DNA fingerprints of *Helicobacter pylori* before and after treatment with omeprazole

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Abstract

Aims: To test whether a hypoacidic environment may potentially "stress" *Helicobacter pylori* DNA, encouraging the emergence of strain variation.

Methods: This hypothesis was tested by inducing prolonged hypoacidity with omeprazole, a potent antisecretory drug. The genomic DNA of *H pylori* was studied by electrophoretic separation of restriction endonuclease fragments followed by rRNA gene hybridisation in seven patients infected with *H pylori* before and after treatment with omeprazole 20–40 mg daily for six to eight weeks. DNA was isolated and purified using the guanidium thiocyanate reagent method. DNA samples were digested with *Hae III*, electrophoresed, vacublotted, and hybridised using a biotinylated cDNA probe prepared from 16S and 23S rRNA from *H pylori* NCTC 11638. Isolates were compared using their ribopatterns (DNA fingerprints).

Results: A total of 26 isolates were obtained; all DNA isolates were cut by *Hae III*, which was the enzyme that gave the best resolved hybridisation patterns for analysis. No two patients harboured the same strain. The isolates from two patients showed evidence of subtypic variation; one patient had two distinct strains and four patients had their own indistinguishable strains before and after treatment with omeprazole. For each patient, the paired ribopatterns of *H pylori* DNA were not affected by treatment with omeprazole for six to eight weeks.

Conclusion: The *H pylori* genome is relatively stable when exposed to the conditions of prolonged hypoacidity that result from treatment with omeprazole.

level of genomic heterogeneity has been shown among *H pylori* isolated from different patients in the United Kingdom, the Netherlands, Australia and Canada.^{4–11} This genomic heterogeneity is more pronounced in *H pylori* than in many other organisms that have been studied,^{12–14} hence the technique of DNA fingerprinting has clear applications for epidemiological research.

The reason for the genetic heterogeneity in *H pylori* isolates is unclear. Although *H pylori* DNA fingerprints are highly stable in the laboratory, *H pylori* may exhibit genetic instability in vivo. Acute infection seems to be associated with a transient achlorhydria: the evidence comes from self ingestion studies¹⁵ and from retrospective analysis of epidemics of achlorhydria in experimental laboratories which may have been due to transmission of *H pylori* by contaminated nasogastric tubes.^{16–19} In contrast, the acid secretion of asymptomatic volunteers found to be positive for *H pylori* on screening (who are likely to have been infected for several years) is normal.²⁰ The mechanism of this transient achlorhydria is unknown, certain strains of *H pylori* can cause profound suppression of acid secretion in isolated human gastric glands.²¹ Achlorhydria causes a luminal bacterial overgrowth which may facilitate the formation of N-nitrosamine compounds²² which may damage the DNA of the original infecting *H pylori*.

This hypothesis was tested in this study by inducing acid suppression with omeprazole. Omeprazole 30 mg daily decreases mean 24 hour intragastric acidity by 97%,²³ an effect that is present both day and night. Omeprazole has been very effective in the treatment of duodenal ulceration and ulcerative oesophagitis that is resistant to standard doses of H₂-receptor antagonists,²⁴ an effect directly related to its profound acid suppression.²⁵ Omeprazole has been shown to increase the N-nitrosamine concentration of the gastric juice after two weeks treatment in healthy volunteers.²⁶ Patients treated with this drug may therefore be a valid model in which to test the hypothesis.

Methods

Patients presenting for endoscopic investigation of their dyspepsia were identified as being infected with *H pylori* and requiring omeprazole treatment, because of either failure to respond to treatment with H₂-receptor antagonists or because of aggressive duodenal ulceration or reflux oesophagitis. Patients taking

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Helicobacter pylori is a microaerophilic bacterium frequently found in the gastric antrum of both asymptomatic subjects and those with peptic ulcer disease.^{1–2} Recent work has shown that ³⁵S-methionine-labelled protein sodium dodecyl sulphate-polyacrylamide acrylamide gel electrophoresis (SDS-PAGE), immunoblot and DNA fingerprinting based on DNA digest patterns can all be used to identify *H pylori*.³ However, ribopatterns provide a simpler, reproducible and sensitive method of discriminating between isolates of *H pylori*.^{4–7} A high

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bismuth, antibiotics, or omeprazole within the previous six weeks were excluded. From these patients, biopsy specimens for culture of *H. pylori* were taken from at least two different sites in the antrum. Follow up biopsy specimens were taken one week after completing a six to eight week course of omeprazole 20 or 40 mg daily.

H. pylori CULTURE

The biopsy specimens for culture were placed directly in a selective enrichment medium. This consisted of Brucella broth (Difco Laboratories, East Molesey, Surrey, England) supplemented with 10% fetal calf serum, 1% (v/v) Isovitalex (BBL Microbiology Systems, Becton Dickinson, Cowley, Oxford), polymyxin B 1000 Units/ml, vancomycin 10 µg/ml, and amphotericin B 2 µg/ml.^{26 27} Specimens were transported to the microbiology laboratory within three hours. Each biopsy specimen was then placed in 5 ml of selective enrichment medium in a 100 ml conical flask. The flasks were incubated at 37°C on a gyratory platform in a variable atmosphere incubator (Don Whitley Scientific Ltd, Shipley, Yorks), under microaerobic conditions (5% oxygen, 5% carbon dioxide, 2% hydrogen, 88% nitrogen). A sample from each flask was subcultured on to Oxoid brain-heart infusion agar, supplemented with 5% horse blood and 1% Isovitalex, after 48 hours. Positive growth was identified by Gram stain and production of urease, and cultures were preserved at -196°C on glass beads in Oxoid nutrient broth No 2 (Oxoid, Basingstoke) containing 10% (v/v) glycerol. Seven patients had at least one positive culture from before and after treatment, and these isolates were analysed using their ribopatterns.

DNA ISOLATION, DIGESTION AND ELECTROPHORESIS, VACUBLOTTING AND rRNA GENE HYBRIDISATION
Chromosomal DNA samples from isolates of

DNA band size windows used for numerical analysis of *Hae* III ribopatterns

Band window (locus)	Size range (kilobases)	Number and percentage of strains with ribopatterns containing the band
1	1.19-1.30	11 (42)
2	1.31-1.55	17 (65)
3	1.56-1.70	6 (23)
4	1.71-1.82	19 (73)
5	1.83-2.01	16 (62)
6	2.02-2.18	7 (27)
7	2.19-2.43	17 (65)
8	2.44-2.69	19 (73)
9	2.70-2.81	6 (23)
10	2.82-3.10	11 (42)
11	3.11-3.38	8 (31)
12	3.39-3.59	15 (58)
13	3.60-3.78	2 (8)
14	3.79-4.01	0 (0)
15	4.02-4.38	20 (77)
16	4.39-4.80	11 (42)
17	4.81-5.80	7 (27)
18	5.81-6.50	11 (42)
19	6.51-7.50	6 (23)
20	7.51-8.99	11 (42)
21	9.00-9.99	8 (31)

H. pylori were prepared using the guanidium thiocyanate reagent method²⁸ and were incubated with the restriction enzyme, *Hae* III, to produce DNA restriction digestion patterns. Twenty six isolates were obtained from the culture of biopsy specimens from the antrum, body and duodenum of the patients in this study, and NCTC 11637 (National Collection of Type Cultures strain) was also included as a reference on all gels. DNA samples (5 µg) were digested for four hours at 37°C and the digested DNA was then electrophoresed at 30V for 16 hours in a horizontal 0.8% (w/v) agarose gel. After electrophoresis the gels were stained with ethidium bromide and photographed for a permanent record.

Agarose gel electrophoresis of all isolates was followed by transfer of the digested DNA on to nylon membranes (Hybond-N, Amersham International, Amersham, Bucks) by means of vacublotting (Vacu-Gene XL, Pharmacia LKB Biotechnology, Uppsala, Sweden). A biotinylated cDNA probe was prepared from *H. pylori* NCTC 11638 16S and 23S rRNA using Moloney mouse leukaemia virus reverse transcriptase (Gibco-BRL, Paisley, Scotland). Biotinylation was achieved by the incorporation of biotin-16-dUTP. The membranes were then hybridised by standard procedures for 16 hours at 42°C using the biotinylated cDNA probe.^{5 29} The membranes were washed and the hybridised probe detected colorimetrically using a nonradioactive detection kit - Blu-GENE (Gibco-BRL).

BAND SIZE ESTIMATION AND COMPUTATION OF STRAIN SIMILARITIES

DNA band sizes in the Southern blot hybridisation patterns were calculated from migration distances using an automated gel reader and analysis system (IBI, New Haven, Connecticut, USA). Biotinylated λ phage (Gibco-BRL) digested with *Hind* III was used to provide the size markers.

To compare the ribopatterns from all the isolates in the study, the bands were coded according to size to minimise errors when determining similarities between the ribopat-

Figure 1 The rRNA gene patterns (ribopatterns) for *Hae* III digests of chromosomal DNA from multiple isolates of *H. pylori* from cases 1-4. DNA digests were probed with biotinylated cDNA from *H. pylori* NCTC 11638. Isolates are labelled to indicate whether they were obtained before (B) or after (A) treatment with omeprazole. Sizes indicated are for bacteriophage λ *Hind* III digest and *H. pylori* NCTC 11637 (type strain) was included as a reference.

Figure 2 The rRNA gene patterns (ribopatterns) for the *Hae* III digests of chromosomal DNA from multiple isolates of *H. pylori* from cases 5–7. DNA digests were probed with biotinylated cDNA from *H. pylori* NCTC 11638. Isolates are labelled to indicate whether they were obtained before (B) or after (A) treatment with omeprazole. Sizes indicated are for bacteriophage λ Hind III digest and *H. pylori* NCTC 11637 (type strain) was included as a reference.

Figure 3 Dendrogram of the cluster analysis to show similarities based on the *Hae* III ribopatterns of *H. pylori* isolates from cases 1–7, and the type strain NCTC 11637. The numbers on the horizontal axis indicate the percentage similarities as determined by the Dice correlation coefficient.

terns by computer assisted methods of analysis. The patterns were screened for bands within 21 different size ranges up to 10 kilobase pairs (table) that were defined by visual inspection, and positive (presence) and negative (absence) results were recorded for each patient isolate. Bands of very faint intensity were excluded and 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 a dendrogram plotted.³⁰ *H. pylori* NCTC 11637 was included as a reference on each gel.

Results

A total of 26 isolates were obtained from the seven patients; each patient had at least one positive culture before and after treatment. The patients had a median age of 53 years (range 31–71 years); all patients were male. At endoscopy, six of the seven patients had an active duodenal ulcer, one patient had duodenitis and reflux oesophagitis. Three patients had been taking low dose H_2 -receptor antagonists prior to the study.

DNA from all the isolates was cut by *Hae* III, which was the enzyme that gave the best resolved hybridisation patterns for analysis. DNA types were defined on the basis of clearly distinct DNA fingerprints with multiple band differences, whereas DNA subtypes were defined where only one or two bands differed between the ribopatterns of different isolates, and the percentage similarity was at least 90%. Four patients had isolates with identical ribopatterns from all their biopsy specimens (fig 1, cases 1 and 3; fig 2, cases 5 and 7). Two patients had evidence of subtypic variation in the pretreatment biopsy specimens and this variation was also evident after treatment (fig 1, cases 2 and 4), and one patient had two different strain types which were present both before and after treatment with omeprazole (fig 2, case 6).

The percentage similarities among all the isolates in the study, based on their ribopatterns, are shown graphically by means of a dendrogram (fig 3). The numbers on the horizontal axis indicate the percentage similarities as determined by the Dice correlation coefficient. Isolate sets from cases 1, 3, 5 and 7 all group at 100% similarity because the isolates in each set share the same ribopattern. Isolates from cases 2 and 4 are seen to group at 90%, demonstrating subtypic variation in their ribopatterns. Isolates from case 6, however, group at just over 50%, showing clear evidence of two different DNA types. Similarities among patients were much lower than similarities within patient sets, at 65% or less.

Discussion

This study does not support the hypothesis that achlorhydria can induce changes in the *H. pylori* genome. Omeprazole treatment has a suppressive effect against *H. pylori*; the bacterium is present in lower numbers and is

difficult to culture while treatment is maintained.^{31 32} This effect may be because of a direct bactericidal action, or may be a direct consequence of the profound acid suppression due to bacterial overgrowth changing the microflora and ecological niche of the bacterium.²⁶ Omeprazole treatment may not be an ideal model for the transient achlorhydria of acute *H pylori* infection, because of the drug's possible direct action on the bacterium. Nevertheless, no genomic change was observed after treatment with omeprazole in the present study.

This study does confirm that each unrelated person is infected with a unique strain, or occasionally, strains, of *H pylori*. Majewski and Goodwin,¹⁰ Langenberg *et al*⁸ and Owen *et al*⁴ have also shown unique patterns in each subject of the groups that they examined. Two out of seven patients showed subtypic variation among their isolates, but one patient harboured different strain types of the bacterium. Other studies have also shown that infection with two or more strain types can occur occasionally in a single patient. Prewett *et al* showed that two of 15 patients were colonised by two different *H pylori* types²⁰ and Beji *et al* described one patient who had three different *H pylori* DNA patterns from organisms cultured from three biopsy specimens at one endoscopy.³³

This study also shows that *H pylori* isolated from an individual subject at different times—for example, before and after treatment with omeprazole—usually have the same DNA type.^{4 10 11} The minor subtypic DNA differences that were seen in *H pylori* isolated from two patients were also stable with time and treatment. DNA digest patterns of *H pylori* cultures maintained in a laboratory are highly stable. This stability, together with the identical ribotypes obtained before and after treatment, suggests that the subtypic differences are genuine minor genomic variations, possibly representing stable point mutations or inversions.

This study supports the evidence from other studies^{4 7} that two or more DNA subtypes—or indeed types, as in the case of case 6—can coexist in the gastric mucosa of a patient at any one time. The reason for the pronounced genomic heterogeneity of *H pylori* remains unclear, but it is unlikely that it is caused by the bacterium's ability to induce transient intragastric hypoacidity.

Ms Doris Elliott typed the manuscript.

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