (iii) evolutionary changes including periodic selection (27) in a clone during its long term residence in its human host.

ACKNOWLEDGMENTS

We are grateful to Drs. C.M.Berg, P.Falk, D.Kersulyte, M.McClelland, S.Normark, T.Wadström and J.G.K.Williams for stimulating discussions and communication of unpublished results, B.R.Midkiff for expert technical assistance, and Perkin-Elmer Cetus Corporation for the loan of a thermal cycler. This work was supported by grants HG00563 from the US Public Health Service and DEFGO2-89ER60862 from the US Department of Energy to D.E.B, a USDA-ARS North Atlantic Area Research Fellowship to S.K. and RR05870 to T.U.W.

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