Biotype and molecular fingerprints of metronidazole-resistant strains of *Helicobacter pylori* from antral gastric mucosa

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Summary. Biotypes, ribosomal RNA gene restriction patterns (ribopatterns), whole-cell protein patterns and plasmid profiles of paired *Helicobacter pylori* isolates from 17 patients were examined. Each pair comprised a pre- and a post-treatment isolate; nine of the 17 post-treatment isolates were obtained after treatment with tripotassium dicitrate bismuthate (De-Nol) and metronidazole. All strains of *H. pylori* had identical biotypes, but exhibited diversity between pairs in their molecular fingerprints. Each of the 17 strain pairs had unique ribopatterns; the pre- and post-treatment isolates in most pairs (16 of 17) were similar or identical, irrespective of metronidazole susceptibility. DNA subtype variants were detected in three patient sets. Although nine post-treatment isolates had acquired resistance to metronidazole, most (six of nine) resembled the pre-treatment isolates in their ribopattern, protein and plasmid profiles. No significant correlation was observed between metronidazole resistance and plasmid content in these *H. pylori* isolates. Emergence of post-treatment metronidazole-resistant isolates of *H. pylori* was associated only rarely with colonisation by a novel strain or acquisition of a plasmid and, in most patients, probably resulted from spontaneous emergence of resistance in the original infecting strain.

Introduction

Helicobacter pylori is a gram-negative micro-aerobic bacterium, originally classified as *Campylobacter*, with a world-wide distribution in the gastric mucosa of man.¹ Interest in the organism has developed over the past decade because of its association with active chronic gastritis² and its identification as a risk factor in peptic ulcer disease³ and gastric cancer.⁴ Considerable research has been directed towards finding the best treatment combination to eradicate *H. pylori*.^{3,5} A combination of tripotassium dicitrate bismuthate (De-Nol) and metronidazole was shown to be an effective treatment for *H. pylori* infection,⁶ and an eradication rate of c. 85% was achieved in patients whose pre-treatment isolates were sensitive to metronidazole. Further investigations of patients in whom treatment failed to eradicate *H. pylori* revealed that the organism had become resistant to metronidazole.⁷ Although, at the moment, some success can be achieved in treating patients whose isolates are sensitive to metronidazole, there is a pressing need to develop a safe, effective and cheap method of eradicating metronidazole-resistant organisms.

The aim of the present study was to investigate

further the strains of *H. pylori* that developed resistance to metronidazole after combination treatment with De-Nol. Although only c. 20% of *H. pylori* isolates in the UK are resistant to metronidazole, the figure in certain third world countries is well over 80%, probably because of the widespread use of metronidazole to treat conditions such as giardiasis and amoebiasis. This paper describes results obtained with biotyping, ribosomal (r)RNA gene restriction digest patterns (ribopatterns), plasmid profiles and one-dimensional electrophoretic protein patterns for high resolution identification of pre- and post-treatment isolates of *H. pylori*. Molecular fingerprinting methods were selected for the present study because their accuracy and discriminatory potential have been validated in several previous studies of *H. pylori*.⁸⁻¹⁰

Materials and methods

Organisms and culture conditions

The organisms studied (34 isolates from 17 patients) were cultivated from pre- and post-treatment endoscopic antral biopsies. Details of the treatment regimens have been published,⁷ but essential information is given in table I. A reference strain (NCTC 11637),

Table I. *Helicobacter* strain designations, ribotypes and plasmid contents

Strain code*	DNA ribotype with			Plasmid carriage
	<i>Hae</i> III	<i>Hind</i> III	<i>Bst</i> EII	

A. <i>Pre- and post-treatment isolates MTZ-sensitive</i>				
(i) <i>Treatment regimen: De-Nol</i>				
C-I	3	...	...	—
C-II	3	...	...	—
D-I	4	...	...	+
D-II	14	...	...	+
Q-I	NC	NC	2	—
Q-II	NC	NC	2	—
(ii) <i>Treatment regimen: cimetidine</i>				
H-I	8	...	...	+
H-II	8	...	...	+
N-I	NC	1a	...	—
N-II	NC	1b	...	—
(iii) <i>Treatment regimen: ranitidine</i>				
K-I	11	...	...	—
K-II	11	...	...	—

B. <i>Pre- and post-treatment isolates MTZ-resistant</i>				
(i) <i>Treatment regimen: MTZ + De-Nol</i>				
G-I	7	...	...	—
G-II	7	...	...	—
(ii) <i>Treatment regimen: MTZ + De-Nol + pivampicillin</i>				
M-I	13	...	...	+
M-II	13	...	...	+

C. <i>Pre-treatment isolate MTZ-sensitive; post-treatment isolate MTZ-resistant</i>				
<i>Treatment regimen: MTZ + De-Nol</i>				
A-I	1	...	...	+
A-II	1	...	...	+
B-I	2	...	...	—
B-II	2	...	...	—
E-I	5	...	...	—
E-II	5	...	...	—
F-I	6a	...	...	—
F-II	6b	...	...	—
I-I	9	...	...	+
I-II	9	...	...	+
J-I	10	...	...	—
J-II	10	...	...	—
L-I	12	...	...	—
L-II	12	...	...	—
O-I	NC	2a	...	(+)
O-II	NC	2b	...	(+)
P-I	NC	NC	2	—
P-II	NC	NC	2	+

MTZ, metronidazole; NC, DNA not cut; (+) indicates plasmid bands of different sizes detected in the restriction endonuclease digests only.

*Letter refers to the patient; I and II signify pre- and post-treatment isolates.

originating from Perth, Australia, was obtained from the National Collection of Type Cultures. The histological and culture methods have been described.¹¹

All isolates of *H. pylori* were grown on Brain Heart Infusion (BHI) Agar (Oxoid) containing horse blood 5% and supplemented with Isovitalex (BBL) 1%. Cultures were incubated for 48 h at 37°C under micro-aerobic conditions (O₂ 5%, CO₂ 5%, H₂ 2%, N₂ 88%) in a Variable Atmosphere Incubator (Don Whitley Scientific Ltd, Shipley, Yorks.). Stocks of all cultures were preserved in glycerol 10% v/v in Nutrient Broth (Oxoid) over liquid nitrogen.

In-vitro tests for metronidazole sensitivity

In all cases, the *H. pylori* strains isolated by culture of biopsies were tested for sensitivity or resistance to metronidazole. In-vitro sensitivity tests were performed by disk diffusion with IsoSensitest Agar (Oxoid) containing lysed horse blood 5% and disks containing metronidazole 5 µg. Plates were incubated for 48 h at 37°C in an atmosphere of O₂ 6%, CO₂ 10%, N₂ 84%. A zone of inhibition (> 10 mm) was regarded as indicating susceptibility.

Motility testing

Isolates were grown on slopes of BHI blood agar with added Isovitalex as described above. The liquid phase culture at the base of the slope was removed at 24 h, and bacterial motility was determined in hanging-drop preparations by standard methods. Strains that were weakly motile or non-motile were re-cultured and re-tested for motility.

Enzyme biotyping

Biotypes were determined from pre-formed enzyme production in API-Zym kits (API bioMérieux, Basingstoke, Hants.) as described.¹²

DNA isolation, restriction digestion, vacuolting and RNA gene probe hybridisation

Chromosomal DNA was isolated and purified by the guanidium thiocyanate reagent method.¹³ DNA samples (5 µg) were digested for 4 h at 37°C with *Hae*III, *Hind*III, or *Bst*EII (2–3 U/µg of DNA) according to the manufacturer's instructions (Northumbria Biologicals, Cramlington, Northumbria). The digested DNA was electrophoresed at 25 V for 16 h in a horizontal agarose 0.7% w/v gel with buffer containing 89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.3. The DNA fragments were then transferred to nylon membranes (Hybond-N; Amersham International) by vacuum transfer blotting, and hybridised by standard procedures for 18 h at 42°C¹⁴ with a biotinylated cDNA probe. This was prepared from 16S and 23S rRNA of *H. pylori* strain NCTC 11638 with Moloney mouse leukaemia virus reverse transcriptase (Gibco-BRL).¹⁵ Biotinylation was achieved by the incorporation of biotin 16-dUTP. The membranes were washed after hybridisation and the hybridised probe was detected colorimetrically with a non-radioactive detection kit (BluGENE Kit; Gibco-BRL). The pattern of bands obtained was designated the ribopattern.

DNA size estimation

DNA band sizes in the blot hybridisation patterns were calculated from migration distances, either by the DNA SIZE program, based on the algorithm of Elder and Southern,¹⁶ as described previously¹⁷, or by an

Fig. 1. Examples of the rRNA gene patterns (ribopatterns) for *Hae*III digests of chromosomal DNA from *H. pylori* probed with biotinylated cDNA from rRNA of strain NCTC 11638. The strain codes (table I) are indicated below the lanes. R, metronidazole-resistant; S, metronidazole-sensitive. Molecular size markers (kb) were provided by λ -*Hind*III digests.

automated gel reader and analysis system (IBI, New Haven, CT, USA). Biotinylated λ phage (Gibco-BRL) digested with *Hind*III was used to provide the size markers. Three lanes (outside left and right, and centre) containing the markers were included on each gel.

Computation of strain similarities

To compare ribopatterns from different membranes, the bands were coded according to size to minimise errors when determining similarities by computer-assisted methods of analysis. The blot hybridisation patterns were screened for bands within 21 different size ranges up to 10 kb; positive (presence) and negative (absence) results were recorded. Bands of faint intensity were excluded. Double bands falling within a given range were scored as a single band. Computed similarities among strains were estimated by means of the Dice coefficient (negative matches excluded), and clustering of strains was based on the unweighted pair group method to facilitate the generation and plotting of a dendrogram.¹⁸ All computations were performed with the DNAGE program, a modification of described software.¹⁹

Plasmid DNA extraction and electrophoresis

For plasmid DNA extraction, organisms were cultured as described above. Single colonies were picked and plasmid DNA was prepared according to the method of Kado and Liu,²⁰ as modified previously.²¹ Samples were electrophoresed in a horizontal agarose 0.7% gel for 2 h at 100 V. After staining with

ethidium bromide, the gels were viewed on a UV-transilluminator and photographed.

PAGE of proteins

The preparation of whole-cell protein extracts and one-dimensional (1-D) electrophoresis by discontinuous 10% SDS-PAGE was performed as described.²² The resulting patterns were compared visually.

Results

Biotype characteristics

All the strains of *H. pylori* were motile in broth cultures and belonged to API-ZYM biotype II (producing leucine arylamidase and phosphohydrolase, but not esterases). Strains differed in their susceptibility to metronidazole, and are listed in table I according to the susceptibilities of the pre- and post-treatment isolates in each pair. Six paired isolates (12 cultures) were sensitive to metronidazole before and after treatment (set A), and two paired isolates (four cultures) were resistant to metronidazole before and after treatment (set B). The majority of the isolates (nine paired sets, 18 cultures) were sensitive to metronidazole before treatment, but were resistant to metronidazole after treatment (set C).

rRNA gene restriction patterns

Chromosomal DNA from each of the 34 strains of *H. pylori* was treated with *Hae*III. DNA from eight

Fig. 2. Similarities between metronidazole-sensitive and -resistant strains of *H. pylori*, based on the *Hae*III ribopatterns. The numbers on the horizontal axis indicate the percentage similarities as determined by the Dice coefficient and unweighted pair group average linkage clustering.

Fig. 3. The rRNA gene patterns for: (A) *Hae*III and *Hind*III digests of chromosomal DNA from *H. pylori* paired isolate set D; and (B) *Bst*EII digests of chromosomal DNA from *H. pylori* paired isolate sets P and Q. The strain codes are indicated by the letters above the lanes. See legend to fig. 1 for other details.

strains was not digested reproducibly by *Hae*III. Examples of the *Hae*III ribopatterns from the other 26 strains are illustrated in fig. 1, and the similarities between isolates derived from a numerical analysis of

the results are shown in fig. 2. The analysis indicated that the pre- and post-treatment isolates in all but two (11 of 13) of the pairs had identical *Hae*III ribopatterns at the 90% similarity (S) level. The differing isolates

Table II. Summary of ribotyping results obtained with *H. pylori* paired isolates

Ribopattern matches between paired isolates (I and II)	Number of pairs according to metronidazole susceptibility		
	R + R	S + S	S + R
Same ribotype (identical)	2	4	7
Same ribotype, different subtype	0	1	2
Different ribotype	0	1	0

R + R, pre- and post-treatment isolates resistant; S + S, pre- and post-treatment isolates sensitive; S + R, pre-treatment isolate sensitive and post-treatment isolate resistant.

were from the following two patients: *Patient D*, in whom the pre- and post-treatment isolates had completely different *Hae*III and *Hind*III ribopatterns (fig. 3A); and *Patient F*, in whom the pre- and post-treatment isolates had similar but not identical (two band differences) *Hae*III ribopatterns (results not shown), indicating that the isolates were probably genomic variants.

The eight DNA samples from the four remaining strain pairs (patients N, O, P and Q) that were not cut by *Hae*III were examined further by digestion with either *Hind*III or *Bst*EII. The resulting ribopatterns following digestion with one or other of these endonucleases showed that paired strains were identical (*Bst*EII digests) from patients P and Q (fig. 3B), but were subtype variants (*Hind*III digests) from patients

N and O (results not shown). DNA from these strains was also treated with *Bam*HI, *Eco*RI and *Sac*I, but the ribopatterns obtained were unclear and unsuitable for comparative purposes. *Bst*EII was the only endonuclease tested that cut DNA from isolate pairs P and Q, and so their similarities had to be inferred from the two band patterns obtained. The ribotype designation ascribed to each strain is given in table I; the results are summarised in table II according to metronidazole sensitivity.

Plasmid profiles

Plasmid DNA (single bands of < 10 kb) was detected in 11 strains (32%) by the modified method of Kado and Liu (table I), and most paired isolates had plasmids of the same size. The paired strains from patient P were atypical because the pre-treatment isolate was plasmid-free whereas the post-treatment isolate carried a plasmid. Although no plasmid bands were detected in paired isolates from patient O by the Kado and Liu method, there was evidence from total chromosomal DNA gel electrophoresis of DNA bands of differing sizes attributable to plasmids in the undigested samples (results not shown).

Protein patterns

All the strains had one-dimensional SDS-PAGE protein patterns containing the six major characteristic protein bands of *H. pylori* of c. 27, 29, 42, 53, 57 and

Fig. 4. Examples of electrophoretic protein patterns of *H. pylori* isolates. The letters above the tracks refer to the patients from whom the isolates were obtained, and the Roman numerals (below) to the time of isolation (I, pre-treatment; II, post-treatment). Molecular size markers (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. R, reference strain NCTC 11638.

Table III. Comparison of *H. pylori* strains with different characteristics before and after treatment

Strain code	Characteristic				
	MTZ susceptibility	Ribopattern (enzyme)	Plasmid presence	DNA digest pattern	Protein pattern
<i>Different ribotypes</i>					
D-I	S	4 (<i>Hae</i> III)	+	U	U
D-II	S	14 (<i>Hae</i> III)	+ ^d		
<i>Different ribo-subtypes</i>					
F-I	S	6a (<i>Hae</i> III)	—	M	M
F-II	R	6b (<i>Hae</i> III)	—		
N-I	S	1a (<i>Hind</i> III)	—	M	M
N-II	S	1b (<i>Hind</i> III)	—		
O-I	S	2a (<i>Hind</i> III)	+	M	M
O-II	R	2b (<i>Hind</i> III)	(+ ^d)		
<i>Different plasmid content</i>					
P-I	R	2 (<i>Bst</i> EII)	—	M	M
P-II S	S	2 (<i>Bst</i> EII)	+		

MTZ, metronidazole; R, resistant; S, sensitive; +, plasmid present; —, no plasmid; M, matching patterns; U, unmatched patterns; ^d, plasmids different in size; (+) evidence of plasmid DNA in *Sac*I, *Bam*HI and *Eco*RI digest samples.

64 kDa, respectively. Members of each pre- and post-treatment pair, except for those from patient D, had identical patterns in the 47–56 kDa hypervariable region as well as in other regions of the profile. However, there were clear protein profile differences between paired isolates from different patients (fig. 4).

Summary of strain differences

Differences in one or other of the above characteristics were detected in paired isolates from five patient sets; the results are summarised in table III.

Discussion

Previous studies have shown that *c.* 20 % of primary *H. pylori* isolates are resistant to metronidazole and unlikely to respond to dual therapy with metronidazole and De-Nol.^{6,23} Similar frequencies of resistance have been reported in other clinical investigations,^{24–26} although the higher frequency of such strains in younger women was attributed to the previous use of metronidazole for gynaecological complaints.^{23,26} In a study of patients with primary metronidazole-sensitive *H. pylori* isolates, patients in whom the treatment failed to eradicate the organism were colonised by resistant strains.⁷ The results of the present study demonstrate that the same strain of *H. pylori* was present before and after treatment in all except one of these patients, although subtype differences were detected in three sets. Ribopatterns of such *H. pylori* isolates indicated no gross genomic DNA changes resulting from treatment with metronidazole, and the results were consistent with the biotype and the total protein pattern, which is a sensitive measure of expressed genomic variation.

Previous studies have demonstrated marked heterogeneity in ribopatterns amongst *H. pylori* isolates from different individuals, and have shown that strains with matching genotypes were almost exclusively from the same individual.^{8–10} These observations were confirmed by the results of the present study. Additional molecular features of the isolates, namely that 25 % (eight of 34 isolates) had DNA that was not digested by *Hae*III, that only 30 % (12 of 34 isolates) carried plasmids, and that some strains were subtype variants, were consistent with *H. pylori* results from other geographical locations in the UK, Italy, Canada, USA and South Africa.^{9,10,27,28}

In five (29 %) of the strain pairs, some differences were observed between pre- and post-treatment isolates (table III). The isolates D-I and D-II were distinct in all characteristics, and were clearly different strains. Differences between the other paired isolates were less marked. Nevertheless, pre- and post-treatment isolates of patient pairs F, N and O were clearly different subtypes, although no differences were evident in other characteristics. Similar subtype variation has been observed in sequential or multiple isolates from other patients,^{8,9,22} indicating that some individuals may be colonised simultaneously by a mixture of several strain subtypes, irrespective of treatment regimens.

The mode of action of metronidazole is through its conversion, under conditions of reduced oxygen tension, to a cytotoxic intermediate compound able to react with DNA and cause strand breakage.²⁹ The cellular mechanisms for resistance to metronidazole in *H. pylori* are not known, but its emergence is probably attributable to either selection *in vivo* during therapy, or to the occurrence of spontaneous resistance which has been shown to occur at a low level *in vitro*.^{28,30} There was no evidence from our study to suggest that the emergence of resistance *in vivo* was linked to any common phenotypic marker based on biotype, or on

bands in total protein profiles, or with any unique DNA bands in the ribopatterns. The metronidazole-sensitive and -resistant strains had identical composite rRNA gene restriction fragment distributions. Furthermore, our results indicated (as found previously by Majewski and Goodwin²⁸) that the emergence of resistance to metronidazole in *H. pylori* was unlikely to be attributable to plasmid-encoded determinants because there was no direct association between emergence of resistance and acquisition of a plasmid. Also, several metronidazole-resistant *H. pylori* isolates carried no plasmid DNA, and only in the strain pair from patient P was there a link between emergence of resistance and acquisition of a plasmid (table III). There is no evidence of any other mechanism for

metronidazole resistance in *H. pylori*, although there is some evidence in *Bacteroides fragilis* that genetic determinants for metronidazole resistance are plasmid-borne.^{31, 32}

In conclusion, in most of the patients studied, the emergence of post-treatment metronidazole-resistant strains of *H. pylori* probably resulted from spontaneous acquisition of resistance in the original infecting strain and not from the acquisition of a novel strain from an exogenous source.

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