ORIGINAL ARTICLE

Amplified fragment length polymorphism genotyping of metronidazole-resistant *Helicobacter pylori* infecting dyspeptics in England

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Objective Intra-specific diversity of *Helicobacter pylori* infecting stomachs of different individuals was investigated by numerical analysis of amplified fragment length polymorphisms (AFLP), to determine the existence of clones within the strain population and the effect that antibiotic treatment, particularly with metronidazole (Mtz), had on the balance of types/subtypes present before and after treatment.

Methods The 92 cultures studied comprised 89 single or multiple (pre- and post-treatment) isolates from gastric biopsies from 35 dyspeptic patients at two geographical locations in England, and three reference strains. *Hind*III restriction fragments tagged with specific adaptors were used as template DNA for AFLP. Patterns were coded in binary format according to deduced sizes of amplified fragments, and numerical analysis was performed.

Results *H. pylori* isolated from different individuals were highly diverse (43 AFLP types) with a continuum of similarities that included three putative strain clusters at the 55% similarity level. Twelve sets each comprised identical isolates but subclonal variants with similarities of 82–99% coexisted in isolate sets from 19 patients. Seven sets contained strains with different AFLP types which for several corresponded with *vac*A/*cag*A genotypic differences. Mtz resistance was a feature of clonal as well as unrelated isolates.

Conclusions AFLP profiling was a robust, reproducible and highly discriminatory means of indexing *H. pylori* strain diversity, and the numerical analysis enabled clonal/subclonal variants infecting an individual to be defined and contrasted with the general species diversity. The majority (65%) of patients had co-infections with different strain types/subtypes but antibiotic treatment apparently did not markedly modify *H. pylori* population diversity in individual stomachs. Mtz sensitivity was generally associated with greater strain diversity as several subtypes often coexisted in sensitive pretreatment strain sets. In contrast, Mtz-resistant strain populations were less diverse, which was attributed to selection by previous exposure to nitroimidazoles in the same or a different host.

Keywords *H. pylori*, AFLP, genotypes, numerical analysis, clones, metronidazole resistance, *vac*A alleles, *cag*A

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INTRODUCTION

Helicobacter pylori is a Gram-negative microaerobic bacterium infecting the gastric mucosal surface of the stomachs of an estimated 40% of the populations of most developed countries and over 75% of many developing countries [1]. The organism causes chronic active gastritis and is a recognized risk factor in the development of stomach ulcers and gastric malignancies such as adenocarcinoma and mucosa-associated lymphoid tissue lymphoma [2].

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A key feature of *H. pylori* is the high degree of genomic diversity, evident in terms of macrodiversity (intergenic variation) and of microdiversity (intragenic variation), amongst strains originating from stomachs of different individuals, and is attributed to mechanisms such as frequent free recombination and *in vivo* point mutation [3,4]. In addition, variations are present in *H. pylori* populations within an individual stomach/duodenum [5–8]. Although *H. pylori* is not implicated in hospital and community outbreaks, precise methods of strain identification are essential for public health purposes, which include investigations of routes of transmission and causes of treatment failure, as well as the identification of potentially hypervirulent strains, such as those associated with peptic ulcers or gastric cancer. *H. pylori* interstrain comparisons are based mainly on the use of genotyping methods that include ribotyping, restriction endonuclease digest analysis of the complete genome, and more

recently, PCR analyses of arbitrary or repetitive sequences, and of defined genes such as *cag*A, *vac*A, *ure*A/*ure*B and *fla*A/*fla*B [9].

Strains of *H. pylori* also can be readily distinguished by means of amplified fragment length polymorphism (AFLP) analysis, a robust high resolution multilocus marker technique in which adaptor molecules are ligated to a subset of restriction fragments, which are subsequently selected as target sites for selected primers in PCR amplification [10]. AFLP provides a means of examining DNA segments distributed over the entire genome, and studies on *Escherichia coli* using fluorescently labeled fragments indicate the approach also could have some phylogenetic significance [11].

Several genetic studies support the hypothesis that *H. pylori* is a species with a panmictic population structure [12,13], which has arisen as a result of frequent free recombination, yet there is some evidence of clonality over short time periods, such as after intrafamilial transmission [4]. Furthermore, four clusters of isolates were evident from alloenzyme electrophoretic analysis, from which the concept of an *H. pylori*–species complex was proposed [14]. Population genetic studies based on multilocus sequence typing (MLST) of *H. pylori* indicated the presence of weakly clonal groupings with evidence for an 'Asian' clone distinct from strains originating from other parts of the world [15]. A limitation of the above analyses in terms of population genetics was the fact that each study was based on relatively small sets of usually less than 40 isolates.

Our aim was to use AFLP combined with numerical analysis as a novel means of gaining an insight into the existence of clones and/or clonal subgroups of strains within the *H. pylori* population in an individual, to define genomic variants, and to investigate the effect that antibiotic treatment might have on the balance of types/subtypes coexisting in a particular gastric site. The study was based on isolates from British dyspeptic patients and comprised paired, and multiple isolates in some cases from different gastric sites, obtained before and after antibiotic treatment. Numerical analysis was used to obtain evidence for clonal relationships amongst multiple isolates from each individual patient as well as for testing for possible associations with strain features relevant in public health, namely emergence of metronidazole (Mtz) resistance after eradication therapy, and the *cag*A+/*vac*A s1/m1genotype, which is possibly linked to increased virulence [16].

MATERIALS AND METHODS

Bacterial strains and culture conditions

A total of 89 isolates of *H. pylori* from 35 dyspeptic patients, who had undergone routine endoscopy for symptoms of dyspepsia, were isolated at two geographically separate locations in England. The isolates from Ipswich Hospital (Suffolk) comprised 32 paired antral isolates from 16 patients. For each patient, there was one premetronidazole treatment and one post-treatment isolate for which details were previously reported [17]. A further 57 isolates (Leeds PHL) were from 19 patients and these included paired/multiple isolates (from gastric antrum and corpus) from pre- and postantibiotic treatment (clarithromycin and a proton pump inhibitor) biopsy samples, as well as a single isolate from one patient. Details on these isolates were reported previously [18,19].

All cultures of *H. pylori* were derived from subculture of multiple colony sweeps to avoid selection bias, and were stored in 10% v/v glycerol in Nutrient Broth (Oxoid) either over liquid nitrogen or at −80 °C. After primary isolation, strains were subcultured on blood agar (Columbia Agar Base, Oxoid) with 10% v/v defibrinated horse blood at 37 °C under microaerobic conditions (4% O_2, 5% CO_2, 3% H_2, and 88% N_2) in a variable atmosphere incubator (Don Whitley Scientific Ltd, Shipley, UK). In addition, the following three reference strains of *H. pylori* originating from dyspeptic patients were obtained from the National Collection Type Cultures (NCTC, London, UK) as lyophilized cultures: NCTC 11637 (the type strain of the species isolated in Perth, Australia), NCTC 12455 (strain 26695 isolated in Southampton, UK), for which the complete genome sequence has been determined [20], and NCTC 11638 (isolated in Perth, Australia).

AFLP profiling

DNA extraction and restriction endonuclease digestion

Bacterial genomic DNA was extracted by a miniprep protocol [21], using cetyl trimethylammonium bromide (CTAB). An aliquot containing from 2 to 10 μg of DNA was digested for 16 h at 37 °C with 20 U of *Hind*III (Boehringer) according to the manufacturer's instructions.

DNA template preparation

All procedures relating to the AFLP technique were performed as described previously [10]. A 5-μL aliquot of predigested DNA sample was used in the ligation reaction containing a final volume of 20 μL with 0.2 μg of each adapter oligonucleotide (ADH1: 5′ACGGTATGCGACAG 3′; ADH2: 5′AGCTC-TGTCGCATACCGTGAG 3′), 1 U of T4 DNA ligase (Boehringer) and single-strength ligase buffer. The reaction was performed at 37 °C for 3–4 h. The ligated DNA sample was heated to 80 °C for 10 min to inactivate T4 enzyme and then was diluted, according to the amount of digested DNA.

PCR reaction

*Hind*III restriction fragments tagged with the specific adaptors ADH1 and ADH2 were used as template DNA for selective PCR amplification. Primer HI-A (5′ GGTATGCGACAGA-GCTTA 3′) was used for PCR of all DNA samples as it was shown previously to yield clear AFLP fragment patterns with

a high discrimination level between strains of *H. pylori* [10]. Samples were also examined with an alternative primer HI-G (5′ GGTATGCGACAGAGCTTG 3′) in order to optimize discrimination. Amplification reactions were performed in a total volume of 50 μL containing 5 μL of template DNA, 2.5 mM $MgCl_2$, 2.7 μL of 20 μM solution of the primer and 1 U of *Taq* DNA polymerase (Gibco-BRL) in 1 × PCR buffer provided by the manufacturer. The amplification steps were an initial denaturing cycle of 94 °C for 4 min, followed by 33 cycles of 94 °C for 1 min, 60 °C for 1 min and 72 °C for 2.5 min. Amplified DNA products were separated by electrophoresis on 1.5% (w/v) agarose gels (ultrapure electrophoresis grade, Gibco-BRL) at 100 V for 6 h and stained in ethidium bromide (0.5 μg/mL) for 1 h. To interpret the resulting patterns, all the amplification fragments that were readily distinguishable on visual inspection, irrespective of relative staining intensities, were sized and included in the numerical analysis.

Fragment size estimation and computation of strain similarities

Sizes of fragments in AFLP patterns were estimated by comparison with a molecular weight marker (123 bp ladder) (Gibco-BRL) as previously described [22]. To compare patterns from different gels, DNA fragments were coded according to size in a binary form, and similarities were estimated by Dice coefficient (negative matches excluded). Clustering of strains was based on the unweighted pair group method with averages (UPGMA). All the computations were performed using NTSYS software [23]. For the purpose of this study, profiles were defined as identical only if they had all fragments in common, i.e. they were 100% similar in the numerical analysis.

Antibiotic sensitivity tests

A suspension of *H. pylori* was prepared to a density equivalent to a McFarland no. 4 standard in maximum recovery diluent (Oxoid, Basingstoke, UK). Swabs were used to innoculate blood agar plates (Columbia agar base, Oxoid) containing 10% (v/v) defibrinated horse blood with the suspensions. Epsilometer (E test) strips (Cambridge Diagnostics Service, Cambridge, UK) or 5-μg Mtz discs (Oxoid) were placed each plate, which was incubated under microaerobic conditions at 37 °C for 2–3 days. A breakpoint minimum inhibitory concentration (MIC) of <2 mg/L and a growth inhibition zone of <20 mm in diameter was interpreted as resistance to Mtz.

RESULTS

Optimization of DNA concentrations for AFLP

Three different concentrations (2, 4, and 10 μg) of genomic DNA from nine strains of *H. pylori* were digested with *Hind*III, and four dilutions (1/2, 1/4, 1/5, and 1/10) of ligated samples were used for PCR with the HI-A primer. The 10 μg DNA was based on our previous study [10] and the purpose here with a larger number of samples was to determine if that amount of DNA could be reduced without affecting either the efficiency of the PCR or the quality of the AFLP pattern. On the basis of these experiments, the median dilutions were selected for testing all further samples: namely, a digestion reaction containing 4 μg DNA and a 1/4 dilution of ligated sample for PCR. These conditions gave a total DNA concentration in the PCR reaction mix of 62.5 ng.

Reproducibility of the AFLP protocol

Reproducibility of the AFLP protocol was assessed in two different ways. DNA was extracted twice from four strains of *H. pylori* following subculture after an interval of several months. For each strain, the AFLP patterns obtained using the different DNA preparations were indistinguishable. DNAs from 10 other strains were used to prepare templates in triplicate for PCR. The AFLP profiles obtained from each repeat sample were identical although the larger sized fragments of about 1700-bp had fainter intensities (indicating a lower concentration). As these were possibly partially digested DNA fragments and were not fully reproducible, they were excluded when compiling the strain fingerprints for numerical analysis.

General features of the AFLP patterns

AFLP patterns were obtained using DNA from all 89 isolates and three reference strains of *H. pylori* (100% typability), and the patterns comprised between eight and 18 distinct fragments (Figures 1 and 2). Deduced sizes of the 61 individual fragments in the patterns were between 200- and 1600-bp. No single fragment was common to all of the strains, although some fragments were present at a higher frequency; for example, the 1120-bp fragment was present in 90 strain profiles (97.8%), fragment 840-bp was present in 86 profiles (96.6%), and fragment 480-bp was present in 80 profiles (88.8%).

Numerical analysis of the AFLP patterns

The results from the numerical analysis of the *H. pylori* AFLP patterns based on the presence or absence of particular fragments in the data set are illustrated in the dendrogram (Figure 2). We emphasize that the numerical analysis was performed to determine levels of similarity between isolates and was not used as the basis for inferring any phylogenetic relationships. Overall, a total of 43 distinct AFLP patterns (defined at the 82% similarity level) were designated for the 92 strains of *H. pylori* from the 38 patients. The key points from the analysis were as follows.

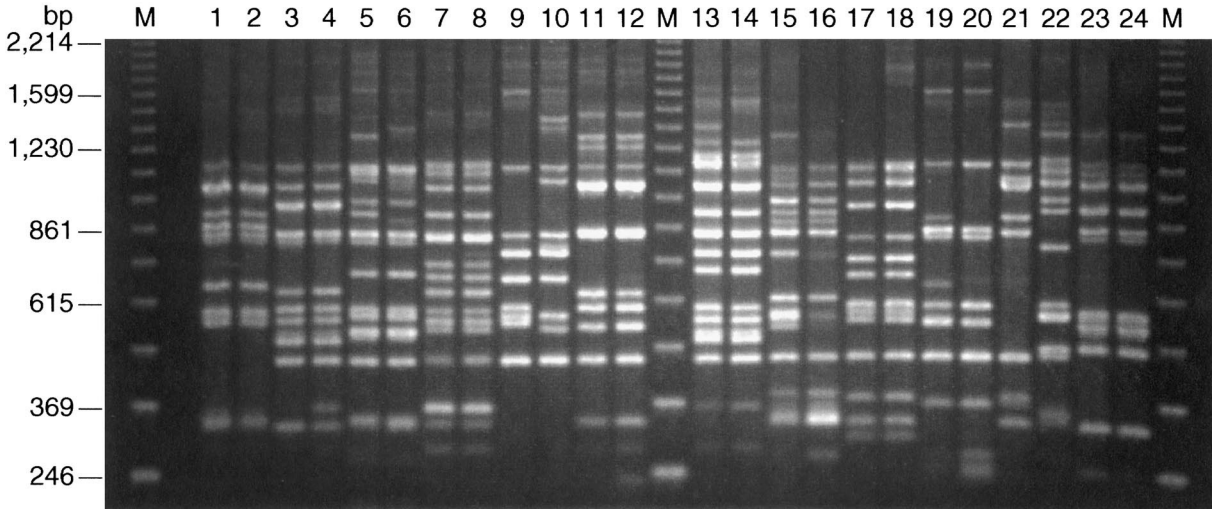

Figure 1 Examples of AFLP patterns (primer HIA) of paired *H. pylori* (Ipswich patient sets). DNA samples (left to right) by isolate identifier: lane 1, A1; lane 2, A2; lane 3, B1; lane 4, B2; lane 5, C1; lane 6, C2; lane 7, D1; lane 8, D2; lane 9, E1, lane 10, E2; lane 11, F1; lane 12, F2; lane 13, G1; lane 14, G2; lane 15, H1; lane 16, H2; lane 17, I1; lane 18, I2; lane 19, J1; lane 20, J2; lane 21, K1; lane 22, K2; lane 23, L1: lane 24, L2. M, molecular size markers.

Isolates from different patients

A high degree of genomic variation was observed amongst isolates of *H. pylori* from different individuals as the AFLP patterns were highly discriminatory, and isolate sets from almost all patients had types that were both different and unique. All isolates were linked at the 28% similarity level, with a continuum of branching linkages up to 100%. There were no readily identifiable groupings that could be mathematically defined as most of the main linkages occurred in the narrow 45–55% similarity region. However, from visual inspection of the pattern of strain clustering, we selected a cut-off at the 55% similarity level, to enable three main clusters, each containing isolates from at least three or more patients, to be discerned within the similarity continuum. Cluster I comprised 32 isolates (12 patients); Cluster II comprised nine isolates (three patients); and Cluster III comprised 20 isolates (10 patients). A further five isolates (two patients) were closely linked at the 50% level and so were also included in Cluster III. The remaining isolates in the dendrogram were not clearly associated with any other distinct clusters. It was notable, however, that the three reference strains [NCTC 11637 (the type strain), NCTC 11638, and NCTC 12455 (the sequenced strain 26695)], were positioned as independent outliers of the three main clusters defined above, with the two latter strains located at the extreme end of the similarity continuum (Figure 2).

Identification of clones

Strains from paired/multiple sets of *H. pylori* from 12 individuals (35%) exhibited identical intraset AFLP patterns (100% similarity). These were interpreted as clonal in origin, which was consistent with their isolation both before and after treatment, as well as being from biopsies taken from different gastric locations (antrum and corpus sites).

Identification of subclones

Nineteen sets of isolates of *H. pylori* provided evidence of minor variations (five or fewer band differences in pairwise comparisons; with three differences on average) with intraset similarities of between 82 and 99%. As isolates in each set were from single individuals (53% of all patients), these findings were interpreted as evidence of subclonal variants within each of those individuals. However, up to three subclones were present in some individuals; see for example strain sets AC and R. Four of the strain sets that contained matched clones also had subclones present (patients W, AD, AG, and AH).

The numerical analysis interpreted on these criteria highlighted several anomalies. In Cluster III (Figure 2), when an 80% cut-off was used as the criterion for separating different clones, identical isolates J1 and J2 were linked both to isolate O1 and to isolate N1, each of which were related as subclonal variants even though from different patients. A cut-off at 82%, however, separated the J isolates as a different type. Likewise, isolate Y3 was separated from isolates Y1, Y2, and Y3 as a different type at the 82% level, whereas at 80% similarity it was considered as a subclonal type. In Cluster III, isolates AF1 and AF2 were linked to isolate O2 as three subclonal variants. Thus patient O appeared to be infected with two different strain types which were more similar to isolates from other patients than to each other. It is not known if there were any family associations between these particular patients to explain the fact they had

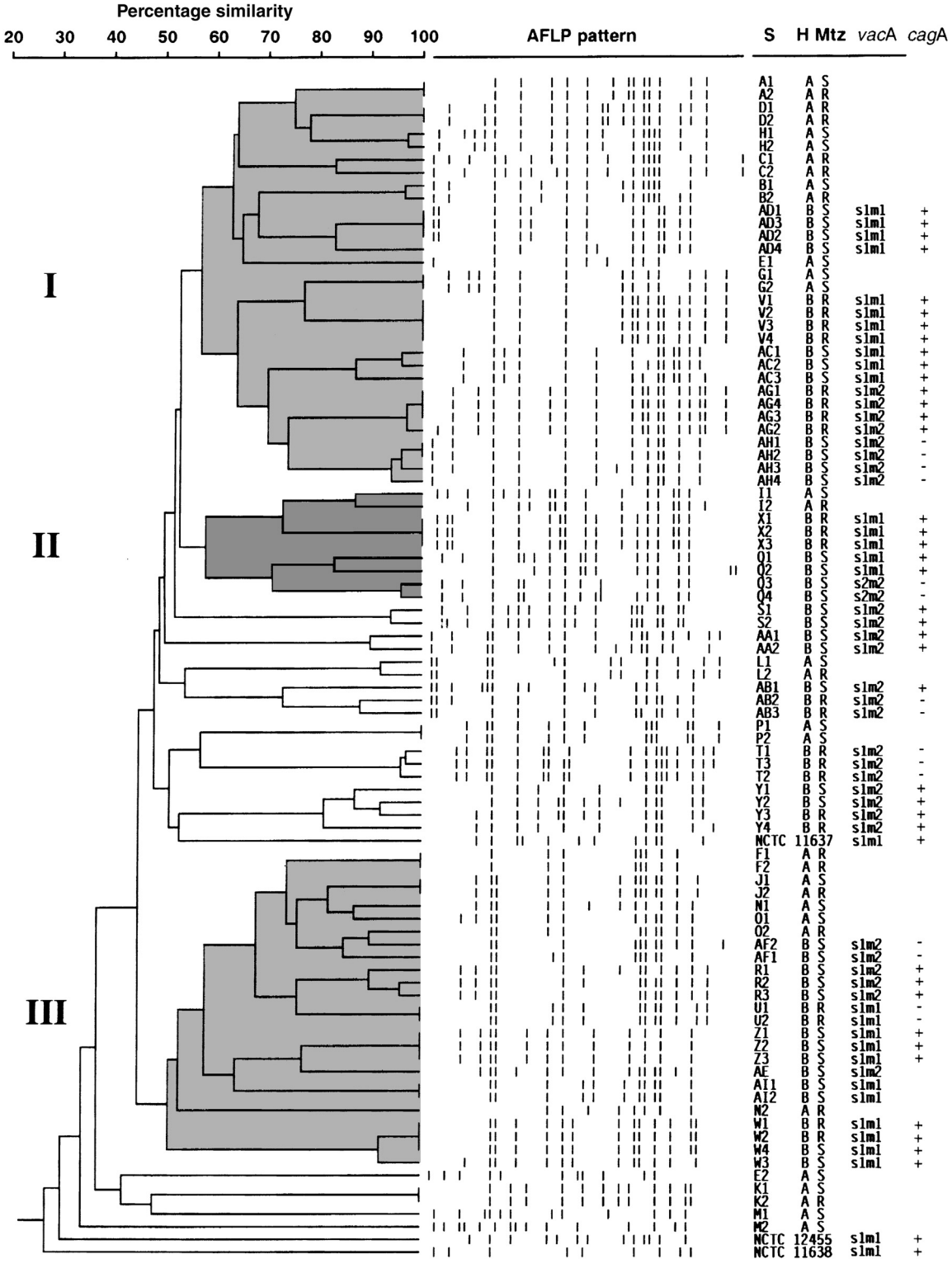

Figure 2 Numerical analysis of AFLP patterns of 92 isolates of *H. pylori* from 38 dyspeptic patients including 34 multiple isolate sets. Main strain groupings are designated as Cluster I (at 55%S), Cluster II (at 55%S), and Cluster III (at 50%S). DNA fragments were sized and levels of similarity between fingerprints were calculated. S, strain designation (see also Tables 1 and 2); H, Hospital/PHL laboratory of origin (A, Ipswich and B, Leeds); Mtz, metronidazole; R, resistant; S, sensitive; vac, *vac*A sm allelic type; *cag*A status.

related subclonal variants in common. Patient Q also was infected by two distinct strain types, each of which was represented by two subclonal variants.

Identification of different strains

Seven isolate sets (E, M, N, O, Q, Y, and AB) of *H. pylori* were identified as comprising different AFLP strain types (linked at <82%), and in several of these sets (Q, Y, and AB), there was also evidence of co-infection with different clonal subtypes (as described above).

Associations between *H. pylori* AFLP patterns and Mtz resistance

Metronidazole susceptibility was determined for all of the *H. pylori* isolates in the study, and resistant isolates were found to be widely distributed amongst the patients. There were no particular AFLP types uniquely associated with resistance to Mtz, as several isolates with identical AFLP patterns had different resistotypes. Within the three main clusters defined in the numerical analysis (Figure 2), there was a mix of resistant and sensitive isolates. However, the AFLP types of isolates were analyzed further with reference to Mtz susceptibility as follows.

Isolates sensitive to Mtz pre- and post-treatment

The genotyping results on such *H. pylori* isolates (S/S set) from 15 patients are listed in Table 1. The proportions of infecting isolates coexisting in the gastric mucosal biopsies from these patients were identified as: identical clonal types in four patients (G, P, Z, AI); different clonal subtypes in eight patients (H, R, S, AA, AC, AD, AF, AH); and different strain types in three patients (E, M, Q).

Isolates sensitive to Mtz pretreatment but resistant post-treatment

The genotyping results on such *H. pylori* isolates (S/R set) from 11 patients are listed in Table 2. The proportions of infecting isolates were identified as: identical clonal types, three patients (A, J, K); different clonal subtypes, five patients (B, I, L, W,Y); and different strain types, three patients (N, O, and AB).

Isolates resistant to Mtz pre- and post-treatment

The genotyping results on such *H. pylori* isolates (R/R) from eight patients are listed in Table 2. The infecting isolates were identified: as identical clonal types, five patients (D, F, U, V, X); and different clonal subtypes, three patients (C, T, AG); different types, none.

Overall, we found that the Mtz sensitive pre/post-treatment isolate sets ($n = 15$) when compared with the resistant pre/post-treatment isolate sets ($n = 8$) were generally more heterogeneous in containing fewer identical strain types/subtypes [27% (4/15) vs. 63% (5/8)]. Even though the numbers of patient sets were small, the diversity of strains in the pretreatment-sensitive/post-treatment-resistant sets was also 27% (3/11). Pretreatment susceptibility to Mtz was therefore a useful indicator of likely strain population heterogeneity, irrespective of post-treatment susceptibility.

Associations between AFLP patterns and *vac*A and *cag*A genotypes

The *vac*A sm-types of 57 isolates of *H. pylori* determined previously [18] are listed in Figure 2. In addition, the three reference strains had the s1/m1 type [R. J. Owen, unpublished

Table 1 AFLP genotypes of *H. pylori*: metronidazole-sensitive isolates pre- and post-treatment

Strain set (see Figure 2)	Genotype pre-treatment		Genotype post-treatment	
	Antrum*	Corpus	Antrum	Corpus
E	7 (1)	–	38 (2)	–
G	8 (1)	–	8 (2)	–
H	3a (1)	–	3b (2)	–
M	40 (1)	–	41 (2)	–
P	22 (1)	–	22 (2)	–
Q	15a (1)	15b (2)	16c (3)	16d (4)
R	–	31a (1)	31b (2)	31c (3)
S	17a (1)	–	17b (2)	–
Z	33 (1)	33 (2)	33 (3)	–
AA	18a (1)	–	18b (2)	–
AC	10a (1)	10 (2)	10c (3)	–
AD	6a (1)	6b (2)	6a (3)	6a (4)
AF	30b (1)	–	30a (2)	–
AH	12a (1)	12a (2)	12b (3)	12c (4)
AI	35 (1)	–	–	35 (2)

*Numbers in parenthesis indicate the strain number in each set; e.g. 1 = E1, and 2 = E2, etc.

Table 2 AFLP genotypes of *H. pylori*: metronidazole-resistant post-treatment strains

Strain set (see Figure 2)	Genotype pre-treatment		Genotype post-treatment	
	Antrum	Corpus	Antrum	Corpus
Metronidazole-sensitive isolates pre-treatment				
A	1 (1)	–	1 (2)	–
B	5a (1)	–	5b (2)	–
I	13a (1)	–	13b (2)	–
J	28 (1)	–	28 (1)	–
K	39 (1)	–	39 (1)	–
L	19a (1)	–	19b (1)	–
N	29a (1)	–	30a (1)	–
O	29b (1)	–	21a (2)	21b (3)
W	37a (1)	37a (2)	37b (3)	37a (4)
Y	24a (1)	24b (2)	24c (3)	25 (4)
AB	20 (1)	–	21a (2)	21b (3)
Metronidazole-resistant isolates pre-treatment				
C	4a (1)	–	4b (2)	–
D	2 (1)	–	2 (2)	–
F	27 (1)	–	27 (2)	–
T	23a (1)	–	23b (2)	23c (3)
U	32 (1)	–	32 (2)	–
V	9 (1)	9 (2)	9 (3)	9 (4)
X	14 (1)	14 (2)	14 (3)	–
AG	11a (1)	11b (2)	11a (3)	11a (4)

results]. The s1/m1, s1/m2 and s2/m2 *vac*A types were distributed amongst the isolate sets with no associations evident either within the main three AFLP clusters (I, II, and III) or elsewhere in the dendrogram. For isolates in 17 patient sets, the *vac*A types were uniform within sets even when there was evidence of type/subclonal variation in the AFLP patterns. The only exception was isolate set Q in Cluster II, where the two pretreatment isolates (Q1 and Q2) were *vac*As1/m1, whereas the two post-treatment isolates (Q3 and Q4) were *vac*As2/m2. The change in strain *vac*A type was particularly interesting as it reflected their diverged AFLP profiles (linked at 70% similiarity). It is generally accepted that strains with different *vac*A types represent different strains because such types appear stable and do not change by simple mutation over time. This result indicated therefore that different strains can have at least 70% similarity based on AFLP patterns. The fact that the Q1 and Q2 isolates were *cag*A +, whereas the Q3 and Q4 isolates were *cag*A –, further supported the AFLP differences between these strains. The majority (42/57) of the strains included in this study were *cag*A +. Interestingly, in isolate set AB, all three isolates had the *vac*A s1m2 genotype but the two that were *cag*A – were more related to each other than to the *cag*A + strain, with all three linked at 72%, indicating two different AFLP types. For set Y, the *cag*A and *vac*A results were the same, so other genomic markers would be needed to confirm the strain differences indicated by AFLP. The other *cag*A – strains sets (T, AF, and AH) were more conserved in both AFLP and *vac*A genotypes. The *vac*A and *cag*A data were not available for other strain sets with mixed AFLP types (E, M, N, and O), to confirm if they were truly different strains.

AFLP analysis of *H. pylori* from different geographical locations

H. pylori were from laboratories in Ipswich and Leeds, and in the case of the former isolates, these were known to have originated from patients in that locality [17]. The Leeds isolates were from more diverse locations as they were collected originally as part of a treatment trial [19]. The isolates from both locations were widely distributed in the continuum of AFLP similarities (Figure 2), and in the case of the Ipswich isolates, there was no evidence to indicate a specific clonal variant or group of strain types infecting individuals in that location.

DISCUSSION

The human gastric mucosa provides a unique environment to which *H. pylori* has become specifically adapted for long-term survival [24]. Epidemiological data indicate that most primary infections occur in early childhood, and so without eradication therapy, the infection is likely to be lifelong [25]. Simultaneous infections with other microorganisms in the same gastric niche

appear to be extremely rare, as is the likelihood of infection with *H. pylori* from other sites, such as the oral cavity, where the species may be a transitory resident [26]. We assert that the current active infecting population of *H. pylori* therefore should closely reflect the strain pool involved in establishing the primary infection. Some, if not all, of the strain population should be clonally descended from members of that original strain pool, possibly with genomic variations accumulated during the course of the continued infection. Failed antibiotic therapy might be expected to have a major impact on the *H. pylori* population dynamics in the stomach, an understanding of which is central to interpretation of strain typing data used for eradication, strain pathogenicity and transmission studies. An interesting feature of the numerical analysis of *H. pylori* AFLP profiles was our finding that 35% (12/34) of patients [note that one patient (AE, cluster III) was excluded from these analyses as only one pretreatment isolate was available] were infected by *H. pylori* with identical strain types in individual biopsies, irrespective of the specific site of isolation (antrum or corpus). For these, the genotyping data (AFLP, *vac*A and *cag*A) could be interpreted to indicate that isolates within each particular patient population were most probably clonally related. Our findings were consistent with the short-term clonal descent concluded from strains isolated from paired family members in Germany analyzed by sequencing of selected pathogenicity-associated gene fragments (*fla*A, *fla*B, and *vac*A) [4]. Likewise, we reported previously that genotypically similar strains of *H. pylori* infected members of three generations of a duodenal ulcer family in England [27], and members of several family groups (parents and child) in Northern Ireland [28].

The question of diversity in pretreatment *H. pylori* strain populations, the so-called baseline diversity, was addressed by examination of the antrum and corpus pretreatment isolates in each individual. A majority of the individuals (19/34, 56%) appeared to have a population comprising two or more subtypes evident from analysis of representative culture sweeps rather than just single colony isolates of these individuals. Similarly, intermediate levels of genomic diversity in *H. pylori* from UK patients were observed in previous studies using multiple pretreatment biopsies [29,30], whereas results on patients in other countries, e.g. Italian subjects, indicated more diversity [31,32]. It is possible that low socioeconomic status, an established risk factor for *H. pylori* infection, may be linked with an increased risk of coinfection with multiple strain types.

A key aim of the present study was to test the impact of antibiotic treatment, particularly Mtz, on the balance of the infecting *H. pylori* strain pool in an individual, as this aspect is not well understood and is important when assessing strain typing data. We first considered the 12 patients infected with a single strain type. In eight of these patients (five in the R/R set and three in the S/S set), we found that the resistotypes of the infecting isolates were unchanged, whereas for four patients in the S/R set, the post-treatment isolates acquired resistance possibly by selection of resistant forms or by rapid genetic modification, presumably as a direct result of exposure to Mtz (Ipswich set) or clarithromycin (Leeds set). The mechanism for Mtz resistance in *H. pylori* is complex and not fully understood and could involve products from multiple genes that include the *rdx*A gene [33]. Overall, we found no direct link between AFLP pattern and Mtz resistance, which is not unexpected as AFLP analysis can be estimated to sample only around 1% of the genome. However, an interesting finding from the analysis was the greater population homogeneity of 'naturally' resistant (pretreatment) isolates suggesting they may have originated from a strain population that had previously undergone selection by exposure to nitroimidazoles. Thus, some diversity may have been filtered out at an earlier stage of the infection or in a previous infected individual(s) before transmission to the present host.

The second main patient group comprised 19 individuals (56% of all the patient sets) in whom we found evidence of clonal variants within the infecting *H. pylori* strain pool. The variants were evident mainly when comparing cultures either from different gastric sites, or before and after treatment. In previous studies, we also observed related variants at different gastric sites using other genotyping methods (ribotyping and RFLP analysis of urease subunit A and B genes) performed on isolates both before and after omeprazole treatment [34] and after triple therapy that included Mtz [30]. When our present data were considered in the context of Mtz treatment, we found five patients (D, F, U, V, and X) who were infected by the same resistant strain before and after treatment. However, variation based on number of types and subtypes was most common in the pretreatment sensitive isolate sets (26 in total), but in only eight patients was genotypic variation associated with a change in Mtz resistotype (namely sensitive to resistant). Finally, for 11 patients, there was evidence of a different strain of *H. pylori* present after treatment, although in only three of these (N, O, and AB) was the new type associated with acquisition of resistance.

The main feature of the information derived from analysis of all the AFLP patterns was the high degree of genomic diversity evident amongst isolates of *H. pylori* from stomachs of different unrelated individuals – a well documented and consistent feature of the species irrespective of the genotyping method used [35,36]. However, genomic comparisons of the two sequenced strains suggest that nucleotide drift in the third position of coding triplets may account for the majority of the DNA-based diversity evident from typing studies, and a comparison of orthologous genes showed a high degree of functional conservation at the protein level [37]. Population genetic studies on *H. pylori*, albeit based on limited numbers of isolates, demonstrated a panmictic, essentially non-clonal, population structure attributable to frequent horizontal gene

transfer between strains and recombination, combined with *in vivo* mutational events accumulated over a long evolutionary history [3,4,14]. Our findings from the AFLP analysis also illustrate the high degree of interstrain diversity of *H. pylori* populations infecting different and unrelated individuals in the UK. However, the sample of individuals investigated by culture and typing remains extremely small in relation to the total number of infected individuals in the UK (currently estimated to be about 40%), and so the diversity data can be viewed only as an approximation for the normal non-dyspeptic population. Nevertheless, there was some evidence from the numerical analysis of AFLP patterns of the existence of two, possibly three, broad strain groupings within the species. These groupings did not relate to any of the other strain features examined in the study, namely Mtz resistance, *vac*A allelic form and *cag*A status, or to the geographical location from where the strains originated. The phylogenetic significance of the groupings cannot be deduced from the present study; however, the occurrence of such groupings may give some credence to the suggestion based on MLEE that *H. pylori* could be viewed as a complex of cryptic species [14] – a concept that needs testing with other genetic criteria such as 16S rRNA gene sequence analysis or MLST.

In conclusion, our findings demonstrated that AFLP provides a reproducible and highly discriminatory technique for investigating *H. pylori* population diversity within individual gastric biopsies, and enables similarities to be expressed in relation to the general diversity of strains from stomachs of different individuals. Our AFLP analysis was technically simple to perform as it did not use fluorescently labeled primers or DNA sequencing. Most importantly, it offered the advantage over other PCR-RFLP-based methods in sampling multiple loci across the genome estimated to represent about 1% of the genome. In a recent study, the AFLP technique proved valuable in demonstrating diversity within *H. pullorum* [10], and resulted in the recognition of a new species named '*H. canadensis*' [38]. We believe AFLP therefore could also have potential as a novel basis for providing criteria for defining future subspecies or quasispecies within *H. pylori*.

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