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Original Article

Genotype Diversity and Quasispecies Development of *Helicobacter pylori* in a Single Host

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SUMMARY:

Infection with different strains of *H. pylori* and emergence of new genomic variants during their long term gastric colonization are assumed as main reasons for eradication failure. This project was designed to detect relatedness and genetic variations among the *H. pylori* single isolates from each patient in Iran. Genotyping and arbitrarily primed PCR fingerprinting (RAPD) were used in this regard. Multiplex-PCR amplification of gene alleles encoding virulence factors *vacA* (m/s), *cagA*, *iceA* (A1/A2) and comparison of RAPD patterns of different singles colonies were performed for each individual patient. The results showed high frequency of diversity among the *H. pylori* strains. Nearly, 23% of the infected patients showed single type infection. The infection types related, unrelated, and related/unrelated were found among 25.6%, 12.8%, and 38.5% of the patients, respectively. Both mixed type infection (77%) and quasispecies development (15.4%) were detected among these patients. Genotype conversion among *vacA* (41.6%), *cagA* (41.6%), and *iceA* (50%) alleles was observed for those strains with identical or related RAPD patterns. Coevolution of different alleles was also detected in a patient infected with strains presenting same RAPD patterns. Collectively, results of this study revealed occurrence of quasispecies development, mixed type infection and changes of virulence properties of *H. pylori* strains among the studied patients.

**Keywords:** *Helicobacter pylori*, RAPD-PCR, Genotyping, Mixed-infection, Quasispecies development.

**Abbreviations:** Random Amplified Polymorphic DNA, RAPD.
INTRODUCTION

*H. pylori* is a Gram-negative, microaerophilic bacterium that was established as an etiological agent of chronic gastritis, peptic ulcer, MALT lymphoma, and gastric cancer (1-4). More than 50% of people in the world are infected with this bacterium (5). *H. pylori* infection can occur in early years of life and remain asymptomatic for long times (6). Changes of the virulence and resistance properties of colonizing strains during the chronic infection assume as main problem for disease management in infected patients. Successful treatment of chronic *H. pylori* infection is difficult and usually requires treatment regimens containing a proton pump inhibitor and two (standard triple therapy) or three antibiotics (concomitant therapy) (7,8). Genetic variation of this bacterium during its chronic infection can be one of the main causes of treatment failure through the emergence of newly evolved resistant *H. pylori* strains in each patient (9). However, this failure could also be caused by co-infection with diverse strains presenting different resistance phenotypes (2).

In addition to antibiotic resistance features of the infecting strains, development of the life threatening complications are also determined by their genetic contents and virulence properties (10, 11). Different manifestations of the *H. pylori* infection among different patients could be explained by genetic diversity of the responsible strains (4). Conversion of non- or weakly pathogenic colonizing strains into pathogenic ones during the chronic infection (i.e. quasispecies infection) or re-infection of asymptomatic patients with more virulent strains, which is known as mixed type infection, could change the patients’ disease outcomes (12).

Given to the mounting body of evidence that explain recombination events among colonized strains of *H. pylori* in a single host, characterization of the newly evolved pathogenic strains that
are ancestry related to their origins is of major importance. Various typing techniques are frequently described to be used for initial screening of genetic diversities among the *H. pylori* variants in each patient. Random Amplified Polymorphic DNA analysis (RAPD profiling) and virulence gene profiling (genotyping) are amongst the common methods have been used in this regard (13). Although similarity or diversity of *H. pylori* strains from each patient could be easily postulated by comparing their RAPD patterns, however this method can not provide any information about their virulence properties and evolved genetic events. Evolution of *H. pylori* virulence genes in human stomach was reported by some studies. In this regard, *H. pylori* genotyping based on its important virulence factors, such as *cagA*, *vacA* (*s*1/2, *m*1/2), *iceA* (*A*1, *A*2), could provide new informative insights concerning their diversities in the pathogenesis and also clinical outcomes. In the present study we investigated genetic relatedness and variations of *H. pylori* single strains in patients with different gastric disorders by using RAPD-PCR fingerprinting and genotyping methods.

**MATERIALS AND METHODS**

**Clinical specimens and culture**

The gastric biopsy specimens were obtained from the antrum and fundus of patients with different gastric disorders after endoscopy in Taleghani Hospital during October 2011 until June 2012. The biopsy specimens were homogenized and cultured on supplemented Brucella Agar with 7% sheep blood and antibiotics (vancomycin 2.0 mg, polymyxin 0.05 mg, trimethoprim 1.0 mg, and amphotericin B 2.5 mg/L). Incubation was performed in microaerophilic conditions at 37 °C for 5-7 days. Suspected isolates were characterized by both biochemical (urease, catalase,
and oxidase reactions) and molecular identification methods. A total of 5-6 single colonies were arbitrarily selected from each culture. Subcultures of these colonies were prepared for DNA extraction and the isolates were stored at -70 °C for further studies.

**DNA extraction and PCR analysis**

Pure cultures of the single colonies were used for DNA extraction by using QIAamp DNA extraction kit (QIAgen®, Hilden, Germany) according to the manufacturer’s instructions. The DNA samples were stored at -20 °C until it was used for gene amplification. The bacterial genus (16s rRNA) and species (glmM) specific primers were used for molecular detection of the *H. pylori* isolates (Table 1) (14). To overcome problems related to DNA concentration of each DNA sample, concentrations of the extracted DNAs were determined by spectrophotometry.

**RAPD Typing**

A volume of 25 μL containing 1X PCR buffer, 1 μM of each primer, 1 μL of genomic DNA (approximately 150 ng), 200 μM of dNTPs mix, 2 mM of MgCl₂, and 0.05 U/μL Taq DNA polymerase was used for each reaction. RAPD-PCR amplification was performed in an automated thermal cycler (AG 22331; Eppendorf, Hamburg, Germany) under the following conditions: 4 cycles of 5 min at 94 °C, 5 min at 36 °C, 5 min at 72 °C followed by 30 cycles of 1 min at 94 °C, 1 min at 36 °C, and 2 min at 72 °C (4).PCR products were electrophoresed in 1.8% agarose gel. Similarity of all RAPD banding profiles was analyzed by GelCompar II Software. Polymorphisms of ≤ 2 and >2 RAPD bands, either by 1283 or 1254 primers, were considered as definitive criteria for detection of related and different strains, respectively.
**Multiplex-PCR genotyping**

The multiplex-PCR genotyping were performed for \textit{vacA} (m1/m2), \textit{vacA} (s1/s2), \textit{iceA} (A1/A2), and \textit{cagA} genes under the following conditions: 35 cycles of 1 min at 94 °C, 40 seconds at 58 °C, and 1 min at 72°C. The PCR products were electrophoresed in 1.8% agarose gel. Primer sequences and expected sizes of the PCR products for each allele are summarized in Table 1. Positive-control DNA samples related to each allelic variant were used in the multiplex-PCR genotyping assays (GenBank accession numbers KC166221, KC166222, KC109194, KC153039, KC180323, KC180324). Genotype diversity of the single strains in each patient were confirmed by allele specific PCR.

**RESULTS**

**\textit{H. pylori} isolates**

Out of the 98 studied patients in this study, only 39 patients (39.8%) were infected with \textit{H. pylori}. A total of 232 clinical isolates (5-6 single colonies from each patients) were obtained from these patients. Mean age of the subjects was 35 years old (in age ranges of 10-70 years). Males and females constituted 36% and 63% of these patients, respectively. All of the isolates showed positive results for both conventional biochemical and molecular identification tests.

**RAPD typing**

To determine genomic relatedness of the \textit{H. pylori} isolates, RAPD-PCR fingerprinting was carried out on the obtained DNA samples from the isolates in each patient. Comparison of the
RAPD-PCR results showed higher sensitivity of primer 1283 versus 1254 in differentiation of mixed type infection in our patients. The infection types related, unrelated, and related/unrelated were distinguished based on primers 1283 and 1254 with sensitivity of 33.7% and 20.5%, 18% and 12.8%, and 18% and 15.3%, respectively. Comparison of the banding profiles among the isolates from different patients demonstrated their distinct origins in all of the cases. Nearly 23% of the patients showed identical RAPD fingerprints for their isolates (no mixed type infection). The remaining patients showed infection with related (25.6%), distinct (12.8%) and both of related and distinct strains (38.5%), simultaneously.

**Multiplex-PCR genotyping**

Multiplex-PCR was performed for all isolates from each patient to determine evolution of their main virulence factors, individually. Analysis of the PCR results revealed frequency of *cagA*, *vacA s1*, *vacA s2*, *vacA m1*, *vacA m2*, *iceA1*, *iceA2*, and *iceA1*/A2 as 86.6%, 94.8%, 4.7%, 59.5%, 37.5%, 5.6%, 28.9%, and 37.1%, respectively. Presence of multiple *H. pylori* strains in a single individual based on their genotype diversity was recognised among 12 out of the 39 studied patients (30.76%) (Figure 1). These divergences were mainly related to allelic forms of *vacA* (41.6%), *iceA* (50%), and *cagA* (41.6%), respectively. Coevolution of *cagA*, *iceA*, and *vacA* alleles was also detected among 25% of the infected patients with the strains presenting mixed genotypes. The results showed simultaneous existence of strains with *cagA+/cagA−* (23.3%), *vacA+/vacA−* (10%), *vacA m1/vacA m2* (10%), *iceA1+/A2−/iceA2+* (6.6%), and *iceA1+/A2+/iceA−* (20%) genotypes. No statistical association was found between severity of the disease conditions and the identified genotypes in each patient.
Genotype conversion among the strains with diverse RAPD patterns

Analysis of the *H. pylori* RAPD patterns among the single colonies showed their genotype diversities in different allelic forms. These diversities were seen among the patients with identical, related, and unrelated RAPD patterns (Table 2). The main diversities were related to iceA2⁺:iceA⁻ (7.7%), vacA m2⁺:vacA m2⁻ (7.7%), and cagA⁺:cagA⁻ (12%). Conversions of vacA m1⁺:vacA m2⁻ (5.1%), iceA1/A2⁺:iceA0 (5.1%) and iceA2⁺:iceA1/A2⁻ (2.5%) were also detected in these patients (Figure 1). Figure 2 represents genotype diversity of *H. pylori* isolates with related, identical, and unrelated RAPD patterns in four patients.

Occurrence of mixed and quasispecies infections

Genetic diversity of strains with related and identical RAPD patterns (quasispecies infection) was determined in this study. A total of six patients (15.4%) showed quasispecies development of *H. pylori* infection, while mixed-infection was illustrated among thirty patients (77%). Changes of the allelic variants vacA m2⁺:vacA m2⁻ (16.6%), cagA⁺:cagA⁻ (50%), and ice A1/A2⁺:iceA1/A2⁻ (16.6%), cagA⁺/iceA1 A2⁻:cagA⁻/iceA1 A2⁻ (16.6%) was observed among these patients. Genotype diversity of the strains in each patient in compare to their differences in RAPD patterns is depicted in Table 2.
DISCUSSION

*H. pylori* exhibits incredible genetic diversity among bacteria colonizing human tissues. Genetic rearrangement and recombination events may occur among *H. pylori* strains during chronic infection in different parts of the gastric tissue (5, 20). These genetic variations can lead to development of diverse *H. pylori* strains in a single host. The genetic variations and conversions may generate more virulent strains that can colonize an individual host preferentially. Several studies indicate that the *H. pylori* mixed infections may occur in single individuals (7, 12, 21). In a study by Finger SA *et al.* in Peru, at least 50% of the patients showed infection with two *H. pylori* strains, however 61% of these patients were infected with the closely related strains (19). In other studies in Korea and India, it was shown that 67.5% and 100% of the studied patients harbored multiple *H. pylori* strains, respectively (21, 22).

Application of accurate molecular methods can be useful in detection of *H. pylori* mixed infections in different individuals. RAPD-PCR is assumed as a valuable high-throughput and low-cost technique to fingerprint relationship and diversity of bacteria (23, 24). Previous reports have shown that RAPD fingerprinting has the ability to detect genetic variations among *H. pylori* strains isolated from individual hosts (25). Salama NR. *et al.* and Sheu *et al.* established diversity of *H. pylori* strains from each patient based on their RAPD profiles that were consistent with their diversities in genetic alleles (26, 27). According to the obtained RAPD PCR results, significant differences were also found in our study among the isolates from a single host. A higher rate of mixed type infection was determined in our patients using RAPD PCR fingerprinting (77%). The results revealed that primer 1283 is more sensitive than 1254 in discrimination of diverse or related strains. This increased sensitivity was also reported by Kim *et al.* (22).
Co-infection with strains presenting identical RAPD patterns, but different genotypic characteristics proposes the occurrence of gene conversion among *H. pylori* strains in each patient. In the case of genes encoding *H. pylori* adhesins (e.g. *babA*, *homA* and *sabB*), conversion of their allelic variants during chronic infection have been demonstrated previously (28). In our study, conversion of *vacA* alleles, *cagA*:cagA’, and *iceA1/A2*:iceA1/2 was proposed in some patients. These virulence factors considered as main proteins involved in pathogenesis of *H. pylori* and their diversity is associated with different disease outcomes (29-32). Results of our study demonstrated presence of great diversity among the isolates based on the genotyping method (30.76%), which is higher than other reports (21). A higher frequency of mixed infection was determined based on *iceA* genetic locus compared with *vacA* and *cagA* genes. Co-infection with the strains presenting *iceA1* and *iceA2* alleles was reported in different studies that ranged between 1.9 and 36.7% (32, 33). In the case of *vacA* alleles, Ghose C. *et al.* reported a frequency of 55% mixed infection for those samples that showed diversity in *vacA* m and s alleles (34). In our study, genotype diversity in *vacA* alleles was observed among 50% of the patients whom equally conferred infection with diverse *iceA* variants, which is similar to that was reported by Figueiredo *et al.* (53.8%) (35). Coexistence of several allelic variants of different genes, including *cagA*:vacA s1/m2/iceA*:cagA’/vacA s1/m1/iceA1A2; *cagA*:vacA s1/m2/iceA2: cagA’/vacA s1/m2/iceA’, and *cagA*:vacA s1/m2/iceA2: cagA’/vacA s1/m1/iceA, was observed in these patients. Quasispecies development of *H. pylori* was detected for 15.4% of the patients with mixed type infection. Similarly, the quasispecies development within an *H. pylori* population was demonstrated by Ernst J. Kuipers *et al.* during long-term host colonization (12). Whole genome sequencing of these strains may provide valuable data about manners of their evolution in one host during a chronic infection.
In conclusion, results of this study showed simultaneous application of multiplex-PCR and RAPD typing assays as useful methods for detection of quasispecies development and mixed type infection in the studied patients. These results showed vast genetic diversity and occurrence of genotype conversion among the responsible strains. The findings showed that quasispecies development can occur among \textit{H. pylori} strains with different genotypes. Comparison of these \textit{H. pylori} strains at genomic level and application of more sensitive methods in this subject (e.g. Multiple locus sequence typing) will provide new insights about the happened genetic events during their evolution in the human stomach.

\textbf{ACKNOWLEDGMENTS}

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\textbf{Conflict of interest statement:} None to declare.

\textbf{References}


Table 1. Primers used in this study

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primers (5′ → 3′)</th>
<th>PCR product (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>vacA (s1/s2)</td>
<td>F:ATGGAAATACAACAAAACACACCGR:TACACGCTCTTTCAATACACTTCC</td>
<td>420</td>
<td>This study</td>
</tr>
<tr>
<td>vacA (m1/m2)</td>
<td>F:CAATCTGTCCAATCAAGCGAGR:GCGTCAAATAATTCCAAGG</td>
<td>567-642</td>
<td>(15)</td>
</tr>
<tr>
<td>cagA</td>
<td>F:AACAGGACAAGTAGCTAGCCR:TATTAATGCCTGTGTGGGCTG</td>
<td>500</td>
<td>(16)</td>
</tr>
<tr>
<td>iceA1</td>
<td>F:TATTTCTGGAACCTTGCGCAACCTGATR:GGCCTACAACCCGATGGATAT</td>
<td>~900</td>
<td>(17)</td>
</tr>
<tr>
<td>iceA2</td>
<td>F:CGGCTGTAGGCACTAAAGCTA R:TCAATCCTATGTGAAACATCGTGGT</td>
<td>~800</td>
<td>(17)</td>
</tr>
<tr>
<td>glmM</td>
<td>F:GGATAAGCTTTAGGGTGGTGGGGG R:GGTACTTTTCAACACTAAGCGGC</td>
<td>296</td>
<td>(14)</td>
</tr>
<tr>
<td>16S rRNA</td>
<td>F:GGCTATGACGGGTATCCGGCR:GCCGTGCAGAACCTTTGTC</td>
<td>764</td>
<td>(18)</td>
</tr>
<tr>
<td>RAPD primer 1283</td>
<td>5′ GCGATCCCA 3′</td>
<td>-</td>
<td>(19)</td>
</tr>
<tr>
<td>RAPD primer 1254</td>
<td>5′ CCGCAGCCAA 3′</td>
<td>-</td>
<td>(19)</td>
</tr>
</tbody>
</table>
Table 2. Frequency of genotype conversion among *H. pylori* isolates in patients with defined RAPD pattern.

<table>
<thead>
<tr>
<th>Infection Type</th>
<th>RAPD patterns&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Genotype diversity&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Related</td>
<td>25.6%</td>
<td>(10%)</td>
</tr>
<tr>
<td>Unrelated</td>
<td>12.8%</td>
<td>(6%)</td>
</tr>
<tr>
<td>Identical</td>
<td>23 %</td>
<td>(44.4%)</td>
</tr>
<tr>
<td>Related/ Unrelated</td>
<td>38.5 %</td>
<td>(26.6%)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Percentages of each infection type based on RAPD-PCR fingerprinting results using the primers 1284 and 1253.

<sup>b</sup> Genotype diversity in each group was estimated based on allelic variants of virulence factors *cagA*, *vacA*, and *iceA*.
Fig. 1. Multiplex PCR results for vacA s, vacA m, cagA and iceA alleles. Panel A indicates presence of vacA s, vacA m, cagA and iceA1 and A2 alleles. Panel B represents genotype diversity of these isolates in vacA m1 and m2 alleles. Panel C shows presence of a single cagA strain among cagA+ ones. Diversity of strains with iceA1 and iceA1/A2 allelic variants is shown in Panel D. M, DNA ladder mix.
Fig. 2. Phylogenetic tree of representative *H. pylori* strains with related, identical, and unrelated RAPD patterns (Primer 1254). Patient number 196 shows infection with related *H. pylori* strains. All of the single isolates are *vacA s*, *vacA m*, *cagA*. However, single isolates 196-1 and 196-4 are *iceA2* and single isolates 196-2 and 196-5 are *iceA1/A2*. Patient number 188 shows infection with isolates presenting identical RAPD patterns. All of the single isolates are *vacA s*, *vacA m*, *iceA1/A2*. However, single isolates 188-3, 188-5 are *cagA* while single isolate 188-4 is *cagA-.* Conversion of *vacA m2*: *vacA m2* and *cagA*: *cagA-* were detected in one isolate inpatient number 180 and patient number 236, respectively. Single strain 236-4 that shows unrelated RAPD pattern is *cagA-.* Scale bar shows similarity percentage of the *H. pylori* strains. Dendrogram was drawn with Gelcompar II software (Version 3.5, Applied Maths).