

ORIGINAL ARTICLE

Analysis of PCR-RAPD DNA and antibiotic susceptibility profiles of antrum and corpus isolates of *Helicobacter pylori* from Malaysian patients

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Abstract

This study was conducted to determine whether there was any genetic heterogeneity among *Helicobacter pylori* strains isolated from the antrum and corpus of the same individual in a Malaysian population and to determine the presence of heterogeneous susceptibility of the isolates by comparing PCR-RAPD and antibiotic profiles. Forty-four *H. pylori* isolates cultured from the antrum and corpus of 22 patients were analyzed. Antibiotic susceptibility testing was carried out by minimum inhibitory concentration determination, using E-Test method strips. PCR-RAPD was carried out on all the strains and the profiles generated were analysed for cluster analysis. Twenty-nine different PCR-RAPD profiles were observed in the 44 isolates. Fifteen pairs of the isolates from the same patients had the same PCR-RAPD patterns while in 7 pairs, the profiles were different. The strains were clustered into 2 separate clusters at a low coefficient of similarity, where most of the strains were in cluster 1. The degree of similarity was very low among most of the isolates. Most of the patients (16 of 22) were infected with strains that have the same antibiotic susceptibility profiles. Out of these, only 10 pairs shared the same PCR-RAPD and antibiotic profiles. Five pairs of isolates with similar PCR-RAPD profiles differed in their antibiotic profiles due to metronidazole resistance in one of the sites. A large degree of genetic heterogeneity was observed among *H. pylori* strains circulating among Malaysian patients. An individual patient can be infected with multiple strains and the strains can be antibiotic resistant.

Keywords: PCR-RAPD, *Helicobacter pylori*, metronidazole resistance, Malaysian patients

INTRODUCTION

Helicobacter pylori has been recognized as the major aetiological agent of chronic gastritis and plays an important role in the pathogenesis of peptic ulceration, a risk factor for the development of stomach adenocarcinoma.

A large genetic heterogeneity among *Helicobacter pylori* strains has been reported among various populations worldwide.^{1,2,3} Molecular fingerprinting technique such as polymerase chain reaction (PCR)-based random amplified polymorphic DNA (RAPD), was able to reveal marked heterogeneity between strains from unrelated individuals. This technique was also shown to be discriminative in determining the similarity of strains isolated from the same

patient in biopsies taken from different sites of the stomach.^{4,5}

The increase in resistant isolates is one of the major reasons why antibiotic susceptibility should be conducted prior to eradication of infection. However, there have been reports that heterogeneity occurs in isolates obtained from different biopsy sites of the same individual. Some strains may be sensitive or resistant in the same individual and this phenomenon has been described for metronidazole and clarithromycin.^{6,7} Isolates that were heterogeneous in their susceptibility to metronidazole also appeared to be heterogeneous at the genome level as determined by randomly amplified polymorphic DNA (RAPD) fingerprinting.⁸

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This study was conducted to determine whether there were any genetic heterogeneity among *H. pylori* strains isolated from the antrum and corpus of the same individual in Malaysian patients and to determine the presence of heterogeneous susceptibility of the isolates by comparing the PCR-RAPD and antibiotic profiles.

MATERIALS AND METHODS

Culture of Helicobacter pylori

Biopsy samples were taken from the antrum and corpus of the stomach of patients with symptoms of gastroduodenal diseases such as gastritis, peptic ulcer disease and gastric cancer who presented at the Gastroenterology Unit, National University of Malaysia Hospital (HUKM) from September 2004 until September 2007. None of the patients had previously been treated for *Helicobacter pylori* infection.

The biopsy samples were cultured onto Columbia agar supplemented with 10% ox blood and the agar plates were incubated at 37°C under microaerophilic condition for 5 to 7 days. Bacterial isolates were identified according to colony morphology, Gram staining, urease, catalase and oxidase tests. The cultures were stored at -80°C in brucella broth supplemented with 15% glycerol and fetal calf serum (Invitrogen, USA) until further investigation.

Only isolates that were obtained from the antrum and corpus of the same individual were included in this study.

Antibiotic susceptibility testing

Antibiotic susceptibility testing was carried out by determining the minimum inhibitory concentration (MIC), using E-Test method (AB Biodisk, Sweden). The E-test strips used were metronidazole, clarithromycin, levofloxacin, amoxicillin, tetracycline and ciprofloxacin. Columbia agar supplemented with 10% ox blood were used as culture media and *H. pylori* culture suspension of 3 McFarland turbidity were used to inoculate the culture plates. After placing the E-test strips of each antibiotic tested, the plates were incubated under microaerophilic conditions for 5 to 7 days. The isolates were considered resistant to the antibiotics tested when the MIC value is $\geq 1\mu\text{g/ml}$ for clarithromycin (CLSI), $> 1\mu\text{g/ml}$ for ciprofloxacin, levofloxacin and amoxicillin, $> 4\mu\text{g/ml}$ for metronidazole and $2\mu\text{g/ml}$ for tetracycline.^{9,10} *H. pylori* strains from American Type Culture Collection (ATCC700392 and ATCC43504) were used as control strains. The

ATCC700392 strain is sensitive to all the tested antibiotics, while ATCC43504 strain is resistant to metronidazole.

DNA extraction and PCR-RAPD

DNA was extracted from bacterial cultures using Nucleospin Tissue kit (Macherey-Nagel). Extraction was carried out following manufacturer's instruction. The extracted DNA was stored at -20°C until used. ATCC43504 was added in the PCR-RAPD analysis for comparison with metronidazole resistant isolates and as an internal control to ensure that the same numbers of fragments were generated for this ATCC strain in each run of the PCR-RAPD analysis.

Primer sequence 5'-CCCCTCAGCA-3' was used for PCR- RAPD of all the isolates. An amount of 0.1 ng of chromosomal DNA was used as DNA template. The PCR was performed using GeneAmp PCR System 2400 thermal-cycler (Perkin-Elmer) for 25 cycles with the following conditions: denaturation at 94°C for 1 min, annealing at 29°C for 1 min and extension at 72°C for 1 min. The amplified PCR products were visualized using 1% agarose gels stained with ethidium bromide. After gel documentation, the stored images were analysed using GelCompar Software version 3.1 (Applied Maths). Dendrograms for cluster analysis were based on similarity matrices calculated from the Pearson product-moment correlation coefficient and the unweighted pair group method using arithmetic averages (UPGMA) algorithm. The percentage of similarity denoted the relatedness of one strain to another where 100% similarity showed that the strains were identical.

RESULTS

Culture and antibiotic susceptibility

A total of 777 patients were biopsied and *H. pylori* was isolated from 119 patients. From these positive patients, 22 pairs of *H. pylori* were obtained from both the antrum and corpus of 22 patients. They were 11 males and 11 females. 17 were Chinese and 5 were Indian. Most (14) were more than 50 years of age, while 7 were in the 30-49 years age-group and 1 was 26 years of age. The other patients had *H. pylori* isolated from either the antrum or corpus only. The susceptibility patterns of *H. pylori* isolated from the antrum were compared with the isolates from the corpus of the stomach of each patient. Based on the MIC values obtained, the susceptibility results were denoted as sensitive or resistant.

Sixteen pairs of *H. pylori* isolated from the antrum and corpus of 16 patients had the same antibiotic susceptibility profiles while in another 6 patients, the antibiotic profiles of the isolates from the antrum were different from those of the corpus. The difference was shown by their resistance to metronidazole in either the antrum or corpus isolates (Table 1). The MIC of metronidazole-sensitive strains ranged

from < 0.016 to 2 µg/ml while the MIC of the resistant strains were 8 µg/ml in 2 strains and >256 µg/ml in 4 strains (data not shown).

PCR-RAPD analysis

The PCR-RAPD of *H. pylori* isolates produced 2-8 bands which ranged in sizes of 500 to 3000 bp. From the 44 isolates, 29 different PCR-RAPD profiles were observed and designated as profiles

TABLE 1: Antibiotic profiles and PCR-RAPD pattern of *H. pylori* strains isolated from antrum and corpus of patients' stomach

Patient No.	Pairs of isolates*	Antibiotics susceptibility patterns						Antibiotic profile	RAPD pattern** A/C	Coefficient of similarity
		MZ	CH	LE	AC	TC	CL			
1.	A1/C1	S/S	S/S	S/S	S/S	S/S	S/S	same	1/1	100%
2.	A2/C2	R/R	S/S	S/S	S/S	S/S	S/S	same	2/3	29.4%
3.	A3/C3	S/S	S/S	S/S	S/S	S/S	S/S	same	4/4	100%
4.	A4/C4	R/S	R/S	S/S	S/S	S/S	S/S	differ	5/5	100%
5.	A5/C5	R/R	S/S	R/R	S/S	S/S	R/R	same	6/6	100%
6.	A6/C6	R/R	S/S	S/S	S/S	S/S	S/S	same	7/8	64.7%
7.	A7/C7	S/S	S/S	S/S	S/S	S/S	S/S	same	9/10	51.3%
8.	A8/C8	S/S	S/S	S/S	S/S	S/S	S/S	same	11/11	100%
9.	A9/C9	S/R	S/S	S/S	S/S	S/S	S/S	differ	12/12	100%
10.	A10/C10	S/S	S/S	S/S	S/S	S/S	S/S	same	13/14	44.4%
11.	A11/C11	R/R	S/S	S/S	S/S	S/S	S/S	same	15/16	14.6%
12.	A12/C12	S/S	S/S	S/S	S/S	S/S	S/S	same	17/17	100%
13.	A13/C13	S/R	S/S	S/S	S/S	S/S	S/S	differ	18/18	100%
14.	A14/C14	S/S	S/S	S/S	S/S	S/S	S/S	same	19/19	100%
15.	A15/15	R/S	S/S	S/S	S/S	S/S	S/S	differ	20/20	100%
16.	A16/C16	R/S	S/S	S/S	S/S	S/S	S/S	differ	21/21	100%
17.	A17/C17	S/S	S/S	S/S	S/S	S/S	S/S	same	22/22	100%
18.	A18/18	S/S	S/S	S/S	S/S	S/S	S/S	same	23/23	100%
19.	A19/C19	R/S	S/S	S/S	S/S	S/S	S/S	differ	24/25	50%
20.	A20/C20	S/S	S/S	S/S	S/S	S/S	S/S	same	26/26	100%
21.	A21/C21	R/R	S/S	S/S	S/S	S/S	S/S	same	27/28	66.7%
22.	A22/C22	R/R	S/S	S/S	S/S	S/S	S/S	same	29/29	100%

*Same number denotes same patient; A- isolate from antrum; C- isolate from corpus. ** Same number denotes same PCR-RAPD pattern. MZ-metronidazole; CH- clarithromycin; LE-levofloxacin; AC-amoxicillin; TC-tetracycline; CL-ciprofloxacin. Coefficient of similarity denotes degree of similarity.

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RAPD

RAPD

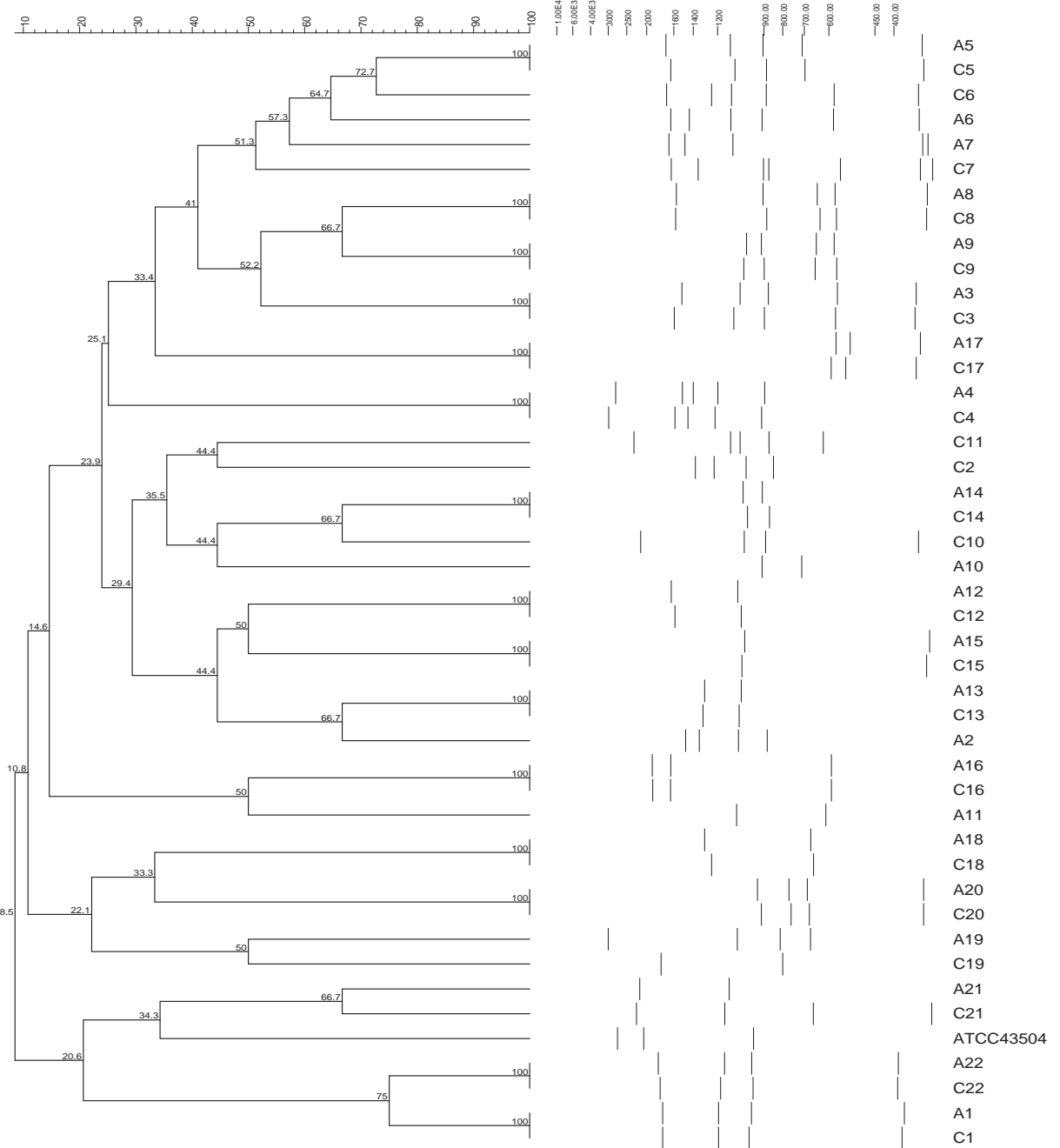


FIG. 1: Dendrogram showing clustering of *Helicobacter pylori* based on RAPD.

1 to 29. PCR-RAPD profile of antrum and corpus isolates showing 100% similarity was observed in 15 patients (Fig. 1).

RAPD dendrograms generated for the 44 isolates demonstrated two clusters of *H. pylori* strains at a similarity coefficient of 8.5%. Isolates in cluster 1 were from 19 patients while isolates

in cluster 2 were from 3 patients. In cluster 1, 13 patients had isolates from antrum and corpus which showed 100% similarity. However when these 13 pairs of isolates were compared, less than 50% similarity level was observed. In the remaining 6 patients, strains isolated from their antrum and corpus strains showed less than 70%

similarity level, suggesting that these strains were different from each other. In cluster 2, 3 pairs of isolates from 2 patients were 100% similar while 1 pair from another patient had only 66.7% similarity level. The degree of similarity was very low among most of the isolates. This showed that there is a large degree of heterogeneity among our local strains.

Comparison of antibiotic profiles and PCR-RAPD pattern

In 10 of the 22 patients, the PCR-RAPD and antibiotic profiles of the antrum isolates were the same as the corpus isolates, thus suggesting that each of the patients were infected with the same strain. Eleven pairs of isolates from 11 patients differed in either the antibiotic profiles or PCR-RAPD patterns while 1 pair differed in both. These results indicated that the patients may be infected with multiple strains. It was also noted that in 5 patients, the isolates from different sites shared the same PCR-RAPD profiles but not their antibiotic profiles. The difference was due to metronidazole resistance in either one of the sites.

DISCUSSION

PCR-based methods had been shown to have higher discriminatory power and typeability than chromosome restriction-based typing method for DNA fingerprinting of *H. pylori*.¹¹ In this study, we applied PCR-RAPD technique to characterize a collection of paired *H. pylori* isolates, from the gastric corpus and antrum of Malaysian patients. We found that PCR-RAPD technique could be reliably used to distinguish one *H. pylori* strain from another. We were also able to determine whether each pair represent isolates with identical (ancestrally related) or different (mixed infection) profiles.

In this study, the antrum and corpus isolates of 15 patients displayed identical fingerprint profiles, whereas isolates from 7 patients displayed distinct fingerprint profiles. However when the identical pairs were compared to each other, a very low coefficient of similarity were noted. The cluster analysis of these strains revealed that there was a large degree of genetic heterogeneity among the *H. pylori* strains.

Different DNA fingerprints of the isolates from different sites of the stomach could be observed in a single host which showed that a patient could harbor more than one strain. The result of this study was in concordance with those obtained in other studies.^{5,12,13}

The prevalence of infection with multiple strains varies geographically. In developed countries, persons are rarely colonized with more than 1 strain.¹⁴ Miehke *et al* showed that in Columbia, which has a high prevalence of *H. pylori* infections, their gastric cancer patients appeared to be infected with a genetically predominant strain throughout the stomach.¹⁵ Jorgensen *et al* also observed that a majority of their patients were infected with a predominant strain.¹⁶ In contrast, multiple colonization by *H. pylori* strains was commonly reported among the Chinese, Taiwan and Korean patients.^{17,18,19} Our data suggest that most of the patients (15 of 22) were infected with a single strain per patient but these strains differed when compared with strains from other patients. This finding suggests that the strains infecting Malaysian population can be due to one predominant strain in the stomach of each patient and the heterogeneity in the strains among patients can be due to the rarity of person-to-person spread. Seven of our patients were shown to harbor more than 1 strain as demonstrated by the different PCR-RAPD profiles. These results suggested *H. pylori*-infected Malaysian patients may be colonized with mixed populations of different *H. pylori* strains. There is a possibility that the proportion of patients infected with more than 2 strains could be higher if more than two sites were sampled.

In some of our patients, their antrum or corpus isolates showed either sensitivity or resistance to metronidazole, while exhibiting the same PCR-RAPD profiles. These results showed that patients can be simultaneously infected with mixed populations of sensitive and resistant strains and is in agreement with other studies.^{7,20} Metronidazole resistance is mainly due to mutations in the *rdxA* gene and the change may not be detected by PCR-RAPD especially if a point mutation is involved. This may be the reason why the resistant strains have identical PCR-RAPD profiles as the sensitive strains.

In conclusion, our local strains have a high degree of heterogeneity. Infections with multiple strains could occur in individual patients and heterogeneous susceptibility to antibiotics could be present in an infected patient.

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