PCR-RFLP AND ANTIMICROBIAL SUSCEPTIBILITY PROFILES OF *HELICOBACTER PYLORI* ISOLATED FROM ANTRUM AND CORPUS OF DYSPEPTIC PATIENTS IN THAILAND

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Abstract. The study determined the genetic heterogeneity of *Helicobacter pylori* isolates from antrum and corpus of the same dyspeptic patients in a Thai population and determined the relationship between the antimicrobial susceptibility (AS) profile (antibiogram) and PCR-restriction fragment length polymorphism (PCR-RFLP) pattern. One hundred and nineteen *H. pylori* isolates comprising 7 single and 56 paired antrum and corpus isolates obtained by gastric biopsy from 160 dyspeptic patients were analyzed. For PCR-RFLP, the 820 bp amplicon of *ure*C was digested with *Sau*3AI and *Hha*I, which revealed 16 (A-Q) and 19 (a- s) different PCR-RFLP patterns after *Sau*3AI and *Hha*I digestion, respectively. Combination of the restriction enzyme digestion patterns resulted in 35 distinct RFLP types. Among the 56 paired isolates, 47 were infected with *H. pylori* having the same AS and PCR-RFLP profiles, 7 with different AS profiles but the same PCR-RFLP profiles and 2 with different PCR-RFLP profiles but the same AS profiles. No patient was infected with *H. pylori* different in both PCR-RFLP and AS profiles. The results indicate that the majority of the paired *H. pylori* isolates displayed identical AS profile and PCR-RFLP patterns suggesting that most patients were infected with a single strain. Some patients could have been infected with single strains that were different in the AS profiles.

Keywords: *Helicobacter pylori,* genetic heterogeneity, antimicrobial susceptibility, PCR-RFLP, dyspeptic patients, Thailand

INTRODUCTION

Helicobacter pylori is associated with gastritis, peptic ulcer, and gastric cancer (Nomura *et al*, 1991). Metronidazole (MTZ), clarithromycin (CLR), amoxycillin (AMX) and tetracycline (TE) are the commonly used drugs for *H. pylori* treatment (Megraud, 1997, 2001; Glupczynski *et al*, 2002). The increase in resistances of *H. pylori* to those antimicrobial agents has resulted in treatment failure (Megraud, 2001; Yakoob *et al*, 2001). Treatment failure may occur from multi-resistance to the

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antimicrobials or from mixed infections of *H. pylori* strains in the patients (Kim *et al*, 2003; Norazah *et al*, 2009) owing to genetic changes in *H. pylori*, thereby resulting in the increase of treatment failure (Simsek *et al*, 2000; Choi *et al*, 2011).

A high level of genetic heterogeneity among *H. pylori* strains isolated from various patients has been reported worldwide (Kim *et al*, 2003; Raymond *et al*, 2005; Norazah *et al*, 2009). *H. pylori* isolated from biopsies taken from different sites of the stomach in individual patients are genetically variable with different antimicrobial susceptibilities (van der Ende *et al*, 1996, 2001; Hua *et al*, 2000; Arents *et al*, 2001). However, studies have shown that the majority of patients had a predominant strain distributed both in the antrum and corpus of the stomach, especially in developed countries (Marshall, 1991; Jorgensen *et al*, 1996).

PCR-restriction fragment length polymorphism (PCR-RFLP) is a reliable and reproducible method for molecular typing of *H. pylori* strains (Fujimoto *et al*, 1994). Thus, the genotyping by PCR-RFLP method is useful for planning of therapeutic regimens and epidemiological studies in *H. pylori* infection. Several studies confirmed that PCR-RFLP analysis of *ure*C is a highly efficient tool to differentiate *H. pylori* strains of clinical isolates (Li *et al*, 1997; Stone *et al*, 1997a,b). As there has not been any report of a simultaneous study of the genetic heterogeneity and antimicrobial profile of *H. pylori* in different sites of stomach among Thai dyspeptic patients, we investigated these parameters in the individual subjects.

MATERIALS AND METHODS

Patients and bacterial isolates

Sixty-three dyspeptic patients undergoing routine upper gastrointestinal endoscopy at the Srinagarind Hospital, Khon Kaen, Thailand, and whose antrum and corpus biopsies were positive for *H. pylori* culture were enrolled in the study. From these biopsies, 119 *H. pylori* were isolated, comprising 7 single isolates from either antrum or corpus and 56 paired isolates from both antrum and corpus. The patients were diagnosed as non-ulcer dyspepsia (NUD), peptic ulcer dyspepsia (PUD) and gastric carcinoma (GCA). These patients had not been treated previously for *H. pylori* infection.

The study was approved by the Ethics Committee of Khon Kaen University and performed according to the Declaration of Helsinki. Written informed consent was obtained from each patient prior to being enrolled in the study.

Identification of *H. pylori* from biopsy specimens

Culture was performed as described previously (Chomvarin *et al*, 2005). In brief, each antrum and corpus specimen was separately homogenized in normal saline and cultured on 7% human blood agar (Difco, Detroit, MI) supplemented with 5 mg/l trimethoprim, 10 mg/l vancomycin, 5 mg/l amphotericin B and 5 mg/l cefsulodin (SR147) (Oxoid, Unipath; Basingstroke, Hamshire, England). Plates were incubated at 37°C under micro-aerophilic condition for 4 and 7 days. Colonies were confirmed by Gram staining, and by oxidase, catalase and urease tests.

PCR-RFLP typing

DNA was extracted from a loopful of cells using a genomic DNA purification kit (Gentra system) according to the manufacturer's instruction as previously described (Chomvarin *et al*, 2008). The PCR primers were based from the known sequence of *ure*C, which encodes

an accessory protein for urease expression (Labigne *et al*, 1991), (forward 5′ TGGGACTGATGGCGTGAGGG 3′ and reverse 5′ AAGGGCGTTTTTAGATTTTT 3′) to amplify a 820 bp product (Fujimoto *et al*, 1994). PCR was performed in a total volume of 50 µl containing 100 ng DNA, 200 µM each of dNTP (Gibco BRL, Gaitherburg, MD), 1 PCR buffer (20 mM Tris-HCl, pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 1 µM of each primer and 1.25 U *Taq* polymerase (Gibco BRL), conducted in thermal cycler (GeneAmp Perkin-Elmers, PCR 2400, Foster City, CA) for 35 cycles of 94°C for 2 minutes, 45°C for 1 minute and 72°C for 1 minute. Amplicons were analyzed by electrophoresis in 2% agarose gel, staining with ethidium bromide and examination under UV illumination.

PCR-RFLP analysis was performed as described previously with modification (Stone *et al*, 1997a,b). A 10 µl aliquot of the PCR amplicon was digested with 5 U either *Sau*3AI (Promega, Madison, WI) or *Hha*I (Promega) at 37°C for 6 hours in the buffer recommended by the manufacturer. The digested DNA was analyzed by gel-electrophoresis on 3% Nusieve agarose gel (BMA, Rockland, ME) at 100 volts in 0.5 x TAE buffer (40 mM Tris, 20 mM acetic acid, and 1mM EDTA, pH 8.0) for 45 minutes, stained with ethidium bromide and examined under UV illumination. A 100 bp DNA ladder was used as a molecular weight marker.

Antimicrobial susceptibility by disk diffusion method

This test was performed as described previously (Boyanova, 1999, 2000). In brief, *H. pylori* colonies were adjusted to a density equal to 3.0 McFarland standard ($1x10^9$ CFU/ml) (Xia *et al*, 1994). The suspension was spread on Mueller-Hinton blood agar plates (Oxoid) and the disks containing metronidazole (5 mg), clarithromycin (15 mg), amoxycillin (10 g), tetracycline (30 mg), erythromycin (15 mg), and ciprofloxacin (5 mg) were placed on the agar surface. Plates were incubated under micro-aerophilic condition for 3 days at 37°C. The zone size ≤ 16 mm was considered as being resistant for metronidazole (Boyanova *et al*, 2000; Mishra *et al*, 2006), ≤ 25 mm for amoxycillin (Lang *et al*, 2004) and ≤ 30 mm for clarithromycin, erythromycin, ciprofloxacin and tetracycline (Boyanova *et al*, 2000).

RESULTS

PCR-RFLP analysis

In order to determine the distribution of *H. pylori* strains isolated from antrum and/or corpus of the dyspeptic patients, the 820 bp PCR amplicons of *ure*C were digested with *Sau*3AI and *Hha*I, revealing that 16 (A-Q) and 19 (a-s) different PCR-RFLP patterns, respectively (Fig 1). Combination of two PCR-RFLP patterns showed that 119 *H. pylori* isolates could be classified into 35 distinct RFLP types (type 1-35) (Table 1). The dominant RFLP types were type 8, 10 and 7 represented in 17 (14%), 14 (12%) and 11 (9%) isolates, respectively.

The *Sau*3AI D- and *Hha*I f RFLP patterns were most frequent in *H. pylori* isolates from gastroduodenal diseases However, no association between gastroduodenal diseases (NUD, PUD, and GCA) and the combined RFLP patterns was observed.

Comparison of *H. pylori* antimicrobial susceptibility (AS) profile and PCR-RFLP pattern

In order to identify whether the antimicrobial resistant *H. pylori* developed from a pre-existing susceptible strain or

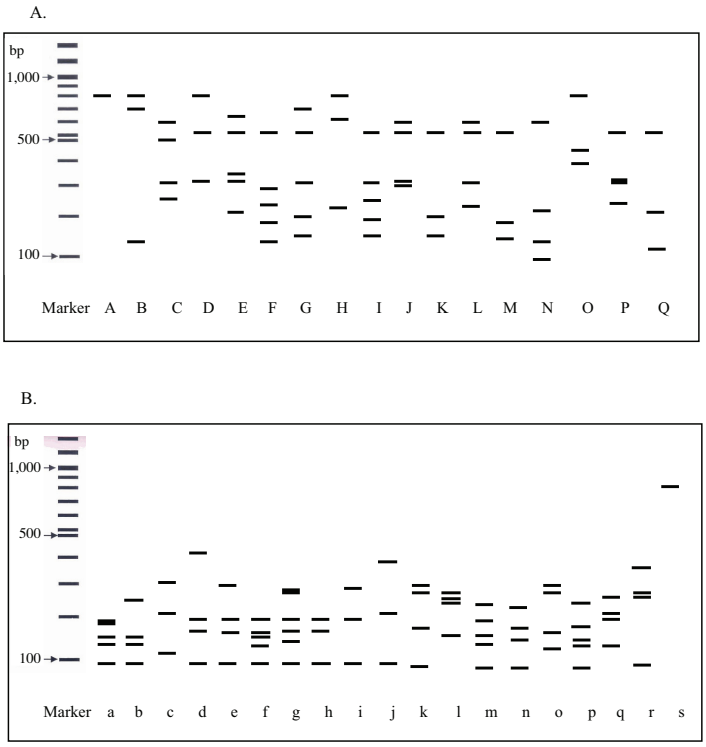

Fig 1–Representative PCR-RFLP of 820 bp amplicon of *H. pylori* *ure*C after digestion with *Sau*3AI (A) and *Hha*I (B). The 17 and 19 RFLP patterns are assigned with upper letters for *Sau*3AI and lower for *Hha*I digestion.

were the results of mixed infection of susceptible and resistant strains, we compared the AS profiles and PCR-RFLP patterns of *H. pylori* isolates obtained from antrum and corpus of 56 pairs isolated from dyspeptic patients. The majority (84%) of *H. pylori* isolated from match antrum and corpus had identical AS profiles and PCR-RFLP patterns. In 2 patients, however, the AS profiles of *H. pylori* isolates were identical but their PCR-RFLP patterns were different and conversely, in another 7 patients, PCR-RFLP patterns were identical but different AS profiles (Fig 2). Examples of discordant AS profiles and PCR-RFLP patterns are shown in Table 2.

DISCUSSION

In order to understand the distribution of *H. pylori* strains circulating in Thai dyspeptic patients in this study, we used *ure*C as the target, and *Sau*3AI and *Hha*I for PCR-RFLP analysis (Fujimoto *et al*, 1994). The result indicates that there were several *H. pylori* strains distributed in our population. Previous studies showed the prevalence of infection of multiple *H. pylori* strains varied according to geographical locations (Marshall, 1991; Jorgensen *et al*, 1996; Kuo *et al*, 1999; Yakoob *et al*, 2001a,b). The prevalence of multiple *H. pylori* strains in this northeastern area of Thailand were in accordance with those in previous reports (Marshall, 1991; Jorgensen *et al*, 1996; Kuo *et al*, 1999; Yakoob *et al*, 2001a,b).

In order to determine whether antimicrobial resistant *H. pylori* developed from a pre-existing susceptible strain or from co-infection with susceptible and resistance strains, we compared PCR-RFLP patterns of 56 paired-isolates from the antrum and the corpus of individual dyspeptic patients. The result indicates that the majority of the paired *H. pylori* isolates displayed identical AS profile and PCR-RFLP pattern suggesting that most patients were infected with a single strain. Our result agrees with previous reports that showed typically one strain of *H. pylori* is responsible for *H. pylori* infection in individual patients (Owen *et al*, 1992;

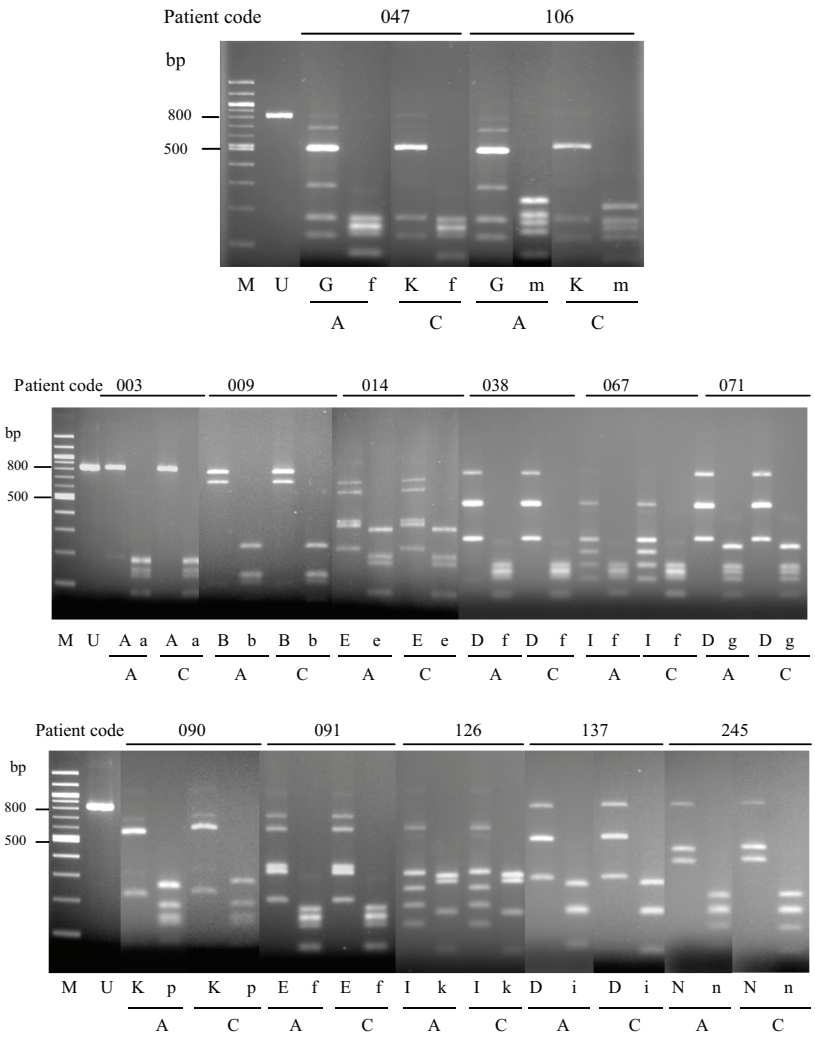

Fig 2–Examples of PCR-RFLP types of *H. pylori ure*C digested with *Sau*3AI and *Hha*I isolated from antrum (A) and corpus (C) of individual dyspeptic patients. The nomenclature of the PCR-RFLP patterns is described in legend to Fig 1. M, molecular standard; U, uncut 820 bp.

Prewett *et al*, 1992; Norazah *et al*, 2009).

The presence of a small number (7/56) patients displaying identical PCR-RFLP profiles but with different AS profiles of *H. pylori* isolated from antrum and corpus could be explained by that drug-resistant *H. pylori* mutant had developed by genomic alterations of a pre-existing susceptible *H. pylori* strain rather than co-infection with susceptible and resistant strains (Jeen *et al*, 2001; Kim *et al*, 2003). The possibility of simultaneous infection with sensitive and resistant strains could not be determined by the present study and needs further exploration.

Only 2 patients displayed identical AS profiles with different PCR-RFLP patterns of *H. pylori* isolates from corpus and antrum. This may be explained by either a low frequency of mixed infection (Owen *et al*, 1993; Jorgensen *et al*, 1996), or a slow rate of changes/mutations (genomic rearrangement, recombination and natural transformation) leading to heterogenous *H. pylori* development during the infection period in a patient (Prewett *et al*, 1992; Taylor *et al*, 1992; Danon *et al*, 1998). Additionally, the antimicrobial agents used for therapy could affect genotypic rearrangement of *H. pylori* (Cellini *et al*, 2003).

In summary, the *H. pylori* strains

Table 1
RFLP patterns of the 119 *H. pylori* isolates from 63 dyspeptic patients.

*Sau*3AI	*Hha*I	RFLP type	Number of isolates (%)
A	a	1	8 (7%)
B	b	2	4 (3%)
B	a	3	4 (3%)
C	c	4	2 (2%)
C	i	5	2 (2%)
D	d	6	4 (3%)
D	g	7	**11 (9%)**
D	f	8	**17 (14%)**
D	h	9	2 (2%)
D	i	10	**14 (12%)**
D	m	11	2 (2%)
D	e	12	4 (3%)
D	j	13	1 (1%)
D	l	14	2 (2%)
E	e	15	5 (4%)
E	f	16	2 (2%)
F	f	17	2 (2%)
G	f	18	1 (1%)
G	m	19	1 (1%)
H	f	20	2 (2%)
I	f	21	2 (2%)
I	k	22	2 (2%)
K	f	23	1 (1%)
K	m	24	1 (1%)
G	s	25	1 (1%)
M	a	26	2 (2%)
M	q	27	2 (2%)
D	r	28	2 (2%)
A	k	29	4 (3%)
D	o	30	2 (2%)
N	f	31	2 (2%)
O	n	32	2 (2%)
P	b	33	2 (2%)
Q	f	34	2 (2%)
L	p	35	2 (2%)

circulating in northeastern Thailand are genetically heterogenous, but the majority of patients were infected with a single strain. Although the frequency was low, mixed infection with antibiotics-susceptible and-resistant strains and the possible conversion of susceptible to resistant strains could have occurred in some patients. Treatment of *H. pylori* infection in both antrum and corpus of the individual patients could still use the same antimicrobial regimens.

Table 2
Comparison of antimicrobial susceptibility and PCR-RFLP patterns of *H. pylori* isolates obtained from antrum and corpus.

Patient code number	Antimicrobial susceptibility pattern						Antimicrobial profile	RFLP pattern		RFLP type	RFLP profile
	MTZ	CLR	E	CIP	AMX	TE		*Sau*3A	*Hha*I		
003	**S/R**	**S**	**S**	**S**	**S**	**S**	**ND**[a]	A	a	1	ID
009	S	S	S	R	S	S	ID[a]	B	b	2	ID
010	S	S	S	S	S	S	ID	D	e	12	ID
011	**R**	**S**	**S**	**S/R**	**S**	**S**	**ND**[a]	C	c	4	ID
014	S	S	S	S	S	S	ID	E	e	15	ID
024	S	S	S	S	S	S	ID	D	g	7	ID
025	R	S	S	R	S	S	ID	D	d	6	ID
028	S	S	S	S	S	S	ID	L	a	26	ID
029	S	S	S	S	S	S	ID	C	i	5	ID
033	R	S	S	S	S	S	ID	D	f	8	ID
035	R	S	S	S	S	S	ID	D	h	9	ID
036	**S**	**S**	**S**	**S**	**R/S**	**S**	**ND**[a]	H	f	20	ID
037	R	S	S	S	S	R	ID	D	i	10	ID
038	S	S	S	S	S	S	ID	D	f	8	ID
039	R	S	S	S	S	S	ID	D	m	11	ID
040	**S**	**R/S**	**S**	**R/S**	**S**	**S**	**ND**[a]	D	e	12	ID
047	R	S	S	S	S	S	ID	**G/K**	**f**	**18/23**	**ND**[b]
048	S	S	S	S	S	S	ID	A	a	1	ID
049	**S/R**	**S**	**S**	**S**	**S**	**S**	**ND**[a]	D	i	10	ID
050	S	S	S	S	S	S	ID	D	r	28	ID
052	S	S	S	S	S	S	ID	D	d	6	ID
067	S	S	S	S	S	S	ID	I	f	21	ID
071	R	S	S	S	S	S	ID	D	g	7	ID
080	R	S	S	S	S	S	ID	A	k	29	ID
090	S	S	S	S	S	S	ID	K	p	35	ID
091	**S/R**	**S**	**S**	**S**	**S**	**S**	**ND**[a]	E	f	16	ID
103	R	S	S	S	S	S	ID	L	q	27	ID
106	S	S	S	R	S	S	ID	**G/K**	**m**	**19/24**	**ND**[b]
107	S	S	S	S	S	S	ID	B	a	3	ID
112	R	S	S	S	S	S	ID	F	f	17	ID
126	S	S	S	S	S	S	ID	I	k	22	ID
128	S	S	S	S	S	S	ID	D	l	14	ID
137	S	S	S	S	S	S	ID	D	i	10	ID
139	R	S	S	S	S	S	ID	D	o	30	ID
187	**R**	**R**	**S**	**S**	**R/S**	**S**	**ND**[a]	D	i	10	ID
245	S	S	S	S	S	S	ID	N	n	32	ID

S, sensitive; R, resistant; MTZ, metronidazole; CLR, clarithromycin; AMX, amoxycillin; TE, tetracycline; E, erythromycin; CIP, ciprofloxacin
ND[a], non-identical of AS profile but identical of PCR-RFLP pattern.
ND[b], non-identical of PCR-RFLP pattern but identical of AS profile.

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