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Genotyping of *Helicobacter pylori* Isolates by Random Amplified Polymorphic DNA and Polymerase Chain Reaction-Restriction Fragment Length Polymorphism

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Helicobacter pylori is thought to show strain diversity. The differences in the pathology of *H. pylori* may have to do with this diversity. We saw the use of polymerase chain reaction (PCR) to amplify the ureC in *H. pylori* and the examination of restriction fragment length polymorphism (RFLP) within the ureC region as a means to type clinical isolates of *H. pylori*, where point mutations in the sequence diversity of the ureC gene can be found. Likewise, we saw the use of randomly amplified polymorphic DNA (RAPD) to type genotypic variation in the gene order on physical maps, mosaicism in conserved gene, non-conserved genes and extragenetic elements. In this study, *H. pylor*i genomic DNA was prepared from strains isolated from 22 unrelated patients and examined by PCR-based typing of *H. pylori* isolates using RAPD analysis of genomic DNA and RFLP analysis of ureC gene with HindIII, EcoRI and Sau3A. From the restriction enzyme digestion of an 820-bp fragment from within the ureC gene in H. pylori strains isolated from the 22 patients, we found only one pattern with HindIII or EcoRI, but 6 different patterns with Sau3A. Nine strains (40.9%) were grouped RFLP pattern 1 with Sau3A digestion of ure C region. We found 21 different RAPD patterns of genomic DNA. The *H. pylori* RFLP patterns of ureC gene gave much simpler profiles than those produced by RAPD analysis of gemonic DNA. In summary, *H. pylori* isolates have great genetic diversity among strains, which can be exploited to type isolates. RAPD on the whole bacterial genome can accurately distinguish between isolates. RFLP analysis with Sau3A of the ureC gene also had a good discriminatory power and group isolates into clusters. Comparing the genetic diversity of *H. pylori* isolated from different patients, these methods yield fast and reliable results on the transmission and pathogenicity of *H. pylori*.

Key words

Helicobacter pylori, polymerase chain reaction, restriction fragment length polymorphism, randomly amplified polymorphic DNA

INTRODUCTION

Helicobacter pylori (*H. pylori*) is a microaerophilic gram-negative bacterium and is recognized as the major etiologic agent of chronic gastritis [1-4], which is a risk factor for the development of peptic ulcer disease and adenocarcinoma of the distal stomach [5-9]. *H. pylori* shows strain diversity and the differences in the pathogenicity of *H. pylori* may be a result of this diversity [10-15]. This heterogeneity can be analyzed at two different levels: genotypic variation among

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strains and variations in *H. pylori* populations within an individual host. Genotypic variation includes point mutations in conserved genes, variation in the gene order on physical maps, and mosaicism in conserved gene or non-conserved genes [16]. Several putative virulence factors, including hemagglutinins, flagella, and cytotoxins [17-19], and urease are thought to enable the organism to survive in the gastric mucosa [20]. *H. pylori* produces a highly active urease that catalyzes the hydrolysis of urea and elicits an immune response in patients. Although the urease enzyme in *H. pylori* has highly conserved amino acid sequence among strains, point mutations in the ureC genes were found. The sequence diversity in the gene encoding of this enzyme provides an opportunity to differentiate *H. pylori* isolates [13, 15, 21-22].Molecular epidemiological studies of *H. pylori* isolates have shown that plasmid profiling [23, 24], analysis of chromosomal DNA by either restriction endonuclease analysis or pulsed-field gel electrophoresis, and ribotyping are all highly discriminatory because *H. pylori* isolates from different patients exhibit unique profiles [10,11,13-15]. However, these established genotyping techniques may yield complex fingerprints resulting in interpretation problems, or may be laborious. The polymerase chain reaction (PCR) allows analysis of chromosomal DNA, and some previous studies had also used PCR to amplify the ureC in *H. pylori* and to examine the restriction fragment length polymorphism (RFLP) within the ureC region as a means of typing clinical isolates of *H. pylori* [21, 22], where point mutations can be found in the sequence diversity of the ureC gene.

Arbitrary primer PCR (AP-PCR) or randomly amplified polymorphic DNA (RAPD) analysis uses short oligonucleotide primers of arbitrary sequence to prime in PCR. In this method, DNA synthesis is primed at low stringency, and the template DNA is required only in nanogram quantities. Tests with one or two primers are sufficient to distinguish related from unrelated strain. Studies had found RAPD as an easy, rapid, and highly discriminatory typing method and more applicable for use in clinical laboratories than other typing methods [14, 25] because it can type genotypic variation in the gene order on physical maps, mosaicism in conserved gene, non-conserved genes and extragenetic elements.

In this study, *H. pylori* genomic DNA was prepared from strains isolated from 22 unrelated patients and examined by PCR-based typing of *H. pylori* isolates using RAPD analysis of genomic DNA and RFLP analysis of ureC gene with HindIII, EcoRI and Sau3A. Using these typing methods, we examined the genetic heterogeneity of *H. pylori* isolates and also compared the typing ability of PCR-based RFLP analysis and RAPD analysis between unrelated *H. pylori* isolates.

MICROBIOLOGIC TECHNIQUES

Subjects

Patients requiring endoscopy were eligible to enter this study. All patients were symptomatic, with abdominal pain, vomiting, weight loss, epigastralgia, tarry stool, abdominal fullness, or hematemesis.

Gastrointestinal Endoscopy and Microscopic Examination

All subjects underwent endoscopy and biopsy of the upper gastrointestinal tract. During endoscopy, three biopsy specimens were taken. The biopsy specimen for histology was fixed in Bouin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. A pathologist determined the presence and degree of gastritis and peptic ulcer. The second specimen was smeared onto a glass slide and stained with Gram strain. Slides were examined for characteristic curved, gram-negative bacterial cells. The third biopsy specimen was used for microbiologic culture.

Microbiologic Techniques

Within a maximum of 2 hours after collection, the biopsy specimens were homogenized in 1 ml saline solution, plated onto anaerobic 5% sheep blood agar plates (BBL Microbiology

Table 1 Clinical data of 22 peptic ulcer disease patients with *H. pylori* infection and RFLP and RAPD typing of the the 22 *H. pylori* isolates

No.	Age (y/o)	Sex	Diagnosis*	RFLP†	RAPD‡
1	32	M	GU, duodenitis	1	1
2	65	M	DU scar, GE	2	2
3	61	F	G, GU, DU scar	3	3
4	41	F	G	4	4
5	69	M	G, DU	5	5
6	35	F	G, DU scar	5	5
7	56	F	GU, DU scar	5	5
8	33	M	DU healing	6	7
9	62	M	G, DU healing	1	8
10	29	M	G, DU scar	1	9
11	31	M	G, DU scar	6	10
12	41	M	G, DU healing	4	11
13	36	M	G, DU healing	1	12
14	42	M	G	1	13
15	56	M	DU healing	1	14
16	63	F	G, DU healing	5	15
17	40	F	G, DU scar	5	16
18	37	M	G, DU scar	6	17
19	46	M	G, DU healing	1	18
20	35	M	GU	1	19
21	39	F	G, DU healing	5	20
22	47	M	DU scar	1	21

*G: gastritis; GU: gastric ulcer; GE: gastric erosins; DU: duodenal ulcer. †Sau3A RFLP pattern of ureC PCR-product. ‡RAPD pattern of genomic DNA.

Systems, Cockeyville, Md.), and cultured for 4 to 7 days at 37℃ under microaerobic conditions (Campypak, BBL Microbiology Systems, Cockeysville, Md. U.S.A.). The isolates were identified by Gram stain, colonial morphology, and positive oxdiase, catalase, and urease reactions.

Amplification of the ureC gene of H. pylori by PCR

Genomic DNA was prepared by boiling the frozen cell pellets in 0.5 ml sterile distilled water at 100℃ for 10 minutes. The boiled suspensions were centrifuged at 12,000 rpm for 10 minutes at 4℃. The supernatants were then stored in sterile vials at −70℃ until use. Two oligonucleotide primers, 5'−TGGGACTG ATGGCGTGAGGG−3' and 5' −AAGGGCGTT TTTAGATTTTT−3' , which amplified a 820−bp product from the ureC gene [21].

Amplification reaction mixtures contained 25 ng of bacterial DNA, 200 ng of both oligonucleotide primers, 0.2 mM (each) deoxynucleoside triphosphates, 2.5 mM $MgCl_2$, and 0.2 U SuperTaq DNA polymerase (BBL, Becton Dickinson Microbiology Systems, Cockeysville, Md, U.S.A.). Amplification was performed with temperature ramping as follows: one cycle at 94℃ for 5 minutes, 45℃ for 5 minutes, and 72℃ for 5 minutes, followed by 35 cycles of 94℃ for 1 minutes, 45℃ for 2 minutes, and 72℃ for 2 minutes. In the final cycle, the polymerization step was extended for 30 minutes. Amplified DNA (5 μl) was separated by gel electrophoresis in 2% agarose

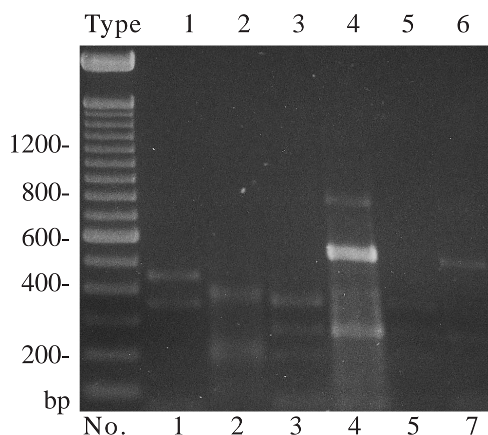

Fig. 1 Types of RFLP from Sau3A digestion of the ure-gene PCR-products of *H. pylori* isolates

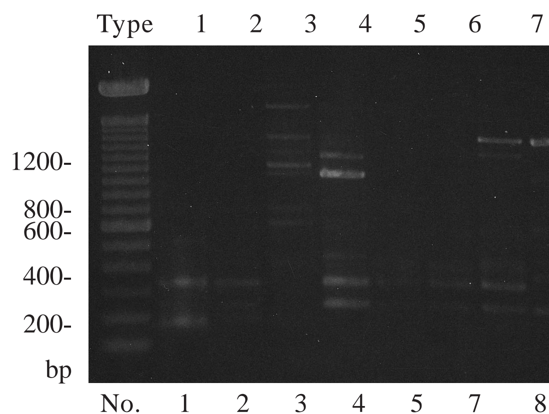

Fig. 2 Types of RAPD from AP-PCR typing of chromosomal DNA of *H. pylori* isolates

and visualized by ethidium bromide staining.

RFLP analysis of ureC

The amplified products obtained by PCR were subjected to restriction endonuclease (HindIII, EcoRI, and Sau3A) digestion for 2 hours at 37℃. The digested samples were analyzed by agarose gel (2.0% ; w/v) electrophoresis.

RAPD analyses of genomic DNA

A 1 μl sample of the genomic DNA was performed for RAPD analysis using the arbitrary primer PJ118 (5'–TGTTCGTGCTGTTTCTG–3'), which gave the well resolution and high discriminatory power [25]. Arbitrary primer polymerase chain reaction was performed using the same cycle profile as described above described PCR.

RESULTS

A total of 22 patients were found to have peptic ulcer disease and *H. pylori* infection. All were unrelated. The clinical data of their endoscopic diagnoses are summarized in Table 1. Gastritis and duodenal ulcer healing was the most common finding (16 patients), followed by gastritis and duodenal ulcer bleeding (3 patients), and duodenal ulcer scar with gastric erosion (2 patients).

PCR with ureC primers amplified an 820-bp fragment from within the ureC gene in all H. pylori strains isolated from the 22 patients (**Table 1**). From the restriction enzyme digestion of these PCR products, we found only one RFLP pattern with HindIII or EcoRI, but 6 different patterns with Sau3A (**Fig. 1**). Nine strains (40.9%) were grouped as RFLP pattern 1 with Sau3A digestion of the ureC region. RAPD typing of genomic DNA of the 22 H. pylori isolates yielded 21 different RAPD patterns with primer PJ118 (**Fig. 2**). Patients 5 and 6 had identical patterns on RAPD patterns of genomic DNA and Sau3A RFLP patterns of ureC-amplified segment. These isolates from 22 unrelated patients were classified on the basis of Sau3A RFLP patterns and RAPD patterns of genomic DNA, and divided into 21 distinct groups (**Table 1**). We found that the pathogenicity of the strains was related to the RFLP of the ureC PCR-amplified product. With regard to incidences by type, patients infected with RFLP pattern 5 exhibited the highest rate of gastritis and duodenal ulcer.

DISCUSSION

The heterogeneity of genomic DNA of *H. pylori* could be of considerable value in epidemiological studies [10, 11, 13–15, 23, 24]. RAPD analysis of genomic DNA and PCR-based RFLP analysis have been widely used in the investigation of the spread of *H. pylori*

within institutions or between family members [14, 21, 22, 25]. In this study, we confirmed the evidence for the considerable genomic diversity of *H. pylori* by RAPD analysis and PCR-based RFLP analysis techniques. However, some studies also reported that the RAPD analysis of genomic DNA in particular does not appear to be suited for tracking epidemiological relatedness between species due to its inability to discriminate between artificial variation and true polymorphism [26].

In our study, both RAPD and RFLP analysis had good discriminatory power. RAPD analysis yielded distinct patterns when genomic DNA was the template. While RFLP analysis with HindIII or EcoRI both yielded one pattern, digestion with Sau3A gave 6 distinct RFLP patterns, which indicates a slightly better discriminatory power than RAPD analysis of genomic DNA in the unrelated isolates. The RAPD typing of genomic DNA and RFLP typing of PCR-amplified ureC gene from the 22 strains showed sequence divergence. With regard to incidences by type, patients infected with RFLP pattern 5 exhibited the highest incidences of gastritis and duodenal ulcer. The results presented in our study demonstrate that RAPD and RFLP analyses of PCR product have a simple, reliable and discriminatory profile and can group isolates, which may suggest differences in pathology due to the strain diversity of *H. pylori.*

In summary, *H. pylori* isolates have great genetic diversity among strains, which can be exploited to type isolates. RAPD on the whole bacterial genome can accurately distinguish between isolates. RFLP analysis with Sau3A of the ureC gene also had good discriminatory power and group isolates into clusters. Comparing genetic diversity of *H. pylori* isolated from different patients, these methods yield fast and reliable results on the transmission and pathogenicity of *H. pylori.*

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AP-PCR與PCR-RFLP對幽門旋曲桿菌分型

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幽門旋曲桿菌被認爲菌株多樣化可能與幽門旋曲桿菌多種臨床病徵有關。幽門旋曲桿菌urease基因之多樣化可提供區別菌株之處。本論文中，我們從二十二位病人分離出幽門旋曲桿菌並抽取染色體DNA，再調查RFLP對PCR放大820-bp ureC基因之分型分析，並與RAPD對菌株染色體分型比較。HindIII及EcoRI切割22位病患消化道切片檢體分離菌株之PCR放大820-bp ureC基因之分型分析只有1種RFLP型式，而Sau3A分型分析有6種不同RFLP型式。Sau3A之第1種RFLP型式有9株，爲22分離菌株的40.9%。我們以RAPD對22分離菌株之染色體之分型分析可區分爲21種RAPD型式。Sau3A之RFLP分型分析圖比RAPD對染色體之分型分析圖簡單、容易判讀。RFLP對PCR產物之分型能力好且可將分離菌分群。比較幽門旋曲桿菌基因之多樣化，RFLP及RAPD可得到快速及可信賴的結果來了解幽門旋曲桿菌之傳染途徑及致病機轉。

關鍵字

幽門旋曲桿菌，聚合酵素鏈反應(PCR)，限制酵素切割片斷分析圖(RFLP)，隨意引子放大片段分析圖(RAPD)

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