Indian Journal of Medical Microbiology, (2003) 21 (3):166-171

Original Article

GENETIC FINE STRUCTURE ANALYSIS OF *HELICOBACTER PYLORI* ISOLATES BEFORE AND AFTER TREATMENT

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Abstract

Background: Eradication of *H. pylori* infection cures peptic ulcer disease and conversely, relapse is associated with reappearance of *H. pylori* infection. However, it is not clear whether the recurrence of ulcers following *H. pylori* eradication is due to recrudescence (identical strain) of the previous infection or as a result of exogenous reinfection (different strain) by another strain. The aim of the present study was to analyze the FAFLP patterns of pre and post treatment *H. pylori* samples to check if the recurrence was due to recrudescence or reinfection. **Materials and Methods:** 24 of 30 duodenal ulcer (DU) subjects screened for *H. pylori* infection were positive for *H. pylori* infection. The treatment regime included pantoprazole, ciprofloxacin and amoxicillin. The patients were called for a repeat endoscopy after one month and screened for *H. pylori* infection. FAFLP analysis and PCR for the cagA and vacA gene was performed for the pre and post treatment samples. **Results:** Of the 24 positive *H.pylori* patients, only 6 were negative after treatment and the remaining 18 were positive for *H.pylori* infection. The analysis of the pre and post treatment samples of the 18 patients showed that the FAFLP profiles of the initial and follow-up pools were similar to one another. **Conclusion:** It can be concluded that in the present series of patients, reinfection was due to recrudescence of infection due to incomplete eradication. The study also suggests that DNA fingerprinting by FAFLP provides discriminatory and complementary data for identifying strains of *H. pylori* while monitoring therapy.

Key words: *H. pylori, FAFLP, cagA, vacA*

Helicobacter pylori is considered to be an important human pathogen causing gastritis and duodenal ulceration (DU).[1,2] Many studies have shown that approximately 80% of the DU patients are infected with *H. pylori* and the bacterial pathogenic factors such as cytotoxin associated gene A (cagA) and the vacuolating cytotoxin gene (vacA) were significantly associated with DU.[3-5] According to Xia and colleagues eradication of *H. pylori* infection cures peptic ulcer disease and relapses of peptic ulcer disease are associated with reappearance of *H. pylori* infection.[6] Data on reappearance of *H. pylori* infection following successful eradication are conflicting and vary from 0 to 47%[7,8] and ulcer relapse rates up to 11.7% have been reported.[9,10] Relapse of gastroduodenal disease occurs in about 80% of cases and is associated with recrudescence of infection.[11] However, it is not clear whether the recurrence of ulcers following *H. pylori* eradication therapy is due to recrudescence of the previous infection or due to exogenous reinfection. A sensitive and feasible genotypic analysis of patient isolates is often required to distinguish different strains associated with mixed infections, relapse, microevolution and quasi species development.[12]

Assessment of *H. pylori* infection, too early after eradication therapy, may result in over estimation of cure rates and a high reappearance rate. A four weeks treatment period is generally accepted as most of the recrudescent infections occur during this period. Despite assessment of cure, four weeks after cessation of therapy, there are reports suggesting a reappearing infection rate of 18.9% (range, 9.2%-47.1%).[6]

Accurate and sensitive genomic fingerprinting techniques are required to help determine if the most common cause of treatment failure is due to the acquisition of antibiotic resistance by the organism or through reinfection with a new strain.[13] In recent years the range of molecular techniques available for epidemiological fingerprinting has expanded, and there are now many genotypic methods that allow high levels of discrimination between bacterial strains. Various typing methods, including ribotyping, restriction digests of genomic DNA [restriction enzyme analysis (REA)],

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Received: 21-10-2002
Accepted: 28-05-2003

random amplified polymorphic DNA (RAPD) analysis, and restriction of PCR-amplified gene segments have documented great diversity among individual *H. pylori* strains.[14,15]

In our study, we have modified and evaluated a PCR-based technique, amplified-fragment length polymorphism (AFLP) analysis, for use in fingerprinting strains of *H. pylori*. The method was originally developed for the typing of crop plants (European patent application Zabeau and Vos, 1993) and has been applied to the typing of plant, animal and prokaryotic DNAs.[16] The aim of this study was to compare the FAFLP patterns of pre and post treatment *H.pylori* samples by Fluorescent Amplified Fragment Length Polymorphism (FAFLP) analysis to check if the recurrence was due to recrudescence or reinfection.

Materials and Methods

Bacterial Isolates

H. pylori were isolated from gastric biopsies obtained after informed consents from patients of both sexes who had undergone upper gastrointestinal endoscopy. A total of 50 dyspepsia patients were subjected to upper gastrointestinal endoscopy (UGIE). Bacterial growth was observed on brain heart infusion (BHI) agar under microaerophilic conditions in case of only 24(80%) patient biopsies. *H. pylori* colonies were identified based on the colony morphology, Gram staining, positive urease test and subsequent gene specific PCR tests. The 24 patients who were confirmed as having *H. pylori* were given a therapeutic dose of pantoprazole 40 mg, ciprofloxacin 500 mg and amoxicillin 500 mg each twice daily. This course was administered for one week followed by pantoprazole once daily for 3 weeks. The patients were called for a repeat endoscopy after one month. Biopsies were collected again during the repeat UGIE and were screened for *H. pylori*.

DNA Isolation

Bacteria harvested after 24 hours of incubation were washed briefly in STE buffer (0.1M NaCl, 10mM Tris-HCl, 1mM EDTA). The pellet was incubated with lysozyme (5mg/mL) for 15 minutes at room temperature. 100mL of 1% SDS and 10mL of 25 mg/mL proteinase K was then added to the reaction mixture and incubated overnight at 37°C. The samples were treated with phenol chloroform and precipitated in absolute ethanol. The resulting pellet was washed twice with 70% ethanol and suspended in TE buffer (10:1). The DNA was stored at -20°C until further use.

Genotyping of cagA and vacA

The cagA and vacA status of all the isolates was determined by PCR by using primers as mentioned in the table 1. The PCR products were analyzed by agarose gel electrophoresis.

Table 1 : Primers used in the study

Primer region	Primer sequences	Product Size
cagA[25]	Forward 5' - GGA AAT CTT TAA TCT CAG TTC GG 3'	349bp
	Reverse 5' - GGC AAT GGT GGT CCT GGA GCT AGG C 3'	
vacA mid region[3]	m1 VA3-F 5' GGT CAA AAT GCG GTC ATG G 3'	290bp
	VA3-R 5' CCA TTG GTA CCT GTA GAA AC 3'	
	m2 VA4-F 5' GGA GCC CCA GGA AAC ATT G 3'	352bp
	VA4-R 5' CAT AAC TAG CGC CTT GCA C 3'	
vacA signal sequence[3]	s1/s2[a] VA1-F 5' ATG GAA ATA CAA CAA ACA CAC 3'	259/286bp
	VA1-R 5' CTG CTT GAA TGC GCC AAA C 3'	

[a] vacA types s1 and s2 are differentiated on the basis of differences in size of the PCR product

FAFLP PCR

FAFLP analysis mainly included simultaneous restriction ligation with EcoRI/MseI -T4 DNA ligase enzymes followed by preselective and selective PCR. This was carried out exactly as described earlier.[17]

Gel electrophoresis and data collection

FAFLP products with formamide loading dye (1.5-2□L final volume) were loaded on to an ABI Prism 377 XL-96 DNA sequencer along with a red colored internal lane standard GS-500 Rox (PE Biosysytems). Fragment

separation was continued for 5 hours on a 5% denaturing polyacrylamide gel. Fragments were detected and compiled by the ABI Data Collection™ (PE Biosystems) software. Gel images were generated and all the lanes were extracted for making individual electropherograms. Fragment analysis was performed with the Genescan Analysis™ 3.1 package (PE Biosystems). Individual sample files were then exported to the Genotyper™ 2.5 software for computer assisted genotyping.

Gel analysis and Genotyping

Based on the presence or absence of monomorphic and polymorphic bands/ peaks, different FAFLP profiles were identified as 'amplitypes'.

Results

FAFLP gel data consisted of precisely amplified fragments in the size range of 50 to 500 bp. The two dimensional gel images (Figure) were transformed with Genescan™ 3.1 software (ABI) into electropherograms; these were color coded, overlaid, and examined for polymorphisms. For every single lane representing the whole genome fingerprint of the corresponding isolate, FAFLP data were found to be highly digitized, precise and reproducible.

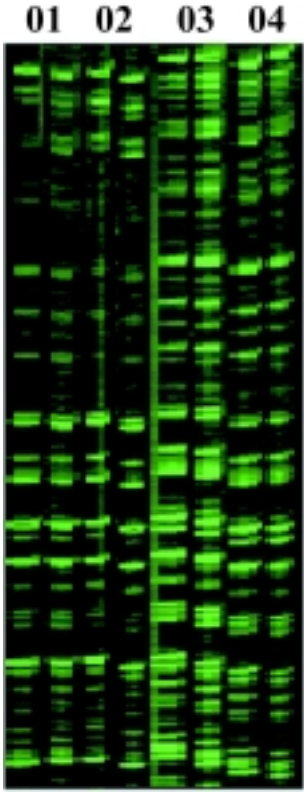

Figure : Genotypic fingerprints of repeat isolates of *H.pylori* from 4 different patients. Flourescent -labelled AFLP profiles were scanned and digitized and a high level of similarity was observed for each patient. Lane 01, 02, 03, 04 represent the pre and post treatment isolates of 4 patients respectively.

Table 2 : Characteristics of patients sampled by endoscopy at 1 month interval and genotypes of *Helicobacter pylori* pools cultured from each sample

Patient S. No	Sex/age (years)	Genotype cagA	Genotype vacA	*H. pylori* status after treatment
1A	M/23	+	S1/M1	+
1B		+	S1/M1	
2A	M/22	+	S1/M1	+
2B		+	S1/M1	
3A	F/45	+	S1/M1	+
3B		+	S1/M1	
4A	M/50	+	S1/M1	+
4B		+	S1/M1	
5A	M/65	+	S1/M1	+
5B		+	S1/M1	
6A	M/40	+	S1/M1	+
6B		+	S1/M1	
7A	M/39	-	S1/M2	
7B		-		-
8A	F/60	+	S1/M1	+
8B		+	S1/M1	
9A	M/36	+	S1/M2	
9B		+	S1/M2	+
10A	F/22	+	S1/M1	-
10B		-	-	
11A	M/45	-	S1/M2	+
11B		-	S1/M2	
12A	F/34	+	S1/M1	+
12B		+	S1/M1	
13A	M/42	+	S1/M1	-
13B		-	-	
14A	M/55	+	S1/M1	+
14B		+	S1/M1	
15A	F/39	+	S1/M2	+
15B		+	S1/M2	
16A	M/36	+	S1/M1	-
16B		-	-	
17A	M/56	+	S1/M1	+
17B		+	S1/M1	
18A	M/23	-	S1/M2	-
18B		-	-	
19A	F/34	+	S1/M1	+
19B		+	S1/M1	
20A	M/54	-	S1/M1	+
20B		-	S1/M1	
21A	F/43	+	S1/M1	+
21B		+	S1/M1	
22A	M/56	+	S1/M1	+
22B		+	S1/M1	
23A	M/64	+	S1/M1	-
23B		-	-	
24A	M/22	-	S1/M1	+
24B		-	S1/M1	

The initial and followup amplitypes were found to be identical in all the cases and did not differ significantly even in the case of a single locus. All the FAFLP fragments were found to be 100% reproducible and were detected each time under similar electrophoretic conditions with a fluorescent intensity of more than 100 and a size variation of less than 0.5bp. All the PCR runs followed by gel electrophoresis on ABI Prism 377 XL-96 sequencer were repeated 5-6 times to ascertain reproducibility.

Of the 24 patients who were positive for *H.pylori* before treatment, only 6 were negative after treatment and the remaining 18 still had the *H.pylori* infection. All the *H. pylori* isolates were subjected to PCR based genotyping for cagA and vacA status. Table 2 describes the genotypic and phenotypic properties of all the pre- and post-treatment isolates. It is clear from the table that the initial and follow up isolates did not differ with respect to cagA and vacA genotype. These findings indicate that the pools of pairs of the 18 isolates represent the same strain within each patient. The total set of results are summarized in Table 2.

Discussion

The pathogenic role of *H. pylori* in chronic active gastritis and its association with DU is well established.[18] The importance of cagA and vacA in *H. pylori* virulence and duodenal ulceration has been observed in various reports. Effective molecular techniques have been identified for fingerprinting *H. pylori* isolates that allow clear characterisation of paired isolates before and after treatment.[19] For cases where strains with an identical DNA pattern can be detected before eradication and after *H. pylori* reinfection, two principal explanations may be suggested; firstly, a relapse i.e. the organism was not completely eradicated, but only transiently suppressed; secondly, reinfection with the same strain; usually possible by transmission from family members or spouses infected with the identical strain.[20] *H. pylori* can live in human gastric pits, where bactericidal activity of antimicrobial agents might not be effective and hence recolonisation occurs after treatment is completed. Incomplete elimination would most probably occur if the organism inhabited a reservoir other than the stomach.[6]

Assessment of *H. pylori* infection too early after eradication therapy, may result in overestimation of cure rates and a high reappearance rate.[8,10] In the present study, four weeks post treatment *H. pylori* assessment was carried. The paired pools of *H. pylori* isolates obtained one month apart were tested using high resolution genotyping method of FAFLP and the results were correlated with cagA and vacA status. Genotyping of the *H. pylori* strains based on presence or absence of cagA and vacA showed that cagA plus and vacA s1m1 genotypes are significantly associated with DU. These results are consistent with other studies[3,4] and further support the role of these two virulence markers in causing disease manifestations.[5,21]

The FAFLP patterns of isolates of *H. pylori* from antral biopsies of an infected patient before and after treatment were generally more similar to isolates corresponding to the same patient than to any isolate from different individuals undergoing a similar treatment regime. In theory, a repeated analysis of such a strain pool gives the impression of a changing genotype, if the individual strains in the pools vary over time.[22] The utility of this approach was supported by the demonstration of $\geq$99% identity in the specific genotyping results within the 18 pairs in which initial and follow up samples representing the same population. The 18 patterns examined were all receiving the same treatment and were all infected by *H. pylori* with unique genotypes according to their FAFLP patterns. These observations are in concordance with already discussed genomic diversity of *H. pylori* isolates from different individuals worldwide.[22]

These results specifically confirm and complement the data from previous comparisons of DNA profiles of pre treatment and post treatment biopsies from other workers in showing that sequential and multiple isolates of *H.pylori* are generally the same.[14,20] The results are also in accordance with the study of Kuipers *et al*,[22] who observed that patients harboured same strain even after a period of 7 - 10 years as was proven by AFLP and RAPD-PCR analysis.

The observed interpatient heterogeneity of strains reflects the epidemiology and ecology of *H. pylori*.[23] It appears unlikely from our study (in contrast to previous studies on triple therapy[24] and omeprazole[25]) that treatment specifically induces genomic changes in *H. pylori*, although treatment may be a factor in promoting minor (subtype) changes. The observed *H. pylori* strain differences alternatively could be interpreted as reinfection by a different strain type or subtype either from another site in the stomach or from an external source.[24] However, the probability of that within the 6 months follow up period is likely to be low.

Therefore, the first explanation, supporting a relapse, holds good in our study as we can suggest that the treatment regime should change and that the drug did not cause any changes in the genetic make up of isolates within 6 months of treatment. A common

exogenous source cannot be ruled out altogether, but seems rather unlikely. The minor subtypic DNA differences that were seen in *H. pylori* isolated from two patients were also stable with time and treatment. This stability, together with the identical FAFLP patterns obtained before and after treatment, suggests that the subtypic differences are genuinely minor genomic variations, possibly representing stable point mutations or inversions as reported earlier.[25]

We conclude that DNA fingerprinting by FAFLP provides discriminatory and complementary data for identifying strains of *H. pylori* while monitoring therapy. Further more, by its application to *H. pylori* isolates from infected persons, it was possible to define subtype variation in more detail, even though the *H. pylori* genome is highly stable after failed eradication treatment. In the present series of patients, failed therapy confirmed by molecular typing methods is due to recrudescence of infection due to incomplete eradication. The increasing application of these molecular techniques in the future for monitoring strain colonisation should enable clinical gastroenterologists with valuable information for developing efficient therapies for *H. pylori* infection and genotyping can be used as a tool for epidemiological purposes and for understanding the pathogenesis of infection.

Acknowledgements:

This work was carried out by the funds provided from The Department of Biotechnology, Government of India BT/PRO149/MED/09/029/96.

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