Evaluation of Fingerprinting Methods for Identification of *Helicobacter pylori* Strains

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Variation amongst strains of *Helicobacter pylori* was examined by ^{35}S-methionine-labelled protein SDS-PAGE (Radio-PAGE), immunoblot and DNA fingerprinting techniques. These methods allowed discrimination amongst isolates and showed total correlation. Pig and baboon gastric *Helicobacter pylori*-like organisms were found to be very similar to *Helicobacter pylori* by both Radio-PAGE and immunoblotting, whereas a *Helicobacter mustelae* isolate was markedly different. The *Hind*III restriction endonuclease digest patterns of *Helicobacter pylori* DNA illustrated the considerable genomic variation of this organism. The Radio-PAGE and immunoblot typing methods both gave precise identification of *Helicobacter pylori* strains, but Radio-PAGE was found to give higher resolution and represents a standardised universally applicable fingerprinting method for *Helicobacter pylori*.

Helicobacter pylori (formerly *Campylobacter pylori*) is now accepted as the most important cause of human chronic active gastritis, one of the most common chronic infections in humans (1). There is mounting evidence that this bacterium has an aetiologic role in the development and recurrence of gastric and duodenal ulcers (2, 3). Antibacterial therapy resulting in eradication of *Helicobacter pylori* has led to normalisation of chronic active gastritis (4) and lower rates of ulcer relapse (5). Relapse following treatment is linked to the re-isolation of *Helicobacter pylori* from biopsy material (5), and it is important to differentiate between a re-infection with a different isolate and recrudescence due to the same organism. A reliable method of typing *Helicobacter pylori* is required. This would permit detailed epidemiological investigations on transmission of the organism and may allow distinction between pathogenic and non-pathogenic strains.

A serotyping scheme for *Helicobacter pylori* based on coagglutination has been reported by Danielsson et al. (6), and strains can also be typed into groups on the basis of agglutination patterns produced with red blood cells and plant lectins (7, 8). Differences between strains have also been detected by pre-formed enzyme profiles (9). Although the agglutination typing schemes are rapid, they are not widely applicable, being difficult to standardise and requiring either extensive preparation of antisera or multiple fresh and costly reagents. Molecular techniques have also been described as a means for strain identification of *Helicobacter pylori*. *Helicobacter pylori* isolates have been found to be relatively homogeneous by SDS-PAGE analysis of whole cell preparations (10, 11), though sarcosyl outer membrane protein profiles show variation between strains and have been proposed by Goodwin and Armstrong (12) as a useful typing scheme. Burnie et al. (13) have shown by immunoblotting that there is antigenic heterogeneity amongst *Helicobacter pylori* isolates and that this can be utilised for typing this organism.

Restriction endonuclease analysis of genomic DNA has been the most widely used molecular method for discriminating between *Helicobacter pylori* strains (14, 15). Simpler fingerprint patterns can be obtained for *Helicobacter pylori* by the use of ribosomal RNA gene probes and Southern hybridisation (16), but the method is time consuming and technically more demanding. ^{35}S-methionine-labelled protein profiles were first developed for the typing of *Clostridium difficile* by Tabaqchali et al. (17) and later applied to other organisms (18, 19). In this paper we report the application of ^{35}S-methionine-labelled protein (Radio-PAGE), immunoblot analysis and

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DNA fingerprinting techniques for strain differentiation of *Helicobacter pylori*. The aim is to identify a simple and reproducible method for the typing of *Helicobacter pylori*.

Materials and Methods

Source of Isolates. The 30 *Helicobacter pylori* strains used in this study were isolated from human gastric biopsy samples and were obtained from the Gastroenterology Clinic, St. Bartholomew's Hospital, London (HPM strains) and from the Public Health Laboratory Service, Gloucester Royal Hospital, Gloucester (HPG strains). The HPG isolates were originally obtained from a placebo, erythromycin ethylsuccinate and bismuth salicylate treatment trial of patients with *Helicobacter pylori* and gastritis at Dudley Road Hospital, Birmingham (20). Seven paired pretreatment and post-treatment isolates were studied: HPG195, 218; 230, 268; 246, 278; 255, 290; 258, 289; 200, 227; 150, 181. The *Helicobacter pylori* strains 200 and 150 were associated with severe histological gastritis (grade 3), strain 255 with moderate gastritis (grade 2) and the other isolates with mild gastritis (grade 1) using the Warren Marshall classification (5).

The *Helicobacter pylori* strains 255 and 290 were isolated before and after erythromycin therapy (500 mg aqueous solution, 3 weeks), and the other isolates were from patients who had received a placebo. Gastric *Helicobacter pylori*-like organisms (GHPLOs) isolated from the gastric mucosa of a pig (P1), a baboon (B3) (D.M. Jones and A. Curry, Fourth International Workshop on Campylobacter Infections, Goteborg, Sweden, 1988, Abstract no. 188) and a ferret (F6), now classified as *Helicobacter mustelae* (21), were also studied and were obtained from the Public Health Laboratory Service, Withington Hospital, Manchester. The biopsy specimens were initially inoculated onto *Helicobacter pylori* selective medium (22) and incubated under microaerophilic conditions at 37 °C for up to five days; *Helicobacter pylori* was identified by accepted criteria (23). After initial isolation a single colony was subcultured from each biopsy and the strains were preserved at -70 °C in nutrient broth No. 2 (Oxoid, UK) containing 15 % v/v glycerol and 15 % v/v horse serum.

Radio-PAGE. The *Helicobacter pylori* isolates were grown on Columbia blood agar (8 % v/v horse blood) for 48 h under microaerophilic conditions. Ten colonies were then inoculated into 3 ml of nutrient broth (Oxoid No. 2) supplemented with horse serum (10 % v/v), vancomycin (10 µg/ml), colomycin (10 U/ml), trimethoprim (5 µg/ml), amphotericin B (5 µg/ml) and 100 µCi of ^{35}S-methionine (Amersham, UK). The cultures were incubated for 36 h at 37 °C under microaerophilic conditions with constant agitation. The bacterial cells were harvested by centrifugation and the pellets resuspended and boiled for 5 min in sample cracking buffer (2.5 % w/v sodium dodecyl sulphate, 1.3 % v/v 2-mercaptoethanol, 5 % v/v glycerol and 0.002 % w/v bromophenol blue in 50 mM Tris, pH 6.7). Samples containing 1–2 µg of protein were applied to 10 % or 7.5 % v/v polyacrylamide gels and electrophoresis performed for 4 h at 270 volts. The gels were fixed for 1 h in glacial acetic acid (20 % v/v) and propan-2-ol (20 % v/v) and then vacuum dried onto filter paper. The dried gels were exposed to X-ray film at room temperature for at least 24 h.

Immunoblotting. Antibody was prepared against *Helicobacter pylori* strain HPM630 by resuspending the bacteria from the culture plate in distilled water, crushing the cells in an X press at -20 °C and immunising 3 kg adult New Zealand white rabbits with the supernatant using the injection schedule of Heard et al. (24). The rabbits were bled out ten days after the last injection and the hyperimmune antiserum pooled.

Helicobacter pylori strains to be analysed by immunoblotting were harvested from blood agar plates, and cells were washed in phosphate buffered saline (PBS) and resuspended in sample cracking buffer. Samples were boiled for 5 min and 1–2 µg of protein applied to 10 % v/v polyacrylamide gels. The proteins obtained after electrophoresis were transferred onto a nitrocellulose membrane in an LKB transblotter (Pharmacia LKB, UK). The buffer contained methanol 20 % v/v, 25 mM Tris and 192 mM glycine at pH 8.3, and transfer was allowed to proceed at 25 °C for 45 min.

Free protein-binding sites were blocked by overnight incubation at 4 °C in bovine serum albumin (3 % w/v) in buffered saline (sodium chloride 0.9 % w/v and 10 mM Tris, pH 7.4). The nitrocellulose was then incubated at 25 °C for 2 h with the rabbit hyperimmune sera diluted 1/50 in buffered saline containing bovine serum albumin 3 % w/v and Tween 20 0.05 % v/v.

The nitrocellulose was then washed five times with 0.05 % v/v Tween 20 in 0.9 % w/v saline over 30 min and was incubated for 1 h at 25 °C with alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G diluted 1/1000 in 3 % w/v bovine serum albumin buffered saline. The nitrocellulose was washed again as described above and was then incubated for 5–15 min at 25 °C with 100 ml of substrate buffer [100 mM Tris-HCl (pH 9.5), 100 mM NaCl, 5 mM MgCl$_2$ containing a mixture of 660 µl of nitro blue tetrazolium (NBT 50 mg/ml in N, N-dimethyl formamide 70 % v/v) and 330 µl of 5-bromo-4-chloro-3 inosolyl phosphate (BCIP 50 mg/ml in N, N-dimethyl formamide 70 % v/v)]. The reaction was stopped by washing in water.

The immunoblots and Radio-PAGE autoradiograms were examined visually and isolates were differentiated by differences in the presence of a single polypeptide band.

Preparation and Restriction Endonuclease Analysis of Genomic DNA. *Helicobacter pylori* isolates were grown on 8 % v/v horse blood agar plates. Cells were scraped from five plates and then harvested by centrifugation, washed once with 50 mM Tris (pH 8.0), 5 mM disodium ethylene diamine tetraacetic acid (EDTA), 50 mM NaCl (TES) and the pellet resuspended in 2 ml of 50 mM Tris (pH 8.0), 25 % w/v sucrose. Lysozyme (1 ml of 10 mg/ml) solution in 0.25 M EDTA (pH 8.0) was added and the mixture incubated at 0 °C for 20 min. The suspension was then lysed by the addition of 0.25 ml of sodium lauryl sarcosine 5 % w/v in 50 mM Tris EDTA (pH 8.0), 0.75 ml of 10 mM Tris 1 mM EDTA and 0.5 ml of Pronase (20 mg/ml) and incubated at 56 °C for 1 h. The released DNA was extracted three times with equal volumes of phenol-chloroform [1:1 (v/v)]. The DNA was extracted

with ether and was then precipitated by mixing with two volumes of absolute ethanol (-20 °C) and incubating at -20 °C for 1 h. The DNA was pelleted by centrifugation (13,000 x g), washed once with 70 % v/v ethanol, dried and dissolved in water to give 100 µg of DNA per millilitre.

To evaluate the applicability of restriction endonuclease analysis of *Helicobacter pylori,* tests were first performed with various endonucleases. Approximately 5 µg of genomic DNA from each isolate was digested to completion with excess *Hind*III enzyme at 37 °C for 16 h (according to instructions of Amersham, UK). The reaction was terminated by the addition of gel-loading buffer (40 % w/v sucrose, 0.25 % w/v bromophenol blue). The DNA fragments were separated by gel electrophoresis in a 0.7 % w/v horizontal agarose gel in a buffer containing 89 mM Tris HCl, 89 mM boric acid and 2 mM EDTA (pH 8.3). The gels were stained with ethidium bromide (1 µg/ml) and photographed with filtered UV illumination on Poloroid type 665 film. Restriction patterns were compared by visual inspection, and any observed differences were interpreted as signifying different strains. For the pre- and post-therapy *Helicobacter pylori* strains, plasmid profiles were compared by electrophoresis of undigested DNA according to conditions described above. Plasmids were also isolated from *Helicobacter pylori* strain HPG255 by the alkaline lysis procedure of Birnboim and Doly (25).


A

Results

A total of ten *Helicobacter pylori* isolates were examined by Radio-PAGE, immunoblot and restriction endonuclease analysis. In addition, seven pairs of pre- and post-therapy *Helicobacter pylori* strains were examined for plasmids and also tested by restriction endonuclease analysis.

Radio-PAGE. Radio-PAGE demonstrated that there were five major polypeptides (molecular weights 66, 56, 54, 48, 29 kDa) conserved amongst the *Helicobacter pylori* strains (Figure 1, A and B). This method showed that in addition to the conserved proteins the isolates also had minor molecular weight polypeptides in the region of 69–200 kDa, best detected by electrophoresis on 7.5 % w/v polyacrylamide gels and by longer exposures to X-ray film. The 69–200 kDa region was the most variable and allowed discrimination between *Helicobacter pylori* strains (Figure 1A). The *Helicobacter pylori* strains that gave identical profiles (Figure 1A, lanes 4 and 5) were isolated from the same patient with a one-month interval between biopsies. The *Helicobacter mustelae* isolate (Figure 1B, lane 1) had 150, 60 and 46 kDa major polypeptides that were not present in *Helicobacter pylori,* but bands migrating at 66 and 54 kDa were shared between *Helicobacter mus-*
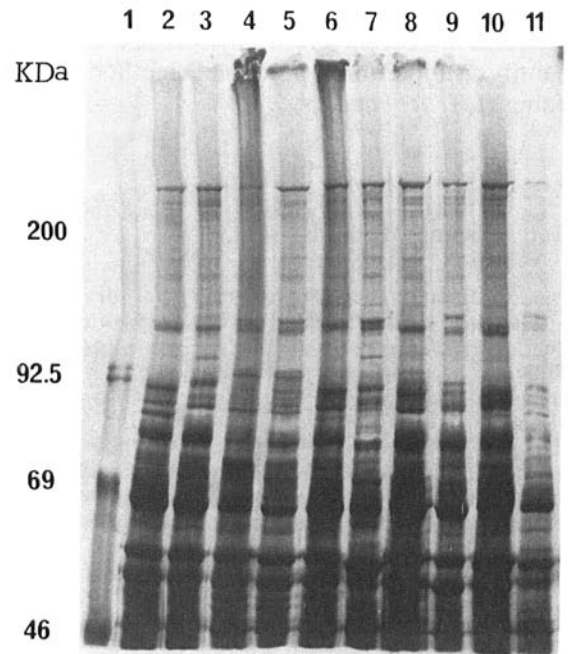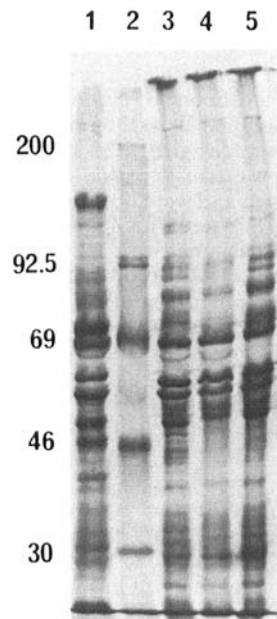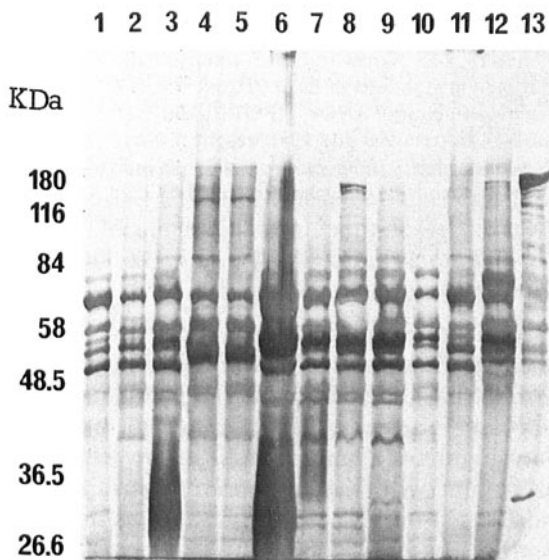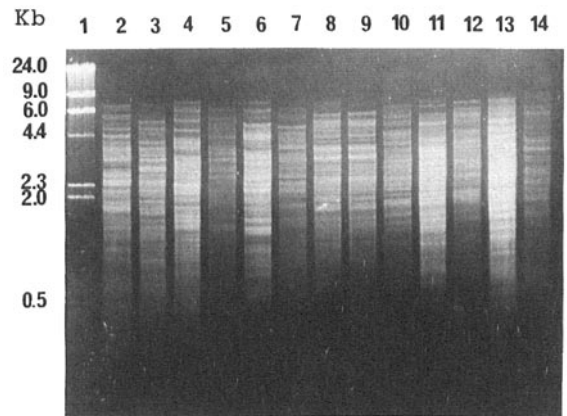

B

Figure 1: Autoradiographs of ^{35}S-methionine-labelled proteins on SDS-PAGE. A: [14]C protein standard (lane 1); and *Helicobacter pylori* HPC29, HPC34, HPM51, HPM192, HPM933, HPM166, HPM167, HPM168, HPM170, HPM169 (lanes 2–11) on 7.5 % v/v polyacrylamide. B: *Helicobacter mustelae* F6 (lane 1); ^{14}C standard (lane 2); and HPM51, GHPLOB3, GHPLOP1 (lanes 3–5) on 10 % v/v polyacrylamide.

telae and *Helicobacter pylori*. The *Helicobacter mustelae* strain had an overall different profile compared to *Helicobacter pylori* isolates. The GHPLOs isolated from a pig and baboon however, were found to have Radio-PAGE profiles very similar to those of the *Helicobacter pylori* strains (Figure 1B, lanes 4 and 5).

Immunoblotting. Immunoblotting revealed that *Helicobacter pylori* has four to six major immunogenic polypeptides in the 48–84 kDa region (Figure 2). The major bands were of molecular weights 84, 74, 66, 56, 54 and 48 kDa. The 54 kDa polypeptide was generally the most immunogenic, and the 66 kDa polypeptide was recognized in all strains examined. Differences in minor antigenic bands could be observed in the 84–180 kDa and 26–48 kDa regions, allowing differentiation between *Helicobacter pylori* isolates. The *Helicobacter pylori* strains isolated from the same patient were found to have identical immunoblot fingerprints (Figure 2, lanes 4 and 5). The pig and baboon GHPLOs gave immunoblot profiles similar to those of the *Helicobacter pylori* isolates (Figure 2, lanes 11 and 12), and *Helicobacter mustelae* exhibited a different fingerprint with 150 and 34 kDa major immunogenic polypeptides (Figure 2, lane 13).

Figure 3: *Hind*III restriction enzyme fingerprints of *Helicobacter pylori* genomic DNA. λ*Hind*III digested standard (lane 1); and *Helicobacter pylori* HPM630, HPM373, HPM814, HPC29, HPC34, HPM933, HPM192, HPM51, HPM166, HPM167, HPM168, HPM170, HPM169 (lanes 2–14).

Figure 2: Immunoblot fingerprints of *Helicobacter pylori* HPC29, HPC34, HPM933, HPM51, HPM192, HPM166, HPM168, HPM167, HPM170, HPM169 (lanes 1–10); GHPLOB3, GHPLOP1 (lanes 11–12); and *Helicobacter mustelae* F6 (lane 13). Developed with rabbit antiserum raised to *Helicobacter pylori* HPM630. Molecular weight standards are shown.
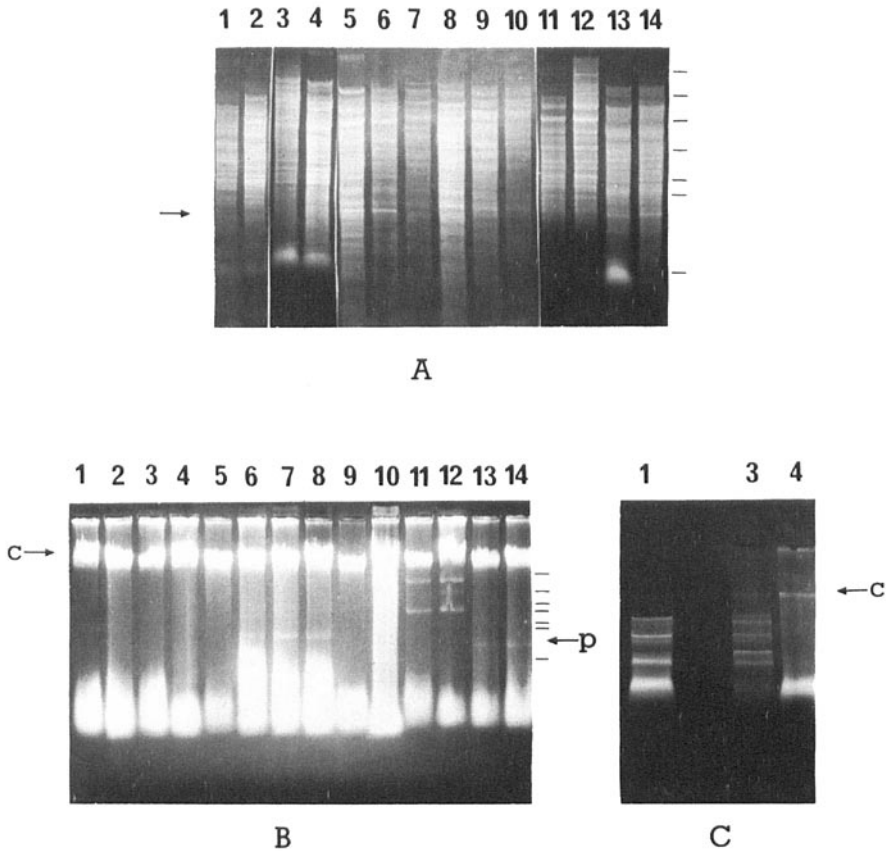
DNA Fingerprinting. *Helicobacter pylori* DNA was found to be digested by the restriction enzymes *Bam*H1, *Eco*R1, *Pst*1 and *Sac*1, but only large fragments were yielded. The enzymes *Hind*III (recognition sequence A.AGCTT) and *Hae*III (recognition sequence GG.CC) gave clear and distinctive patterns of bands. *Hind*III was selected for study as it gave numerous fragments between 14 and 0.5 kb, and reproducible DNA fingerprints were obtained with this enzyme from numerous subcultures of isolates.

Except for the strains isolated from the same patient, which gave identical profiles (Figure 3, lanes 8 and 9), the *Helicobacter pylori* isolates each exhibited unique *Hind*III DNA fingerprints.

Plasmid Profiles and DNA Fingerprinting of Treatment Trial Helicobacter pylori Isolates. Plasmids were detected in four of eight (50 %) of the paired pre- and post-therapy *Helicobacter pylori* isolates and were mostly of small size (Figure 4B, lanes 6–8 and 11–14). *Helicobacter pylori* strains HPG258, 290 and 255 contained 1.5 kb plasmids, HPG268, 230 contained 12.0, 9.0 and 5.0 kb plasmid bands and HPG218, 195 possessed 1.2 kb plasmids. The *Helicobacter pylori* strain HPG268 (Figure 4B, lane 11) probably contains at least two plasmids, since the highest molecular weight band of 12.0 kb is not a linear, open circular form or dimer of the lowest molecular weight 5.0 kb covalently closed circular plasmid band.

Figure 4: *Hind*III restriction enzyme fingerprints and plasmid profiles of pre- and post-therapy *Helicobacter pylori* isolates. A: *Hind*III fingerprints of *Helicobacter pylori* HPG289, 258 (lanes 1–2); HPG227, 200 (lanes 3, 4); HPG290, 255 (lanes 5, 6); HPG278, 246 (lanes 7, 8); HPG181, 150 (lanes 9, 10); HPG268, 230 (lanes 11, 12); and HPG218, 195 (lanes 13, 14). Arrow indicates intense band in lanes 5 and 6 due to plasmid DNA. λ*Hind*III molecular weight standard of 24.0, 9.0, 6.0, 4.4, 2.3, 2.0 and 0.5 kb indicated by –. B: Undigested *Helicobacter pylori* genomic DNA, HPG181, 150, 227, 200, 289, 258, 290, 255, 278, 246, 268, 230, 218, 195 (lanes 1–14). Arrows P and C represent plasmid and chromosomal DNA. – = *Hind*III molecular weight standard. C: *Helicobacter pylori* plasmid DNA isolated by alkaline lysis. HPG290 and HPG255 (lanes 1 and 3); and plasmid-free HPG278 (lane 4).

Helicobacter pylori plasmids were shown to exist as multiple forms by using alkaline lysis plasmid extraction (Figure 4C, lane 1). The plasmids detected were relatively stable in vivo, as plasmids of identical size were present in the *Helicobacter pylori* strains isolated both before and after therapy. The post-therapy *Helicobacter pylori* strain (HPG258) was found to contain a 1.5 kb plasmid that was not present in the *Helicobacter pylori* strain HPG289 isolated prior to treatment (Figure 4B, lanes 5 and 6). *Helicobacter pylori* strain HPG258 contained a 1.5 kb plasmid similar to that of *Helicobacter pylori* strains HPG290 and HPG255 (Figure 4B, lanes 6, 7 and 8) isolated from a different patient.

DNA fingerprinting of the treatment trial *Helicobacter pylori* isolates revealed that *Helicobacter pylori* strains HPG255 and HPG290, isolated before and after erythromycin therapy, were the same (Figure 4A, lanes 5 and 6). The relapse of this patient with the same *Helicobacter pylori* strain was also indicated by plasmid profile (Figure 4B, lanes 7 and 8). Paired pre- and post-placebo *Helicobacter pylori* isolates were found to be identical in six of seven patients (Figure 4). The *Helicobacter pylori* strain HPG258, isolated from one patient after receiving a placebo, produced a fingerprint different from that of the *Helicobacter pylori* strain HPG289 isolated before treatment (Figure 4A, lanes 1 and 2). This

infection with a new *Helicobacter pylori* strain was also suggested from the plasmid profile results (Figure 4B, lanes 5 and 6). The *Helicobacter pylori* strain HPG258 was, however, found to have a DNA fingerprint different from that of HPG290 and HPG255 (Figure 4A, lanes 2 and 5–6), showing that the two patients were colonized by different strains. The DNA fingerprint patterns of some *Helicobacter pylori* strains contained intense bands which are probably due to the presence of high copy number plasmids (Figure 4A, lanes 5 and 6 arrowed).

Discussion

Radio-PAGE analysis of *Helicobacter pylori* detected five major conserved polypeptides of sizes similar to those previously reported by Megraud et al. (11) and Perez-Perez and Blaser (26) on SDS-PAGE examination of *Helicobacter pylori* whole cells. The cloning and sequence analysis of *Helicobacter pylori* urease enzyme genes (22, 27) indicate that the conserved 66 and 29 kDa proteins are urease structural polypeptides and that the 54 and 56 kDA conserved proteins probably represent flagellin polypeptides as reported by Newell (28). Computer analysis of SDS-PAGE *Helicobacter pylori* protein patterns has allowed strain differentiation by this method (29), with the greatest variation amongst *Helicobacter pylori* strains being observed in the 47–56 kDa region. In our study Radio-PAGE was also very sensitive in detecting other *Helicobacter pylori* proteins, thus allowing discrimination between strains, primarily based on the variation of high molecular weight polypeptides in the region of 69–200 kDa. The 47–56 kDa region, however, appeared to be relatively similar among *Helicobacter pylori* strains.

Immunoblotting revealed that the conserved urease and flagellin polypeptides (66 and 54 kDa) are major immunogenic proteins of *Helicobacter pylori;* they have also been reported to react with the majority of human sera from *Helicobacter pylori*-infected patients (30, 31). Burnie et al. (13) based an immunoblot fingerprinting system on antigenically variable *Helicobacter pylori* polypeptides of 29, 34, 37, 40, 44, 59 and 74 kDa, allowing 150 isolates to be typed into nine groups. Two groups accounted for the majority of the isolates, and the results implied that not all isolates were equally virulent, as one of the major groups was associated with the most severe histological chan-

ges, an observation yet to be confirmed. Immunoblot analysis in our study showed that low molecular weight bands in the region of 26–48 kDa were the most variable, whereas the 29–44 kDa polypeptides allowed the best discrimination between isolates. These results are based on the use of a single antiserum; more distinct variations may be observed by using antisera raised to several *Helicobacter pylori* strains.

The GHPLOs isolated from a pig and baboon were found to give profiles very similar to those of the *Helicobacter pylori* strains when analysed by Radio-PAGE and immunoblotting. As expected, the *Helicobacter mustelae* strain isolated from a ferret was found to be significantly different from *Helicobacter pylori* by these fingerprinting methods, which is in agreement with the recent classification of this organism as a species separate from *Helicobacter pylori* (21). The pig and baboon GHPLOs have been previously shown to have *Helicobacter pylori*-related urease genes (22), and they probably represent human *Helicobacter pylori* strains that have infected these animals. *Helicobacter pylori* are probably also present in the gastric antrum of rhesus monkeys, as organisms structurally and biochemically similar to *Helicobacter pylori* isolated by Newell et al. (32) have also been found by us to contain *Helicobacter pylori*-related urease encoding genes (unpublished results).

Each of the *Helicobacter pylori* strains isolated from patients had its own unique *Hind*III DNA fingerprint. Our observation of considerable genomic variation of *Helicobacter pylori* at the subspecies level confirms results previously reported by Langenberg et al. (14), Majewski and Goodwin (15) and Simor et al. (33). The high level of genomic variation may be explained by both the natural competence of *Helicobacter pylori* (34), which allows easy uptake of extracellular DNA, and the high rate of genomic recombination events occurring in this organism. Our findings that 50 % of *Helicobacter pylori* isolates contained plasmids agree with the results of other studies that have examined higher numbers of *Helicobacter pylori* strains (15, 33, 35). Many isolates have been found to contain two or more plasmids which are generally small (1.8–22 kb), and it has not been possible to ascribe a phenotype to a particular plasmid. We have performed initial work on the characterization of *Helicobacter pylori* plasmids, the 1.5 kb plasmid of *Helicobacter pylori* HPG255 having recently been cloned and sequenced (H. Kleanthous, unpublished data). The low percentage of plasmid

carriage in *Helicobacter pylori* limits the useful-ness of this technique as an epidemiologic typing tool by itself. Plasmid profiles obtained in this study identified relapsing infections with the same strain and detected new *Helicobacter pylori* isolates, illustrating the usefulness of combining plasmid profile determination with chromosomal restriction endonuclease DNA analysis to con-firm the identities of strains.

The Radio-PAGE, immunoblot and DNA finger-printing methods showed good correlation with each other for the *Helicobacter pylori* strains ex-amined.

Repeat isolates from the same patient were found to be identical by all three fingerprinting methods, and unique profiles were found with the *Helicobacter pylori* strains isolated from different patients. The new *Helicobacter pylori* strain detected by restriction endonuclease analysis in one patient after receiving a placebo could be ex-plained either by a new infection or, since only single colonies were examined, by the presence of multiple strains in the original infection, as has been reported for some patients (36). It has also been reported that patients may also be colonized by subtypes of *Helicobacter pylori*, which probab-ly arise by mutation in vivo and exhibit similar restriction digest patterns (37, 38). In future *Helicobacter pylori* treatment trials it is important that multiple gastric biopsies be obtained from different sites for each patient and that several colonies of *Helicobacter pylori* be examined by restriction endonuclease analysis. The hetero-geneous profiles obtained with DNA fingerprint-ing of *Helicobacter pylori* means that a typing scheme for *Helicobacter pylori* cannot be devised, in contrast to satisfactory DNA restriction en-donuclease typing schemes produced for other or-ganisms such as *Campylobacter coli* (39) and *Campylobacter jejuni* (40).

The Radio-PAGE and immunoblot fingerprint-ing methods may allow the grouping of *Helico-bacter pylori* strains into types and need to be further evaluated by analysing a larger number of *Helicobacter pylori* isolates. Immunoblot finger-printing depends on the raising of antisera to *Helicobacter pylori*, and with all the inherent anti-genic variations, the method is difficult to stan-dardise in different laboratories. ^{35}S-methionine, however, is simple and reproducible and may pro-vide a more standardised approach. It has been successfully applied and evaluated in the typing of several bacterial species and is generally accepted as the definitive method for the typing of *Clostri-dium difficile* (41). More detailed information on

the application and standardisation of ^{35}S-methionine-labelled proteins has been reported elsewhere (19). It would be useful to apply these fingerprinting methods to the analysis of *Helico-bacter pylori* strains isolated from both asymp-tomatic individuals and patients with varying degrees of histological gastritis and peptic ulcers to determine if pathogenic *Helicobacter pylori* strains can be separated from non-virulent iso-lates.

References

1. **Blaser MJ:** Epidemiology of *Helicobacter pylori* in-fections. In: Malfertheiner P, Ditschuneit H (ed): *Helicobacter pylori*, gastric and peptic ulcer. Springer Verlag, Berlin, 1990, p. 38–40.
2. **Dooley CP, Cohen H:** The clinical significance of *Campylobacter pylori*. Annals of Internal Medicine 1988, 108: 70–79.
3. **Graham DY:** *Campylobacter pylori* and peptic ulcer disease. Gastroenterology 1989, 96: 615–625.
4. **Rauws EAJ, Langenberg W, Hoeuthoff HJ, Zanen HC, Tytgat GNJ:** *Campylobacter pyloridis* associated chronic active antral gastritis. A prospective study of its relevance and the effects of antibacterial and an-tiulcer treatment. Gastroenterology 1988, 94: 33–40.
5. **Coghlan JG, Gilligan D, Humphries H, McKenna D, Dooley C, Sweeney E, Keane C, O'Morain C:** *Cam-pylobacter pylori* and recurrence of duodenal ulcers – a 12 month follow-up study. Lancet 1987, ii: 1109–1111.
6. **Danielsson D, Blomberg B, Jarnerot G, Kosunen TU:** Heterogeneity of *Campylobacter pylori* as demon-strated by co-agglutination testing with rabbit anti-bodies. Scandinavian Journal of Gastroenterology 1988, 23: 58–63.
7. **Huang J, Smyth CJ, Kennedy NP, Arbuthnott JP, Keeling PWN:** Haemagglutinating activity of *Cam-pylobacter pylori*. FEMS Microbiology Letters 1988, 56: 109–112.
8. **Ascencio F, Guruge JL, Ljungh A, Megraud F, Wei S, Wadstrom T:** Lectin typing of *Helicobacter pylori*. In: Malfertheiner P, Ditschuneit H (ed): *Helicobacter pylori*, gastritis and peptic ulcer. Springer Verlag, Ber-lin, 1990, p. 104–109.
9. **Kung JSL, Ho B, Chan SH:** Biotyping of *Campylobac-ter pylori*. Journal of Medical Microbiology 1989, 29: 203–206.
10. **Pearson AD, Ireland A, Bamforth J, Walker C, Booth L, Hawtin P, Holdstock G, Millward-Sadler H:** Polyacrylamide gel electrophoresis of spiral bacteria from the gastric antrum. Lancet 1984, i: 1349–1350.

11. Megraud F, Bonnet F, Garnier M, Lamouliatte H: Characterization of *Campylobacter pyloridis* by culture, enzymatic profile and protein content. Journal of Clinical Microbiology 1985, 22: 1007–1010.

12. Goodwin CS, Armstrong JA: Microbiological aspects of *Helicobacter pylori*. European Journal of Clinical Microbiology and Infectious Diseases 1990, 9: 1–13.

13. Burnie JP, Lee W, Dent JC, McNulty CAM: Immunoblot fingerprinting of *Campylobacter pylori*. Journal of Medical Microbiology 1988, 27: 153–159.

14. Langenberg W, Rauws EAJ, Widjojokusumo A, Tytgat GNJ, Zanen HC: Identification of *Campylobacter pyloridis* isolates by restriction endonuclease DNA analysis. Journal of Clinical Microbiology 1986, 24: 414–417.

15. Majewski SIH, Goodwin CS: Restriction endonuclease analysis of the genome of *Campylobacter pylori* with a rapid extraction method: evidence for considerable genomic variation. Journal of Infectious Diseases 1988, 157: 465–471.

16. Morgan DD, Owen RJ: Use of DNA restriction endonuclease digest and ribosomal RNA gene probe patterns to fingerprint *Helicobacter pylori* and *Helicobacter mustelae* isolated from human and animal hosts. Molecular and Cellular Probes 1990, 4: 321–334.

17. Tabaqchali S, Holland D, O'Farrell S, Silman R: Typing scheme for *Clostridium difficile*: its application in clinical and epidemiological studies. Lancet 1984, i: 935–938.

18. Stephenson JR, Tabaqchali S: New method for typing coagulase negative staphylococci. Journal of Clinical Pathology 1986, 39: 1271–1275.

19. Tabaqchali S, Silman R, Holland D: Automation in clinical microbiology: a new approach to identifying microorganisms by automated pattern matching of proteins labelled with ^{35}S-methionine. Journal of Clinical Pathology 1987, 40: 1070–1087.

20. McNulty CAM, Gearty JC, Crump B, Davis M, Donovan IA, Melikian V, Lister DM, Wise R: *Campylobacter pyloridis* and associated gastritis: investigator blind, placebo controlled trial of bismuth salicylate and erythromycin ethylsuccinate. British Medical Journal 1986, 293: 645–649.

21. Goodwin CS, Armstrong JA, Chilvers T, Peters M, Collins D, Sly L, McConnell W, Harper WES: Transfer of *Campylobacter pyloridis* and *Campylobacter mustelae* to *Helicobacter* gen. nov. as *Helicobacter pylori* comb. nov., and *Helicobacter mustelae* comb. nov., respectively. International Journal of Systematic Bacteriology 1989, 39: 397–405.

22. Clayton CL, Wren BW, Mullany P, Topping A, Tabaqchali S: Molecular cloning and expression of *Campylobacter pylori* species-specific antigens in *Escherichia coli* K12. Infection and Immunity 1989, 57: 623–629.

23. Blaser MJ: Gastric campylobacter-like organisms, gastritis, and peptic ulcer disease. Gastroenterology 1987, 93: 371–383.

24. Heard SR, Rasburn B, Matthews RC, Tabaqchali S: Immunoblotting to demonstrate antigenic and immunogenic differences among nine standard strains of *Clostridium difficile*. Journal of Clinical Microbiology 1986, 24: 384–387.

25. Birnboim HC, Doly J: A rapid extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Research 1979, 7: 1513–1523.

26. Perez-Perez GI, Blaser MJ: Conservation and diversity of *Campylobacter pyloridis* major antigens. Infection and Immunity 1987, 55: 1256–1263.

27. Clayton CL, Pallen MJ, Kleanthous H, Wren BW, Tabaqchali S: Nucleotide sequence of two genes from *Helicobacter pylori* encoding for urease sub units. Nucleic Acids Research 1990, 18: 363.

28. Newell G: Identification of the outer membrane protein of *Campylobacter pyloridis* and antigenic cross reactivity between *C. pyloridis* and *C. jejuni*. Journal of General Microbiology 1987, 133: 163–170.

29. Owen RJ, Costas D, Morgan DD, On SLW, Hill LR, Pearson AD, Morgan DR: Strain variation in *Campylobacter pylori* detected by numerical analysis of one-dimensional electrophoretic protein patterns. Antonie von Leeuwenhoek 1989, 55: 253–267.

30. Newell G: Human serum antibody responses to the surface protein antigens of *Campylobacter pyloridis*. Serodiagnosis and Immunotherapy 1987, 1: 209–217.

31. von Wulffen H, Grote HJ, Gatermann S, Loning T, Berger B, Buhl C: Immunoblot analysis of immune response to *Campylobacter pylori* and its clinical associations. Journal of Clinical Pathology 1988, 41: 453–459.

32. Newell DG, Hudson MJ, Baskerville A: Isolation of a gastric campylobacter like organism from the stomach of four rhesus monkeys and identification as *Campylobacter pylori*. Journal of Medical Microbiology 1988, 27: 41–44.

33. Simor AE, Shames B, Drumm B, Sherman P, Low DE, Penner JL: Typing of *Campylobacter pylori* by bacterial DNA restriction endonuclease analysis and determination of plasmid profile. Journal of Clinical Microbiology 1990, 28: 83–86.

34. Nedenskov-Sorensen P, Bukholm G, Bovre K: Natural competence for genetic transformation in *Campylobacter pylori*. Journal of Infectious Diseases 1990, 161: 365.

35. Tija TN, Harper WES, Goodwin CS, Grubb WB: Plasmids in *Campylobacter pyloridis*. Microbios Letters 1987, 36: 7–11.

36. Beji A, Vincent P, Darchis I, Husson MO, Cortol A, Leclerc H: Evidence of gastritis with several *Helicobacter pylori* strains. Lancet 1989, ii: 1402–1403.

37. Oudbier JH, Langenberg W, Rauws EAJ, Bruin-Mosch C: Genotypical variation of *Campylobacter pylori* from gastric mucosa. Journal of Clinical Microbiology 1990, 28: 559–565.

38. Owen RJ, Fraser F, Costas M, Morgan D, Morgan DR: Signature patterns of DNA restriction fragments of *Helicobacter pylori* before and after treatment. Journal of Clinical Pathology 1990, 43: 646–649.

39. Kakoyiannis CK, Winter PJ, Marshall RB: Identification of *Campylobacter coli* isolates from animals and humans by bacterial restriction endonuclease DNA analysis. Applied and Environmental Microbiology 1984, 48: 545–549.

40. Bradbury WC, Pearson AD, Marko MA, Congi RV, Penner JL: Investigation of *Campylobacter jejuni* outbreak by serotyping and chromosomal restriction endonuclease analysis. Journal of Clinical Microbiology 1984, 19: 342–346.

41. Tabaqchali S: Epidemiologic markers of *Clostridium difficile*. Reviews of Infectious Diseases 1990, 12: 192–199.