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Ribotyping Patterns and Emergence of Metronidazole Resistance in Paired Clinical Samples of *Helicobacter pylori*

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Metronidazole-susceptible pretreatment isolates and metronidazole-resistant posttreatment isolates of *Helicobacter pylori* from 11 patients before and after unsuccessful triple therapy consisting of metronidazole, amoxicillin, and colloidal bismuth subcitrate were studied. Ribotyping (rRNA gene restriction pattern analysis) of the isolates demonstrated that all patients except one had identical digest patterns for pre- and posttreatment isolates.

Helicobacter pylori is known to cause active chronic gastritis (9, 12), and its association with peptic ulcer disease has also been confirmed (16). *H. pylori* infection is chronic and persists for years unless treated with antimicrobial agents. So far the most successful treatment, with eradication rates of over 90%, has been achieved with triple therapy consisting of amoxicillin or tetracycline, metronidazole, and a bismuth compound (4, 17). Metronidazole resistance plays an important role in treatment failures in *H. pylori* infections, as *H. pylori* can be eradicated significantly more often from patients with metronidazole-susceptible isolates and posttreatment isolates of *H. pylori* in nonresponding patients are resistant to metronidazole (15).

The present study was undertaken to determine whether *H. pylori*-positive patients not responding to triple therapy consisting of amoxicillin, metronidazole, and bismuth subcitrate were reinfected with novel metronidazole-resistant isolates or whether a metronidazole-resistant population was selected from the original infecting isolates. rRNA gene restriction pattern analysis (ribotyping), which has been used successfully elsewhere to identify *H. pylori* strains (11, 20), was chosen to compare pre- and posttreatment isolates from 11 such patients.

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***H. pylori* isolates.** Pre- and posttreatment isolates of *H. pylori* were cultivated from 11 patients before and after unsuccessful triple therapy consisting of colloidal bismuth subcitrate, amoxicillin, and metronidazole for 2 weeks (17). The 11 patients, 7 of whom had peptic ulcer disease, were selected on the basis of having metronidazole-susceptible pretherapy isolates and metronidazole-resistant posttherapy isolates. All the patients were symptomatic before and after the unsuccessful therapy. Relevant information, including susceptibility of the isolates to metronidazole and date of specimen collection, is given in Table 1. The recurrence of *H. pylori* infection was verified (as the primary infection) on the basis of culturing and histological examination of gastric biopsy specimens and examination of serum antibodies. The *H. pylori* isolates were identified on the basis of colony appearance, Gram staining, and positive reactions in biochemical tests (catalase, oxidase, and urease) and were preserved at −70°C prior to use. The MICs of metronidazole for the isolates were determined by the agar dilution method (15). Consecutive isolates from the same patient were tested in parallel. After therapy failure, metronidazole resistance (MIC, ≥32 mg/liter) was demonstrated for all posttreatment isolates. As a control, *H. pylori* NCTC 11637 was included in the study.

Ribotyping method. Chromosomal DNA was isolated from *H. pylori* isolates grown in 6% sheep blood agar for 7 days at 37°C under microaerobic conditions. Extraction and purification of chromosomal DNA were performed as described previously (20). The concentration and purity of DNA samples were estimated by comparing the electrophoretic patterns with those displayed by a standard DNA. DNA samples (2 μg) were digested overnight at 37°C with restriction endonucleases *Hin*dIII and *Dra*I under conditions recommended by the supplier (Boehringer GmbH, Mannheim, Germany). Restriction endonuclease *Hae*III was not used in this study because previous studies (14, 20) showed that a considerable proportion of *H. pylori* isolates were not cleaved by this enzyme into detectable fragments. Southern blot hybridization was carried out under the same conditions and by the same procedures as those described previously (20). Plasmid pKK 3535, kindly provided by Altwegg and collegues (1), was digested with restriction endonuclease *Pst*I and labelled with digoxigenin by use of a nonradioactive DNA labelling and detection kit from Boehringer according to the manufacturer's instructions. This plasmid DNA probe encoded 5S RNA, 16S RNA, 23S RNA, and $tRNA^{Glu2}$ genes. The pattern of bands obtained after hybridization was designated the ribopattern.

The digest patterns obtained after Southern hybridization with rRNA probe pKK 3535 are shown in Fig. 1, 2, and 3. Ribopatterns obtained from *Hin*dIII digestion consisted of 2 to 10 bands ranging from 1 to 20 kbp, as shown in Fig. 1, while only 2 to 3 bands ranging from 1 to 6 kbp were observed when restriction endonuclease *Dra*I was used for digestion (Fig. 2). Both restriction enzymes appeared to be highly discriminatory in differentiating *H. pylori* isolates, producing distinct ribopatterns for the 11 patients, thus revealing the high degree of heterogeneity among *H. pylori* isolates from individual patients.

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TABLE 1. Pre- and posttreatment isolates of *H. pylori* cultivated from patients who had recurrent infections after triple therapy

Patient	Isolate	Metronidazole susceptibility[a]	Date of specimen collection (day.mo.yr)
1	277/90	S	19.2.90
	633/90	R	7.5.90
	1151/90	S	12.11.90
2	105/88	S	28.3.88
	1314/89	R	28.12.89
3	604/87	S	29.7.87
	511/89	R	20.6.89
4	1080/91	S	24.9.91
	1443/91	R	17.12.91
5	446/90	S	20.3.90
	857/90	R	31.7.90
6	1439/91	S	16.12.91
	279/92	R	6.3.92
7	1037/90	S	10.10.90
	1244/91	R	4.11.91
8	170/90	S	25.1.90
	708/90	R	31.5.90
9	1195/90	S	21.11.90
	1294/91	R	11.11.91
10	386/90	S	8.3.90
	635/90	R	7.5.90
11	1034/90	S	10.10.90
	1417/91	R	10.12.91

[a] S, susceptible; R, resistant.

Analysis of the ribopatterns of the 23 isolates demonstrated that all patients except one had identical digest patterns for pre- and posttreatment isolates, suggesting that the development of metronidazole resistance may have been an important factor in the recurrence of *H. pylori* infections in these patients. Similar results for 20 patients have been reported by others (10, 13).

One patient in our study (patient 1) had three isolates submitted for ribotyping. The third isolate of *H. pylori*, recovered from antral biopsies taken 9 months later than the first ones, was found to be susceptible to metronidazole. All three isolates from this patient had similar ribopatterns after *Hin*dIII and *Dra*I digestion, although a minor band variation was observed in the second isolate. This result may suggest that mixed populations (both susceptible and resistant to metronidazole) of the same isolate coexisted in this particular patient during this period but that only one isolate was selected from cultures for ribotyping. The band variation observed in the second isolate (*Hin*dIII digest; Fig. 1) may have represented a genomic variant of the original isolate or may have been a result of cross-contamination during culturing. Similar results were observed for the pre- and posttreatment isolates (*Dra*I digest; Fig. 2) from patient 4. Another patient (patient 10) had two distinct pre- and posttreatment ribopatterns (lanes 5 and 6 in Fig. 3), indicating that this patient had probably been reinfected with a novel isolate of metronidazole-resistant *H. pylori*. It is also possible that more than one isolate was present in his stomach mucosa. The existence of several original isolates of *H. pylori* infecting a single person has been demonstrated (2, 8, 20). Some of these multiple infecting isolates may be missed when a single colony is used for purification during subculturing. To avoid this problem, more than one colony from the primary culture should be examined. However, this practice may prove to be too time-consuming and costly as a routine procedure when a large sample of patients are involved in a study.

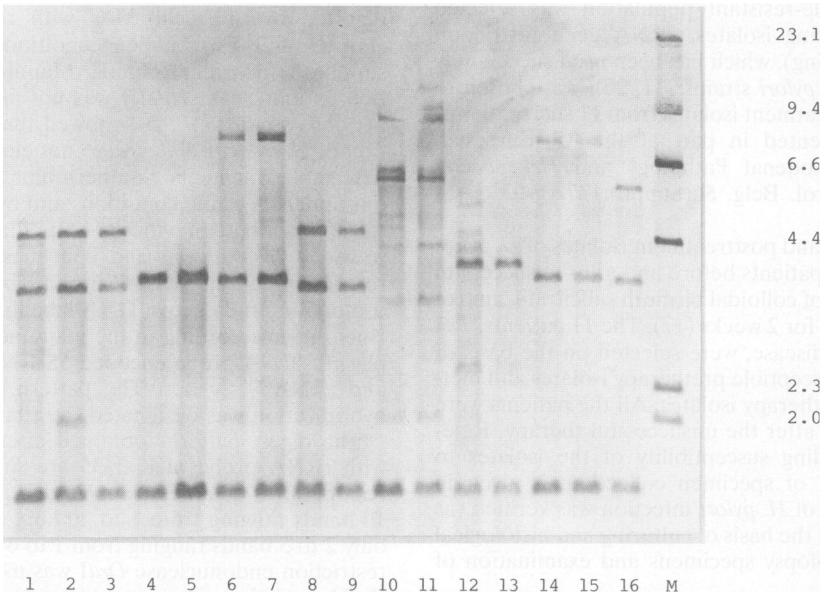

FIG. 1. rRNA gene restriction patterns (*Hin*dIII digests) of *H. pylori* DNA from pre- and posttreatment isolates recovered from patients treated with triple therapy. Lanes (respectively): 1 to 3, patient 1 isolates 277/90, 633/90, and 1151/90; 4 and 5, patient 2 isolates 105/88 and 1314/89; 6 and 7, patient 3 isolates 604/87 and 511/89; 8 and 9, patient 4 isolates 1080/91 and 1443/91; 10 and 11, patient 5 isolates 446/90 and 857/90; 12 and 13, patient 6 isolates 1439/91 and 279/92; 14 and 15, patient 7 isolates 1037/90 and 1244/91; 16, *H. pylori* NCTC 11637; M, molecular size markers (in kilobase pairs).

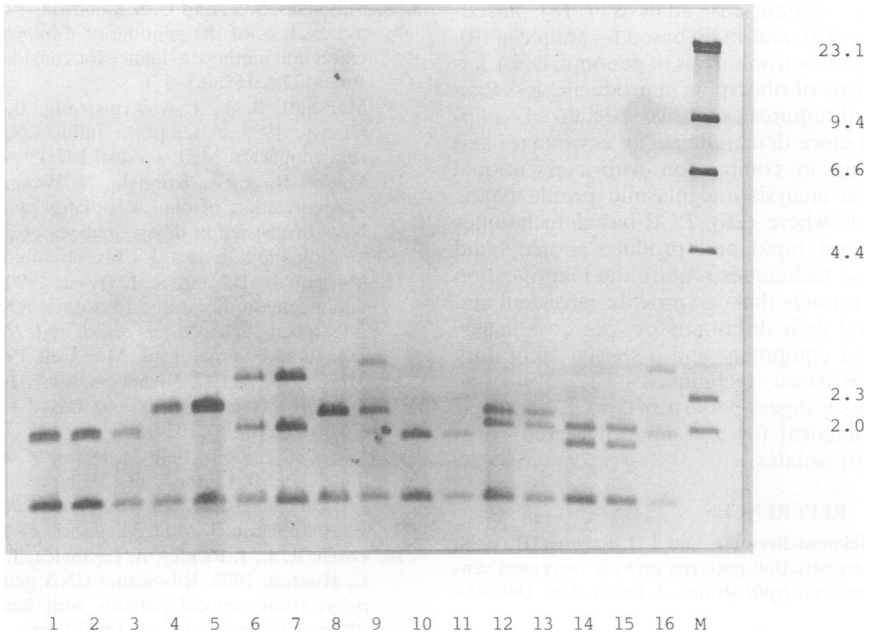

FIG. 2. rRNA gene restriction patterns (*Dra*I digests) of *H. pylori* DNA from pre- and posttreatment isolates recovered from patients treated with triple therapy. Lanes are as in the legend to Fig. 1.

Not only have metronidazole-resistant *H. pylori* strains been isolated before treatment of such infections, but also the rapid development of resistance has been demonstrated in vitro (6) and in vivo (5). It has been suggested that the development of resistance to metronidazole in *H. pylori* during treatment may be the reason for the failure to eradicate the organism (15). The precise identification of strains by ribotyping has made it possible to differentiate between a reinfection with a new strain and recrudescence due to the original strain of *H. pylori* that has developed drug resistance. This study demonstrated that ribotyping is a sensitive and discriminatory typing method for *H. pylori* strains and has a useful role in clinical applications for monitoring treatment regimens, so that more effective intervention procedures can be introduced.

Several other molecular typing techniques were recently developed for *H. pylori* isolates; these include plasmid profile

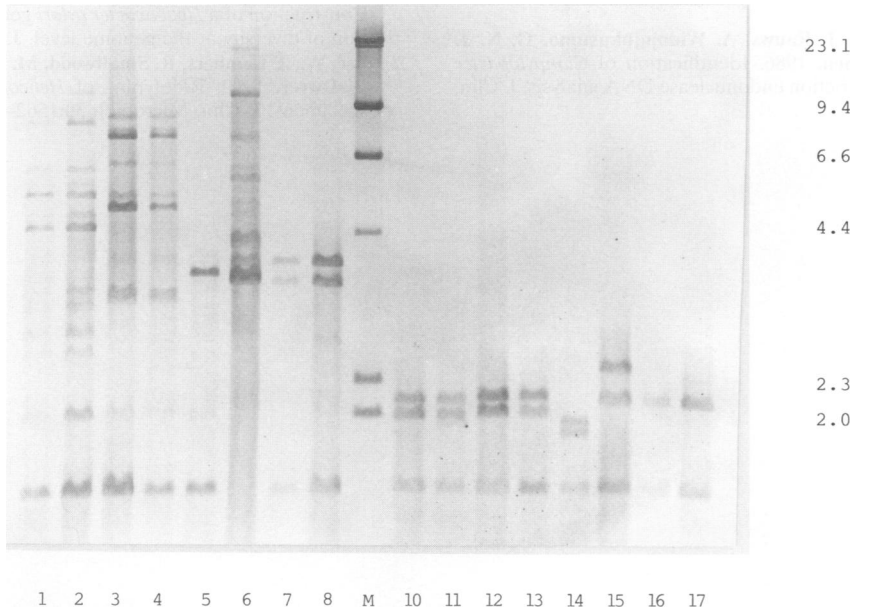

FIG. 3. rRNA gene restriction patterns (*Hin*dIII [lanes 1 to 8] and *Dra*I [lanes 10 to 17] digests) of *H. pylori* DNA from pre- and posttreatment isolates recovered from patients treated with triple therapy. Lanes (respectively): 1 and 2, patient 8 isolates 170/90 and 708/90; 3 and 4, patient 9 isolates 1195/90 and 1294/91; 5 and 6, patient 10 isolates 635/90 and 386/90; 7 and 8, patient 11 isolates 1417/91 and 1034/90; M, molecular size markers (in kilobase pairs). Lanes 10 to 17 correspond to lanes 1 to 8.

typing (3, 18), restriction endonuclease analysis (7, 18), pulsed-field gel electrophoresis (19), and PCR-based techniques (10). Although pulsed-field gel electrophoresis of genomic DNA has advantages similar to those of ribotyping in producing less than 30 well-resolved bands, it requires expensive specialized equipment and is technically more demanding. The advantages and limitations of ribotyping in comparison with conventional restriction endonuclease analysis and plasmid profile typing have been discussed elsewhere (20). PCR-based techniques appear to be simple and rapid and produce simple band patterns. However, these techniques require the identification of a suitable primer or primers that can provide consistent and reproducible results, and their discriminatory power is uncertain. In addition, special equipment and a special laboratory are required for PCR-based techniques. The ribotyping method, which produces a digest pattern of 2 to 10 bands, is thus convenient and practical for a comparative study of a large sample of *H. pylori* isolates.

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