Hao Yu, Yingjia Mao, Lijie Cong, Zhiyong Wang, Hua Zhang and Lei Wang*

Prevalence and genotyping of *Helicobacter pylori* in endoscopic biopsy samples from a Chinese population

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Abstract

Background: *Helicobacter pylori* inhabit the gastric mucosa of humans and are associated with several gastrointestinal diseases which include gastric cancer, peptic ulcer, chronic gastritis and gastric mucosa-associated lymphoid tissue lymphoma. *Helicobacter pylori* exhibit a high degree of genetic variability and are associated with its epidemiological, pathological characteristics and dynamics of transmission. The objective of the study was to determine the prevalence and genetic heterogeneity of *H. pylori* isolated from endoscopic biopsy samples from a Chinese population.

Methods: Gastric biopsy samples from 86 patients (males, 55; females, 35) who presented to the endoscopic section for various gastrointestinal abnormalities were collected. The samples were subjected to a real-time polymerase chain reaction (PCR) and microbial culture for the isolation of *H. pylori*. Further, the isolates were subjected to randomly amplified polymorphic DNA (RAPD) and restriction fragment length polymorphism (RFLP) analysis.

Results: Of the 86 gastric biopsy samples, 61 (70.9%) samples were positive for rapid urease test and 37 (43%) samples (28 from male and nine from female) grew *H. pylori*. Among the biopsy samples subjected to real-time PCR, 39 (45.3%) samples were found to be positive for *H. pylori*. The RAPD analysis yielded 15 different

patterns (four to 17 different sized fragments per strain). The phylogenetic analysis of RAPD yielded 22 clusters at a similarity level ranging from 63% to 100%. RFLP analysis yielded nine different patterns (two to six different sized fragments per strain). Two major restriction patterns were identified, of which 14 (37.8%) strains forms the most common pattern (genotype I) followed by five (13.5%, genotype II) strains with an intra-strain similarity of 100%.

Conclusions: The overall prevalence of *H. pylori* was 45.3%. Despite reports on the declining trend in the prevalence of *H. pylori* infections, our prevalence rate was still higher than those reported from other developed countries. However, further studies involving a large sample size and covering more regions of China is highly warranted.

Keywords: biopsy sample; *Helicobacter pylori*; randomly amplified polymorphic DNA (RAPD); restriction fragment length polymorphism (RFLP).

Introduction

Helicobacter pylori inhabit the gastric mucosa of humans and are associated with several gastrointestinal diseases which include chronic gastritis, peptic ulcer, gastric cancer and gastric mucosa-associated lymphoid tissue lymphoma [1, 2]. It has also been implicated in extra-gastrointestinal diseases such as cardiovascular diseases, diabetes mellitus and autoimmune diseases [3]. The prevalence of *H. pylori* varies based on age, ethnicity, geography and socio-economic status [1]. A systematic review and a meta-analysis which assessed the worldwide prevalence of *H. pylori* infection reported a large variation among different countries and found more than half of the world's population to be infected [4]. In many developed countries due to the improved socio-economic standards of living, the incidence of *H. pylori* tends to be low. However, it remained high, especially in developing countries which has a very low socio-economic status and poor sanitation practice [5, 6]. In developed countries,

*Correspondence: Lei Wang, Department of Oncology, Chifeng Hospital, Chifeng 024000, P.R. China, Phone/Fax: 0086-0476-8331476, E-Mail: LouisemCollinsy@yahoo.com

Hao Yu and Yingjia Mao: Medical Laboratory, Chifeng Hospital, Chifeng, P.R. China

Lijie Cong: Department of Digestive Internal Medicine, Chifeng Hospital, Chifeng, P.R. China

Zhiyong Wang: Endoscopy Room, Chifeng Hospital, Chifeng, P.R. China

Hua Zhang: Pathology Laboratory, Chifeng Hospital, Chifeng, P.R. China

the prevalence of *H. pylori* is significantly lower, accounting for up to 25%–50%, compared to that in developing countries, accounting for 70%–90% of the population [7, 8]. In China, due to rapid urbanization, the prevalence of *H. pylori* is most likely decreasing [9]. However, the implications of *H. pylori* infection in the clinical health status of diseased patients and also healthy subjects, emphasize the importance to understand its prevalence at any given time point.

Helicobacter pylori exhibit a high degree of genetic variability and are associated with its epidemiological, pathological characteristics and dynamics of transmission [10]. Studies have reported that due to the diversity there is a difference in the pathogenicity of *H. pylori* and certain genotypes were detected in certain populations [1, 11]. The recurrence of *H. pylori* infection after apparent eradication has been reported; however, it was unclear whether the recurrence of the ulcer was due to exogenous re-infection by another strain or due to previous infection [12]. Hence, it is highly important to accurately detect and differentiate *H. pylori* strains for better patient management and to quell the long-term consequences of continued asymptomatic diseases [13]. Conventional typing methods such as biotyping, hemagglutination, plasmid profiling, immunoblotting and cytotoxin activity assay were available. They exist with some inherent difficulties which limit their widespread use for the precise differentiation of *H. pylori* strains [13–15]. Molecular methods such as multilocus sequence typing, pulsed-field gel electrophoresis and ribotyping were reported to be highly discriminatory [11, 16]. However, they are time-consuming, laborious, require technical expertise and are expensive. The polymerase chain reaction (PCR)-based restriction fragment length polymorphism (RFLP) and randomly amplified polymorphic DNA (RAPD) analyses have been developed for *H. pylori* genotyping [17, 18]. The RAPD analysis which uses short oligonucleotide primers is a low stringent technique and requires DNA in nanogram quantities [11]. The RFLP analysis involves a restriction enzyme to fragment the specifically amplified region of the DNA to produce a genetic profile. The urease and its accessory proteins encoded *UreA*, *UreB*, *UreC* and *UreD* genes were used for the RFLP analysis of *H. pylori* [19]. Both RFLP and RAPD methods of genotyping are reported to be rapid, easy and highly discriminatory and can be used in clinical laboratory settings than other typing methods [11]. The aim of the present study is to determine the prevalence and genetic heterogeneity of *H. pylori* isolated from endoscopic biopsy samples from a Chinese population by real-time PCR, RFLP and RAPD techniques, respectively.

Materials and methods

Patients

A total of 86 consecutive gastric biopsy samples from 86 patients who were presented to the endoscopic section for various gastrointestinal abnormalities at the Chifeng Hospital, Chifeng, China between November 2016 and October 2017 were collected. The Chifeng Hospital situated at the urban city of Chifeng has two affiliated hospitals consisting of up to 1400 beds, covering a population from four surrounding provinces. Patients who received prior antibiotic therapy within 2 weeks and who had previous gastric surgery were excluded from the study. The hospital Ethical Committee approved the study and informed consent was obtained from all the patients. Samples were collected in a sterile container, sent immediately for microbiology analysis and the remaining samples were frozen at -80°C until used for real-time PCR.

Isolation and Identification of *H. pylori*

The obtained samples were dissected under aseptic conditions into three parts for a rapid urease test, *H. pylori* culture and real-time PCR. A rapid urease test was performed using the modified rapid urease agar medium as described by Shahidi et al. [20]. Briefly, immediately after collection, biopsy samples were inoculated into urea agar and incubated at room temperature. The change in color of the medium from yellow to red at a maximum incubation period of 2 h indicates a positive reaction. *Helicobacter pylori* culture was done as described by Farshad et al. [21]. Briefly, biopsy samples were inoculated on the modified Columbia agar plates (Merck, Germany) supplemented with 10% lysed horse blood. The plates were then incubated in an anaerobic jar at 37°C for 2–3 days under microaerobic conditions. After incubation, based on the colony morphology, gram staining, positive catalase, oxidase, and urease tests the organisms were identified as *H. pylori*. The isolates were stored in Columbia broth (Merck, Germany) supplemented with 20% sterile glycerol at -70°C until used.

DNA extraction

DNA from the biopsy samples for real-time PCR was extracted using QIAamp DNA FFPE Tissue Kit (Qiagen, Hilden, Germany) as per manufacturer's protocol. DNA from *H. pylori* isolates for RAPD was extracted using the

boiling lysis method, briefly, 200 µL of *H. pylori* culture was incubated at 95 °C for 10 min. Then samples were centrifuged at 14,000 *g* for 10 min and the supernatant containing DNA was collected. Both the DNA samples were stored at –20 °C until DNA quantification and purity was determined using spectrophotometry (NanoDrop ND-1000, USA).

Real-time PCR

A real-time qualitative PCR (QuantStudio™ 7 Flex Real-Time PCR System, Applied Biosystems) to detect the presence of *H. pylori* in the biopsy samples was performed using the following 16S rRNA primers 5'-GCGACCTGCTGGAACATTAC-3' and 5'-CGTTAGCTGCATTACTGGAGA-3' [1]. The PCR cycling conditions were as follows: 40 cycles of 95 °C for 50 s, 60 °C for 50 s and 72 °C for 50 s. Using an inbuilt intuitive software, a melt-curve analysis was performed to detect non-specific amplifications. The sample was considered positive when two of the three triplicate showed a positive result within the <35-cycle cut-off.

RAPD

The RAPD was done as described by Lin et al. [11] with a slight modification in the cycling condition. The 25 µL reaction mixture contains 5 µL of the template, 10 pmol of primer PJ1118: 5'-TGTCGTGCTGTTCTG-3' and 12.5 µL of 2X PCR master mix (Promega, USA). PCR cycling conditions were initial denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 1 min, the annealing temperature of 45 °C for 2 min, extension at 72 °C for 2 min and a final extension at 72 °C for 10 min. After PCR, the amplicons were resolved in 1.5% (w/v) agarose gel and the pattern of amplicons were analyzed in comparison with a DNA marker.

RFLP

The *H. pylori* phosphoglucosamine mutase encoded *UreC* (glmM) gene, was amplified for RFLP analysis as described by Ozbey et al. [22]. The 50 µL reaction mixture contains 5 µL of the template, 10 pmol of each primer (forward: 5'-AAG AAG TCA AAA ACG CCC CAA AAC-3' and reverse: 5'-CTT ATC CCC ATG CAC GAT ATT CCC-3') and 25 µL of 2X PCR master mix (Promega, USA). PCR cycling conditions were initial denaturation of 94 °C for 5 min, 45 cycles of denaturation at 94 °C for 45 s, annealing temperature of 59 °C for 30 s, extension at 72 °C for 90 s and a final extension

at 72 °C for 10 min. After PCR, 15 µL of the amplicons were digested with 10 U of *HhaI* (Promega, USA) restriction enzymes as per manufacturer's instructions for 4 h at 37 °C. The digested products were resolved in 2% (w/v) agarose gel and the patterns of amplicons were analyzed in comparison with a DNA marker.

Phylogenetic tree analysis

A phylogenetic tree was constructed for RFLP and RAPD; fingerprints obtained with specific primers were analyzed separately as a single data set to obtain a single dendrogram. They were coded in binary form 1 or 0, respectively. The NTSYSpc 2.11p software (Exeter Software, Setauket, USA) was used for clustering and phylogenetic tree construction.

Statistical analysis

Continuous variables were presented as mean and ranges; categorical variables as numbers and percentages. Chi-squared (χ^2) tests were performed to determine the statistical significance using Minitab statistical software (Minitab version 13.1; Minitab Inc, PA, USA). A *p* value of <0.05 was considered as statistically significant.

Results

Of the 86 patients, 51 (59.3%) were male and 35 (40.7%) were female. The mean age was 39.5 ± 7.2 years (range 19–59 years). A majority of the patients had gastric ulcer; 37 (43.0%) followed by gastritis 26 (30.2%) (Table 1). Of the 86 gastric biopsy samples, 61 (70.9%) samples were positive for rapid urease test and 37 (43%) samples (28 from male and nine from female) grew *H. pylori*. All the samples which isolated *H. pylori* were found to be positive for rapid urease test. Among the biopsy samples subjected to real-time PCR, 39 (45.3%) samples (29 from male and 10 from female) were found to be positive for *H. pylori*. Although more number of samples were positive for *H. pylori* by real-time PCR, they did not differ significantly ($p > 0.05$). However, significantly higher numbers of gastric ulcer biopsy samples were positive for *H. pylori* when tested using real-time PCR ($p = 0.003$) and using the culture method ($p = 0.034$) (Table 2).

The RAPD analysis yielded 15 different patterns with a minimum of four different size amplicons per strain to

Table 1: Demographic detail of the patients.

Demographic details	n (%)
Male	59 (59.3)
Female	35 (40.7)
Age, years (mean $\pm$ SD)	39.5 $\pm$ 7.2
Gastritis	26 (30.2)
Gastric ulcer	37 (43.0)
Duodenal ulcer	18 (20.9)
Gastric cancer	5 (5.8)
Smokers	31 (36.0)
Alcohol consumption	57 (66.3)

Table 2: Real-time PCR and biopsy culture results.

Description (n)	Real-time PCR, n (%)	<i>H. pylori</i> culture positive, n (%)
Male (59)	29 (49.2)	24 (40.7)
Female (35)	10 (28.6)	13 (37.1)
Gastritis (26)	15 (57.7)	11 (42.3)
Gastric ulcer (37)	29 (78.4)	23 (62.2)
Duodenal ulcer (18)	9 (50.0)	3 (16.7)
Gastric cancer (5)	0 (0)	0 (0)
Smokers (31)	18 (58.1)	12 (38.7)
Alcohol consumption (57)	27 (47.4)	24 (42.1)

a maximum of 17 different size amplicons per strain. The amplicons size ranged from 50 bp to >1500 bp (Figure 1). The phylogenetic analysis of RAPD yielded 22 clusters at a similarity level ranging from 63% to 100%. A total of ten (27%) strains (strain numbers 2, 16, 19, 20, 21, 23, 25, 26, 29, 37) showed 100% intra-strain similarity. Of these, eight were isolated from gastric ulcer patients and two were isolated from gastritis patients. A total of nine (24.3%) strains

Figure 1: A representative gel picture showing the PCR-RAPD patterns, M: 100 bp DNA ladder, 1–7: Samples showing different DNA patterns.

(strain numbers 3, 5, 6, 10, 13, 15, 30, 31, 35) showed an intra-strain similarity of $\geq 90\%$. Of these, six were isolated from gastric ulcer patients; two were isolated from gastritis patients and one was isolated from duodenal ulcer patient. Overall, the RAPD analysis revealed high heterogeneity among our isolates (Figure 2).

All the 37 isolates were positive for *UreC* gene (1169 bp) using PCR. The amplicons were digested with *HhaI* restriction enzyme and yielded nine different patterns (designated as genotype I to IX) with a minimum of two fragments per strain to a maximum of six fragments per strain (Figure 3). Two major restriction patterns were identified, of which 14 (37.8%) strains (strain numbers 10, 11, 16, 20, 21, 23, 25, 26, 28, 29, 31, 33, 35, 37) forms the most common pattern (genotype I) followed by five (13.5%, genotype II) strains (strain numbers 3, 6, 9, 15, 36) with an intra-strain similarity of 100%, respectively. Of 14 strains which showed the most common pattern (genotype I), seven were isolated from gastric ulcer patients, five were isolated from gastritis patients and two were isolated from duodenal ulcer patients. All the five strains which showed the second most common pattern (genotype II) were isolated from gastric ulcer patients. Although 37.8% of our isolates were identified with genotype I, the RFLP analysis of the remaining strains revealed high heterogeneity (Figure 4).

Discussion

Recent studies suggest that the prevalence of *H. pylori* is declining in many countries especially in the developed countries [9, 23], while in the developing countries it remains high and varies from 70% to 90% [5–8]. In our study 45.3% of our biopsy samples were positive for *H. pylori* by real-time PCR, compared to the culture method (43%). As these rates did not differ significantly and the fact that real-time PCR is highly sensitive, we considered the rate yielded by real-time PCR as our prevalence rate and discussed below. *Helicobacter pylori* are associated with various gastrointestinal diseases and continue to be a major health problem worldwide [1, 2, 4]. The overall prevalence of *H. pylori* in our study was reported to be 45.3%. As there were limited studies on the prevalence of *H. pylori* isolated from gastric biopsy samples, we have compared our results with studies that reported its prevalence in the normal population as well as with different disease conditions. A recent study from urban China which determined the seroprevalence of *H. pylori* in a normal population, reported an overall prevalence of 31.9% and it varied from 46.8% to 80.4% in other parts of China [23–27]. In this study, the prevalence was

Figure 2: A phylogenetic tree analysis of PCR-RAPD.

slightly higher than that reported from the normal population of urban China [23], and lower than that reported from other parts of China [24–27]. Other studies which included urban Chinese population (migrant/maritime workers) reported comparable prevalence rates ranging from 41% to 44.9% [28–30]. A recent study which included dyspeptic patients from all parts of Mongolia reported an overall *H. pylori* infection rate of 80%, which is much higher than that reported in our study [31]. Rapid urbanization in China has resulted in a likely decrease of *H. pylori* infections [9]. Although our study included both urban and rural populations, a majority of our patients were from the urban area

(62.5%) which might be the reason for a lower prevalence than that reported from other parts of China [23–27]. When compared to other countries, our results are consistent to those reported in healthy subjects from Japan (44.3%) and in children and in adolescent subjects who underwent upper gastrointestinal endoscopy with gastric biopsies from Brazil (41.1%) [32, 33]. The prevalence of *H. pylori* in our study is lower than that reported from some developing Asian countries; in patients who underwent upper gastrointestinal endoscopy from India (62%), in dyspeptic patients from Bhutan (86%) and Kazakhstan (76.5%) [34–36]. The prevalence of *H. pylori* infection in dyspeptic patients from African countries including Ethiopia (65.7%), Morocco (75.5%) and Nigeria (93.6%) were higher than that reported in our study [37–39]. The prevalence of *H. pylori* in our study is higher than that reported in normal subjects from western countries including the Netherlands (31.7%) and Canada (37.9%) while lower than those reported from Portugal (84.2%), Turkey (82.5%) and Mexico (52.2%) [40–43]. Of the 39 (45.3%) samples which were positive for *H. pylori* using the culture method, 29 samples from males were found to be positive compared to females (n=10). In our study, males were predominantly infected by *H. pylori* which is consistent with that reported from China [23]. Smoking is considered as one of the independent risk factors for *H. pylori* infection which was higher in males compared to females in China [44]. Other postulated explanations include relative immunodeficiency and lower levels of plasma IgM in males [45].

Figure 3: A representative gel picture showing the PCR-RFLP patterns, M: 100 bp DNA ladder, 1–7: Samples showing different DNA patterns.

Figure 4: A phylogenetic tree analysis of PCR-RFLP.

The genomic heterogeneity is considered as a valuable tool in epidemiological studies [11]. The RAPD and RFLP techniques have been widely used in several epidemiological studies of *H. pylori* [17, 18, 46]. In our study, the RAPD analysis yielded a maximum of 17 different patterns whereas the RFLP yielded a maximum of six different patterns. The phylogenetic analysis of RAPD yielded 22 clusters while the RFLP yielded 10 clusters. In our study, both the RAPD and RFLP had good discriminatory power and yielded different patterns of strain variability. Our results were consistent with those reported by Lin et al. [11]. We found that most of the strains isolated from gastric ulcer patients showed almost similar patterns when tested using the RAPD and RFLP, respectively. We found that the RFLP pattern VI was associated with gastric ulcer, while the patterns III and VII were associated with gastritis. Apart from the isolates from gastric ulcer samples, other isolates showed diverse DNA fingerprints associated with diverse disease conditions. Thus, the majority of our stains showed high genetic variability yielding several different DNA fingerprints. This could indicate that *H. pylori* have epidemiological diversity and showed varied prevalence rates compared to other studies from different geographical locations [23–27, 32–43]. Although several reports indicate that the *H. pylori* infection is declining [7, 8, 23], our results suggest that it is still higher compared to the developed countries [40–43]. In addition, the genetic variation among our isolates reiterates the requirement

to investigate the clinical outcomes on a continues basis and a geographical-specific approach should be adopted for a better prophylaxis. We have included patients from the Chifeng and its affiliated hospitals and although this hospital recruits patients from four nearby provinces, the prevalence in this study may not truly reflect the entire population of China. Hence, further studies involving a large sample size and covering more regions of China are highly warranted.

Conclusions

The overall prevalence of *H. pylori* was 45.3%. Despite reports on the declining trend in the prevalence of *H. pylori* infections, our prevalence rate was still higher than those reported from other developed countries. We suggest that one should not be complacent citing the reports on the declining trend on the prevalence; rather we should continuously monitor the trend in the prevalence of *H. pylori* due to their high genetic variability.

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