

Differentiation of strains of *Helicobacter pylori* by numerical analysis of 1-D SDS-PAGE protein patterns: Evidence for post-treatment recrudescence

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SUMMARY

Twenty-three pre- and post-treatment isolates of *Helicobacter pylori* from the antral mucosa of eight patients with dyspepsia and gastritis were compared using 1-D SDS PAGE of proteins. The protein patterns were highly reproducible and were used as the basis for two numerical analyses. The first, based on the total protein patterns, showed that a number of the strains did not cluster with their respective patient set. This was thought to be due to differences in both mobility and intensity of proteins in the major band region. The second analysis, based on partial patterns, excluding the major band region (51–68 kDa), divided the clinical isolates into clearly defined groups corresponding to the patient sets. Although there was a degree of heterogeneity with respect to protein pattern between the pre- and post-treatment isolates of some patients, there was nonetheless clear evidence that each patient was harbouring strains of only a single type. These results suggested that patients were not being reinfected with a different strain but that there was recrudescence of the pre-treatment strain. Protein 'fingerprints' provided a precise and reproducible means of strain differentiation, and revealed that in each patient the same strain persisted after drug therapy even though there was marked patient-to-patient strain variation.

INTRODUCTION

Helicobacter pylori (formerly *Campylobacter pylori*) has been implicated in the etiology of gastritis and peptic ulcer disease [1–3]. The organism is widely prevalent in human populations [2] but little is known about natural reservoirs or routes of transmission although some evidence supports person-to-person transmission [2, 4]. Currently, detailed epidemiological surveillance of *H. pylori* is impossible because there are no generally suitable serotyping, phage typing or biotyping schemes for precise strain identification. The species appears phenotypically to be relatively homogeneous with respect to biochemical activity [5, 6], antimicrobial susceptibility [7, 8] and pre-formed enzyme profiles [5, 6]. Insufficient variability in plasmid DNA content [9, 10], surface antigens [11] and lectins

[12], precludes any of these characters from serving as the bases for typing schemes. The lack of a reliable, easily applicable method for differentiation of strains has hindered the understanding of the ecology and epidemiology of this species.

High-resolution polyacrylamide gel electrophoresis of bacterial proteins has been used extensively for identification at the species, sub-species and infra sub-specific levels [13, 14]. The technique using either conventionally stained or radiolabelled proteins, has been applied increasingly to the typing of a variety of clinically important species that have included *Clostridium difficile* [15], methicillin-resistant *Staphylococcus aureus* [16, 17], *Enterobacter cloacae* [18] and *Acinetobacter calcoaceticus* [19]. Preliminary studies, using one-dimensional sodium dodecyl sulphate-polyacrylamide gel electrophoretic (1-D SDS-PAGE) protein patterns [20, 21] and DNA restriction endonuclease digest patterns [9, 22] have shown extremely good discrimination and high reproducibility for *H. pylori*. A considerable degree of electrophoretic heterogeneity in protein profiles was demonstrated and the diversity between strains of *H. pylori* appears to provide a sensitive and reproducible means for the discrimination of isolates for epidemiological purposes and for assessing the effects of drug therapy.

The aim of the present study was to compare objectively, using a computerized numerical analysis, the high-resolution SDS-PAGE protein patterns of a number of pre- and post-treatment isolates of *H. pylori* from eight patients who did not respond to the course of treatment. The ultimate utility of this data would be in the investigation of relapse following treatment with antimicrobial therapy and could provide evidence for either reinfection from an exogenous source or recrudescence of the same strain.

MATERIALS AND METHODS

Bacterial cultures, media and growth conditions

The 23 clinical isolates from eight patients (designated A to H in this study) were obtained from gastric tissue collected during endoscopy and biopsy of patients with symptoms of dyspepsia and histologically confirmed gastritis. Cultures were provided by the Public Health Laboratory, Withington Hospital, Manchester. When complete, the strain sets comprised two isolates (I and II) collected on the same day from two adjacent sites in the antrum (pre-treatment) and two further isolates (III and IV) from the antrum on subsequent biopsy 1 month later (post-treatment). Treatment was with one of two forms of nitrofurantoin (50 mg of microcrystalline suspension or 75 mg as a macrocrystalline capsule, per dose) four times daily for 28 days. Strain NCTC 11638 (human stomach, Australia) was included in all gels as a study reference.

Organisms were grown routinely on Oxoid Brain Heart Infusion (BHI) agar supplemented with 5% v/v horse blood and 1% Isovitalex for 2 days at 37 °C in microaerobic conditions (5% O₂; 5% CO₂; 2% H₂; 88% N₂) in a Variable Atmosphere Incubator (Don Whitley Scientific Ltd, Shipley, Yorks). Strains were preserved at -196 °C on glass beads in Nutrient Broth No. 2 (Oxoid: CM67) containing 10% v/v glycerol.

Preparation of protein samples and electrophoresis

For each protein sample, approximately 0.02–0.04 g wet weight of the bacteria were harvested directly from the BHI blood agar plates and suspended in about 50 μ l of double-strength lysis buffer (20% v/v glycerol, 2% v/v 2-mercaptoethanol, 4% w/v sodium dodecyl sulphate [SDS] and 70% v/v stacking gel buffer). The protein samples were then extracted as described previously [23].

Samples were run on discontinuous SDS-polyacrylamide gels which were cast to allow for a 10 mm stacking gel. The final polyacrylamide concentrations were 10% for the separation gel and 5% for the stacking gel. Full details of the methods used in gel preparation and electrophoresis were described previously [16].

Scanning of gels and computation of similarities

The stained protein patterns in the dried gels were scanned using an LKB Ultrosan XL laser densitometer (Pharmacia-LKB Biotechnology, Sweden) as described previously [16]. Absorbance was recorded at 160 μ m intervals along the gel yielding 625 values per 10-cm gel. The absorbance range was set from 0.1–1.5 absorbance units (full scale). The initial (stacking gel/separation gel interface) and final (bromophenol blue marker) bands were deleted and protein patterns corrected for gel-to-gel variation using a reference bacterial standard (NCTC 11638). Replicates of the reference bacterial standard on the subsequent gels was used to calibrate patterns against the reference on the first gel. Segmented linear correction was performed using a total of 19 discernible marker positions (usually peaks) on the reference pattern and by marking the same positions on the calibration pattern replicate. Linear correction (expansion or compression) to the reference distances was carried out within each of the 18 defined segments for each track by three point quadratic interpolation [24]. The length-corrected traces on the reference gel were composed of 556 absorbance values after removal of the initial and final bands.

Two analyses were performed. The first utilized the whole of the protein pattern and the second was based on the partial patterns after removal of the major bands in the molecular size range of 51–68 kilodaltons (kDa). The partial patterns were composed of 482 absorbance values. A general background trend (0.4: fraction of absorbance) in each trace was removed to increase discrimination between patterns. Similarity between all possible pairs of traces was expressed as the Pearson product-moment correlation coefficient, which was converted for convenience to a percent value. The best fit between each pair of traces was obtained by laterally shifting one corrected trace with respect to the other in single point steps of 160 μ m up to four points on either side of the initial alignment. A dendrogram was constructed to reflect the similarities between strains included in the matrix. Strains were clustered by the method of unweighted pair-group with arithmetic averages (UPGMA). Computations were carried out on a microcomputer (Compaq 386) using a program package written in Turbo Pascal [16, 24]. Molecular sizes were calculated from migration distances by the DNA SIZE program as described previously [25].

RESULTS

General features of PAGE-protein patterns

One dimensional SDS-PAGE of whole-cell protein extracts of the isolates of *H. pylori* produced patterns containing 35–40 discrete bands with molecular weights of 20–100 kDa. Proteins of < 20 kDa were not resolved under the electrophoretic conditions used in this study. PAGE patterns are illustrated in Fig. 1. A distinctive feature of all of the *H. pylori* profiles was the presence of six major bands with molecular sizes of approximately < 27, 29, 42, 53, 57, and 64 kDa. Qualitative differences in pattern both within and between isolates from the respective patient sets were evident mainly in two regions; the first ranged from 68–~100 kDa and the second from 42–53 kDa. There were in addition, both qualitative and quantitative differences in the major bands (53–64 kDa), both within and between the isolates of the patient sets.

Reproducibility

The protein patterns of the strains examined were highly reproducible both within and between gels. Samples of the reference strain (NCTC 11638), which were run on each gel, were used to estimate the reproducibility and gave an average similarity of $98.2 \pm 0.7\%$. As an additional check on between gel reproducibility, molecular weight protein standards were included in each gel, their calculated average similarity was $96.0 \pm 2.2\%$ although they provided a less objective measure of reproducibility as they were based on only four bands. The groups established by the cluster analyses proved to be extremely stable when the computations were repeated using different levels of trace alignment and background subtraction.

Numerical analysis

Numerical analysis of PAGE-protein patterns based on the determination of the Pearson correlation coefficient and UPGMA clustering, and including all the protein bands, revealed that a number of the isolates failed to cluster with other members of their respective patient sets as shown in the dendrogram (Fig. 2). Isolate G-IV was linked to the cluster representing the patient H isolates at the 84% similarity level (S). However, the three isolates of patient H clustered together at a significantly higher level (91% S). Isolate C-III clustered with both isolates of its own patient set and another isolate at a low level (87% S). The sole isolate from patient F (F-II) was found within the cluster representing patient C, whereas isolate E-II clustered with isolates of both its own patient set and with those of patient B at a high level of similarity (94%). Visual inspection of the protein patterns of isolates C-III and G-IV showed that they differed from the other isolates of their respective sets in having a less dense band with a higher molecular size in the 53 kDa region.

When the second, partial pattern analysis, which excluded the bands in the 51–68 kDa range, was performed the anomalies described above were no longer evident as shown in the dendrogram (Fig. 3). The isolates of the eight patient sets were clustered separately in individual groups which contained only strains of a single patient. A universally applicable cut-off for delineation of clusters could not

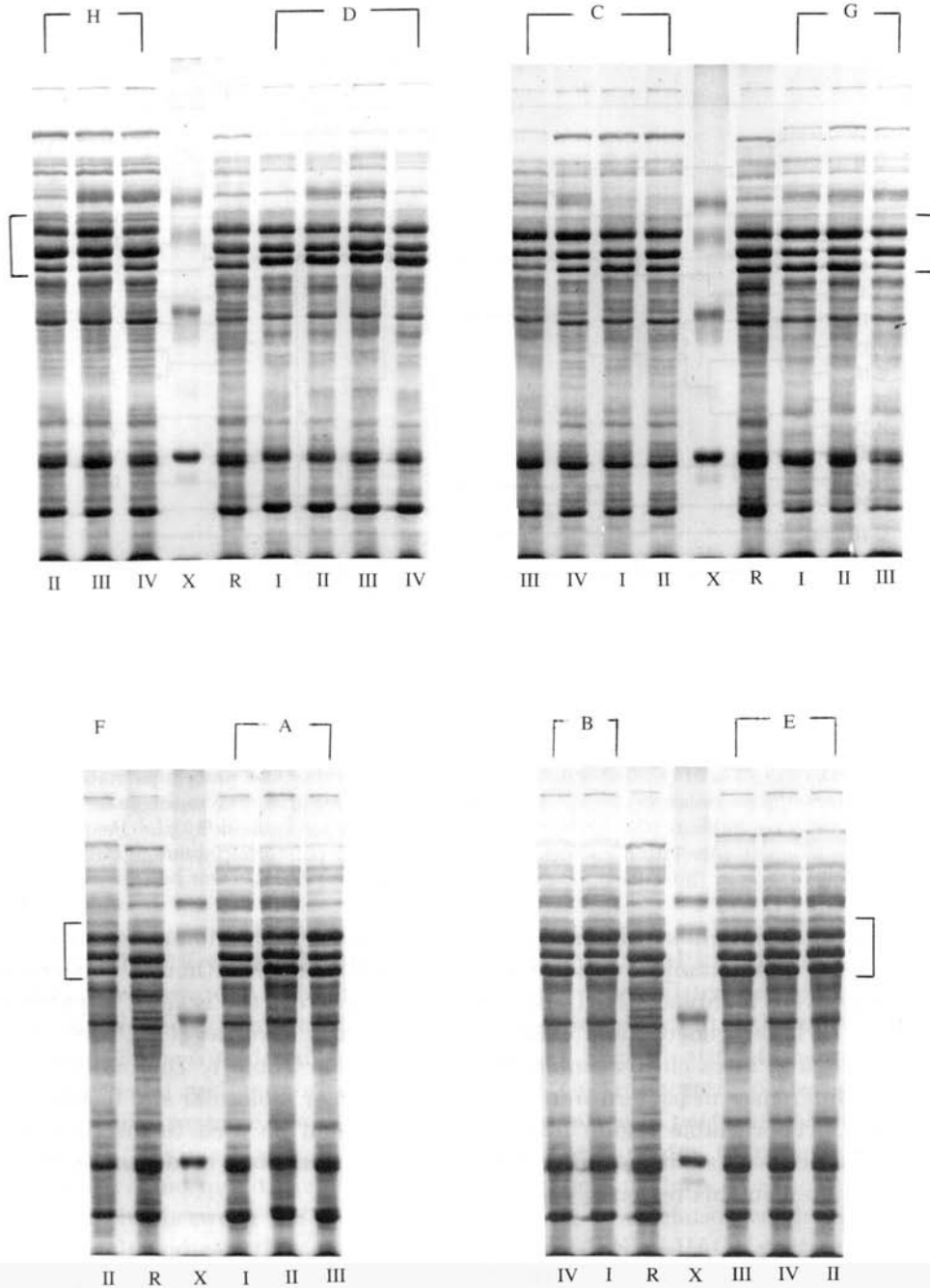


Fig. 1. Electrophoretic protein patterns of isolates of *Helicobacter pylori*. The letters above the tracks in the figures refers to the patient (A-H) from which the isolates were obtained and the Roman numerals (below) to the time of isolation (I and II, pre-treatment; III and IV, post-treatment). Molecular weight markers (track labelled X) are from top to bottom: ovotransferrin, 76-78 kDa; albumin, 66.25 kDa; ovalbumin, 42.7 kDa; carbonic anhydrase, 30 kDa; myoglobin, 17.2 kDa. The brackets on the vertical axis indicate the major protein band region (51-68 kDa) omitted in the second cluster analysis (see Fig. 3). R, reference strain (NCTC 11638).

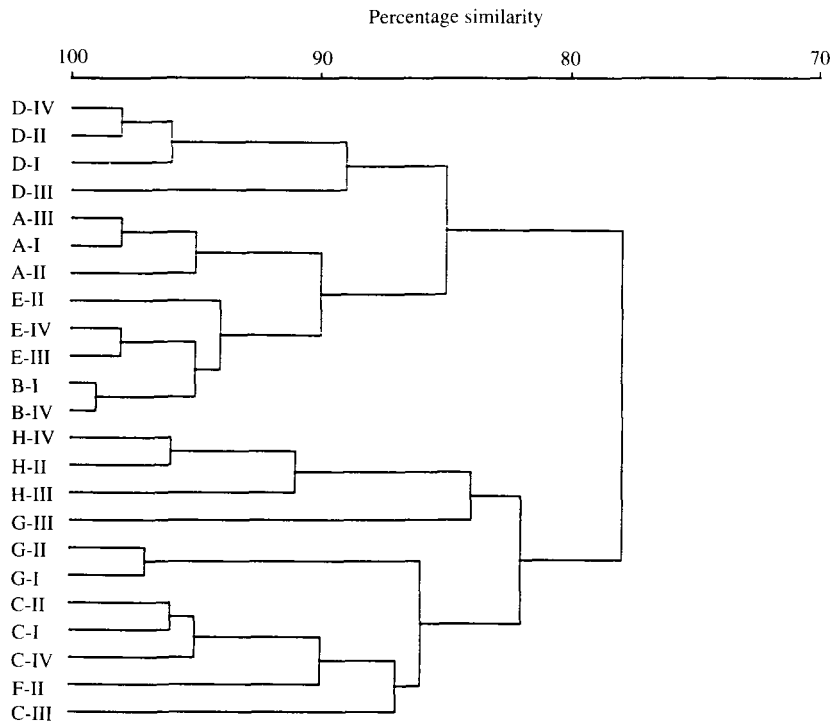


Fig. 2. Dendrogram of the first cluster analysis based on the total protein patterns of *Helicobacter pylori* isolates (vertical axis) as designated in Fig. 1. The numbers on the horizontal axis indicate the percentage similarities as determined by the Pearson product-moment correlation coefficient and unweighted pair group average linkage clustering.

be used as intra-patient set similarity was often as great as that found between the patient sets. For example, isolates of patients B and E, although clustering separately, were highly similar (linked at 96% S) whereas the isolates of patient C were more heterogeneous and clustered together at a lower level (92% S). The mean inter- and intra-cluster similarities are shown in Table 1. The majority of intra-set differences in pattern were found in the higher molecular size bands, i.e. > 68 kDa. The variable region from 42–53 kDa could be used to discriminate between patients sets but few differences in band pattern were evident in isolates of the same set in this region

DISCUSSION

The analysis of bacterial SDS-PAGE protein patterns is an effective means of differentiating medically important bacteria at both the species and infra sub-specific levels and can provide a novel method of typing, especially where there are no other typing methods available [15–18, 23].

The data available on protein profiles of isolates of *H. pylori* [20, 21] provide

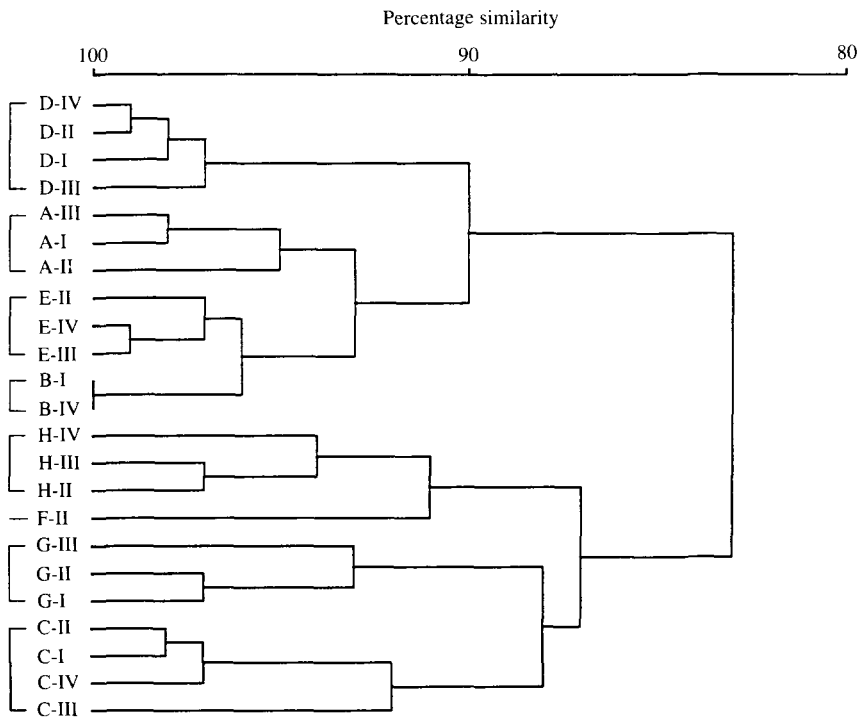


Fig. 3. Dendrogram of the second cluster analysis based on partial protein patterns (excluding major bands in 51–68 kDa range) of *Helicobacter pylori* isolates (vertical axis) as designated in Fig. 1. The numbers on the horizontal axis indicate the percentage similarities as determined by the Pearson product-moment correlation coefficient and unweighted pair group average linkage clustering.

clear evidence of their potential value in epidemiological investigations. These studies demonstrated that a high level of heterogeneity existed between members of *H. pylori*, and it was concluded that isolates with apparently the same protein pattern could be considered to be identical strains. Such identifications were based on both visual and numerical comparison of electrophoretic patterns. In the present study, we have avoided a visual interpretation of patterns, which may be subjective, but used a high resolution laser densitometer to record both the quantitative and qualitative data needed to define a pattern. We then expressed the similarity between the protein patterns using a correlation coefficient.

Our results indicate that numerical analysis of 1-D SDS-PAGE protein patterns provides a useful basis for defining the similarities between multiple isolates from the same patient. It is clear, however, that care must be taken in the interpretation of such data where the analysis utilizes a correlation coefficient. When analyses are based on total patterns, anomalous grouping of strains may arise and can be due to major differences in the quantity of just a single protein band. The disproportionate effect of such bands on the overall clustering of strains can be

Table 1. Mean inter- and intra- patient set percentage similarities ($\pm SD$) as determined on partial patterns by the Pearson product-moment correlation coefficient (r) and UPGMA clustering

Patient Set	A ($n = 3$)	B ($n = 2$)	C ($n = 4$)	D ($n = 4$)	E ($n = 3$)	F ($n = 1$)	G ($n = 3$)	H ($n = 3$)
A	96.3 \pm 1.2							
B	94.2 \pm 1.7	100						
C	81.6 \pm 1.9	85.4 \pm 1.9	94.2 \pm 3.4					
D	89.5 \pm 1.8	91.4 \pm 0.9	79.7 \pm 2.4	98.0 \pm 1.0				
E	91.9 \pm 2.5	96.5 \pm 0.9	88.2 \pm 2.5	87.5 \pm 1.4	97.7 \pm 1.2			
F	85.0 \pm 0.8	87.5 \pm 0.5	88.5 \pm 0.9	82.3 \pm 0.4	88.7 \pm 0.5	(100)		
G	75.2 \pm 2.6	83.5 \pm 2.9	88.1 \pm 1.8	72.1 \pm 4.7	84.8 \pm 3.0	87.3 \pm 2.5	94.0 \pm 2.9	
H	83.6 \pm 1.3	86.3 \pm 2.2	86.3 \pm 4.8	83.7 \pm 2.1	87.6 \pm 3.3	91.0 \pm 1.4	83.7 \pm 5.4	95.0 \pm 1.6

n, number of isolates in each patient set.

overcome, as demonstrated in this study, by the deletion of such bands and the analysis of the remaining partial protein band patterns. The successful application of this procedure has been used for the analysis of patterns from an extensive range of other groups of bacteria where there has been evidence of 'intruding' or 'interfering' bands [14, 16, 23] and has been discussed in detail elsewhere [26].

No significant differences between the pre- and post-treatment isolates of *H. pylori*, which could lead to them being identified as different, were demonstrated by numerical analysis of the protein patterns. Minor differences in some patterns were evident. This homogeneity in isolates from the same patient has been confirmed in a separate study using restriction endonuclease digest patterns of the genomic DNA on the same collection of isolates [27], although further subdivisions could be made on the basis of DNA signature pattern analysis. Our analysis of the partial protein patterns showed unequivocally that the isolates of *H. pylori* from all eight patients had quite different patterns but revealed high levels of similarity between multiple isolates from the same patient. These results were consistent with previous data demonstrating marked genomic variability between *H. pylori* isolates from different patients, and even members of the same family group [9, 22, 28]. It was evident from our results that the same strains of *H. pylori* persisted in the patients after 4 weeks of nitrofurantoin therapy because the pre- and post-treatment sets of isolates had highly similar protein patterns. However, it was of interest that some minor variations in pattern between isolates from the same patient were apparent on visual inspection. These minor differences were masked in the numerical analysis as computations of similarities were based on the numerous bands that make up each of the profiles. Heterogeneity was detected in the higher molecular size bands (> 68 kDa) of many of the patient sets (from both different sites and/or in time). No complete explanation of this phenomenon is apparent beyond the possibility that a mixed, but highly related, population of strains (i.e. originating from a single clone) exists in the gastric mucosa at any one time, and/or that the drug treatment may have effected changes in the expression of some bacterial proteins.

It is clear that patients in this study, all of whom were considered as treatment failures, were not reinfected with a different strain of *H. pylori*. It is, however, still possible that during the 4-week period of treatment they may have been re-exposed to strains of the same type from the same source as the original infection and thus may have been reinfected. The most likely explanation for our results, however, is that the strain(s) causing the infection were not completely cleared during treatment and that this resulted in recrudescence of the original *H. pylori* infection.

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