Original Article

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Analysis of Genomic Diversity among *Helicobacter pylori* Strains Isolated from Iranian Children by Pulsed Field Gel Electrophoresis

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Abstract

Objective: Presence of genomic diversity among *Helicobacter pylori (H. pylori)* strains have been suggested by numerous investigators. Little is known about diversity of *H. pylori* strains isolated from Iranian children and their association with virulence of the strains. Our purpose was to assess the degree of genomic diversity among *H. pylori* strains isolated from Iranian-children, on the basis of *vacA* genotype, *cagA* status of the strains, sex, age as well as the pathological status of the patients.

Methods: Genomic DNA from 44 unrelated *H. pylori* strains isolated during 1997-2009, was examined by pulse-field gel electrophoresis (PFGE). Pathological status of the patients was performed according to the modified Sydney-system and genotype/status of *vacA/cagA* genes was determined by PCR. PFGE was performed using XbaI restriction-endonuclease and the field inversion-gel electrophoresis system.

Findings: No significant relationship was observed between the patterns of PFGE and the *cagA/vacA* status/genotype. Also no relationship was observed between age, sex, and pathological status of the children and the PFGE patterns of their isolates. Similar conclusion was obtained by Total Lab software. However, more relationship was observed between the strains isolated in the close period (1997-2009, 2001-2003, 2005-2007, and 2007-2009) and more difference was observed among those obtained in the distant periods (1997 and 2009).

Conclusion: *H. pylori* strains isolated from children in Iran are extremely diverse and this diversity is not related to their virulence characteristics. Occurrence of this extreme diversity may be related to adaptation of *H. pylori* strains to variable living conditions during transmission between various host individuals.

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Key Words: *Helicobacter Pylori*; Children; Genomic-Diversity; PFGE; Iran

Introduction

Infection by *Helicobacter pylori (H. pylori)* is associated with gastritis, duodenal and gastric ulcer, gastric adeno-carcinoma and mucosa-associated lymphoid tissue lymphoma. In developing countries infection occurs predominantly in childhood and the infected individuals maintain *H. pylori* strains in their stomach for the decades[1-2]. Numerous studies have suggested presence of genetic difference between *H. pylori* strains isolated from various geographical areas[3-7]. However, some relatedness was observed between the *H. pylori* strains isolated in one geographical area[8-11]. Concerning pathogenicity-associated markers (*cagA* and *vacA*), correlation was observed between *vacA* s1 genotype and *cagA* status of *H. pylori* and more

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severe gastroduodenal diseases. However, some discrepancies related to either the geography of strains, or generation of variants during infection, was observed[12-14]. The studies investigating genomic diversity among *H. pylori* strains have employed restriction-endonuclease patterns, ribotyping, sequencing of housekeeping genes, and PFGE[15-21].

PFGE is a rapid method for characterization of individual strains of bacterial species and to demonstrate the clonal relation between the bacterial strains regardless of the year of isolation[22]. This method may also be the most suitable for a local epidemiological study of the molecular relatedness among *H. pylori* strains isolated from unrelated patients.

Previous studies on molecular relatedness of *H. pylori* isolated from the members of the family have shown the identical alleles found in some strains isolated from the children and parents, but not in the strains isolated from unrelated patients[23].

The purpose of this study was to look for the degree of genomic diversity among *H. pylori* strains isolated from unrelated Iranian children, on the basis of *vacA* genotype, *cagA* status of the strains, and age, sex, as well as the pathological status of the patients.

Subjects and Methods

Strains were isolated from 44 pediatric patients during the periods of 1997-1999 (group I), 2001-2003 (group II), 2005-2007 (group III), and 2007-2009 (group IV). The reason of this strain selection was to study the molecular relatedness of *H. pylori* strains isolated in this area from non related patients in avoiding their clonal relation. So the patients admitted to Children's Medical Center, Tehran for their persistent upper gastrointestinal problems during 1997-2009, were selected.

Local ethics committees approved the protocols under which the biopsies for histology and culture were obtained, and informed consent has been obtained. They were all in accordance with the Helsinki Declaration of 1975.

For isolation of primary *H. Pylori* strains, the antral biopsies have been processed according to the previously described protocol[24]. Briefly, the isolates were cultivated on campy-blood agar plates containing brucella agar base, 10% sheep blood, and antibiotics after enrichment in modified campy-thio medium (Merck, Germany). Following the initial growth in campy-blood agar plates, the pure cultures were produced from each isolate and identification was performed by Gram staining, positive urease, oxidase and catalase tests. Biochemical identification was confirmed by PCR amplification of *H. pylori 16S rRNA* and *UreC*. Strains identified as *H. pylori*, were stored in skim milk containing 15% glycerol (Merck), and 10% fetal calf serum (Gibson) at -70°C.

Histological examination of the biopsies, has been performed after H&E, and Giemsa staining; *H pylori* density, gastritis, and inflammation were graded according to the modified Sydney system to mild (MIC), moderate (MAC) and severe active chronic (SAC) gastritis, as previously described[25,26].

Chromosomal DNA was extracted from 72-hour-old confluent cells by using the previously described procedure[26]. PCR primers and protocol for amplification of *16S rRNA*, *cagA* genes as well as *vacA (s1, s2, m1,* and *m2)* alleles, were those previously described [27].

DNA preparation and PFGE was performed using the protocol adopted from the previously described procedure, with some modifications[16,20,28,29]. In brief, two days bacterial cultures were harvested and suspended in one ml phosphate buffer saline, resuspended in 1-2 ml TE buffer to obtain a turbidity equivalent to that of McFarland no. 8. Cell suspension was warmed to 37° C, and 150 µl was mixed with an equal volume of 2% low-melting point (LMP) agarose to prepare the agarose plugs. The solidified plugs were incubated for 48 h in the lysis buffer (0.25M EDTA [pH 8.0], 0.5% lauryl sarcosine, 50 µl proteinase K) at 50 °C. The plugs were washed three times in 10 mM TE buffer containing 1 mM phenyl methyl sulfonyl fluoride for 20 min, followed by three times washing in TE buffer each at 4 °C. For the subsequent enzyme reaction, the TE buffer was removed; the plugs were incubated with 100 µl of the XbaI enzyme buffer for 15 min at 36 °C. The enzyme buffer was replaced with 100 µl fresh enzyme solution containing 15 U of enzyme and incubated at 36° C for 4 h. After the incubation

period, the plugs were washed once in TE buffer and were loaded into the 1% pulsed field certified agarose gel (Invitrogen). For PFGE analysis, a Field Inversion Gel Electrophoresis (FIGE) system was used for 16 h at 4 °C and 130 V. The pulse times varied from 2.4 to 3 s to examine various-sized fragments. DNA obtained from *Staphylococcus aureus* NCTC 8325 strain was used as the size marker[30]. Agarose gels were stained with ethidium bromide and photo-graphed.

Findings

Forty four pediatric patients with no more than one *H. pylori* strain regarding *vacA* genotypes were used in this study. Information related to the age, sex, the date of *H. pylori* isolation, as well as the *vacA* genotype/*cagA* status is provided in Table 1.

The 26695 standard *H. pylori* strain produced 9 reproducible fragments on Xba1 PFGE gels,

Table 1: Patients and strains-related data

Strain n	Group	Age	Sex	vacA	cagA status genotype
1	I	09	boy	s1m2	negative
2	I	12	girl	s1m1	negative
3	I	06	boy	s1m2	positive
4	I	10	girl	s1m2	positive
5	I	13	boy	s1m1	positive
6	I	04	girl	s2m2	negative
7	I	11	girl	s1m2	positive
8	I	05	girl	s2m1	positive
9	II	14	boy	s2m1	negative
10	II	09	boy	s1m1	positive
11	II	07	boy	s2m2	positive
12	II	11	girl	s1m2	negative
13	II	10	girl	s2m2	positive
14	III	12	boy	s1m1	positive
15	III	10	girl	s2m2	positive
16	III	13	Boy	s2m1	negative
17	III	11	girl	s1m2	positive
18	III	05	boy	s2m2	negative
19	III	11	boy	s1m2	positive
20	III	05	boy	s1m2	positive
21	III	11	boy	s2m1	positive
22	III	11	boy	s1m2	positive
23	III	10	girl	s2m1	negative
24	III	11	girl	s1m2	positive
25	III	07	girl	s2m1	positive
26	III	07	boy	s1m1	negative
27	IV	15	boy	s1m2	positive
28	IV	11	boy	s2m1	positive
29	IV	06	girl	s2m2	negative
30	IV	7.5	girl	s1m1	positive
31	IV	09	girl	s1m1	negative
32	IV	11	boy	s2m1	positive
33	IV	09	girl	s1m2	negative
34	IV	05	Boy	s1m1	negative
35	IV	8.5	boy	s2m2	positive
36	IV	13	Boy	s1m1	positive
37	IV	15	boy	s2m2	positive
38	IV	08	girl	s1m2	negative
39	IV	10	boy	s1m1	positive
40	IV	06	girl	s2m1	negative
41	IV	08	boy	s1m2	positive
42	IV	10	boy	s1m1	positive
43	IV	09	girl	s1m2	positive
44	IV	07	girl	s1m2	positive

Group I, II, III and IV corresponded to the strains isolated during 1997-1999, 2001-2003, 2005-2007, and 2007-2009, respectively

whereas 35 (80%) of the isolates showed 8 to 12 fragments, 4 isolates contained 5 to 7 fragments and 5 isolates showed 13-14 fragments. The strains were classified according to the numbers and the size of XbaI fragments on PFGE gels. Their comparison showed a marked genomic diversity. Association between the PFGE patterns of the strains and their *cagA* status, as well as *vacA* (*s*,*m*) alleles was evaluated. Relationship between the PFGE pattern of the strains and sex, age and pathological status of children was also assessed. No significant relationship was observed between the patterns of PFGE (number and size of the fragments) and the *cagA* status or *vacA*/*cagA* genotype (Fig 1, Table 2). Also, no significant relationship was observed between age, sex, and pathological status of the children and the PFGE patterns of their isolates. The PFGE patterns of the strains were also compared on the basis of isolation date (Fig. 2). To confirm the results of visual analysis, the patterns of PFGE were also analyzed using Total Lab software (Total Lab and Phoenix software, www.totallabs.com). The same conclusion was obtained concerning absence of significant relationship between the PFGE patterns of the isolates and their *vacA*/*cagA* genotype/status, and pathological status of the children. Fig 3 represents the relationship between PFGE patterns and *vacA*/*cagA*, as well as the pathological status among 12 isolates of group III (Total Lab and Phoenix software).

Comparison of the PFGE patterns between the isolates of group I (1997-99), II (2001-2003), III (2005-2007), and IV showed the considerable genomic changes over time.

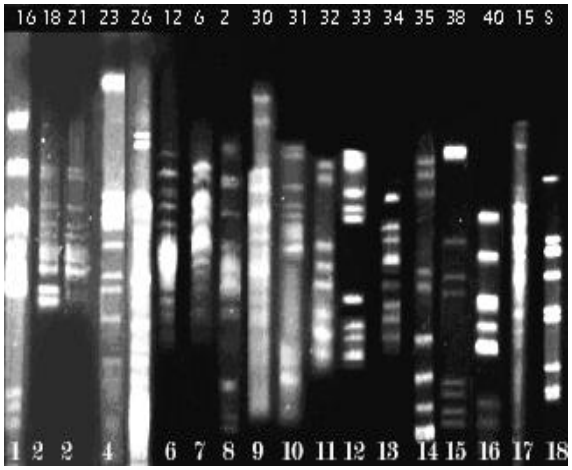

Fig. 1A: PFGE Profile comparing the *cagA*-positive with *cagA*-negative isolates

Numbers above of the fig represent the number of strains according to Table 1. Lanes 1, 2, 4-8, 10, 12-13, and 15-16 represent the *cagA*-negative isolates. Lanes 3, 9, 11, 14, and 17 represent the cagA-positive isolates. Lane 18: 26695 standard strain (sizes of XbaI digested fragments: 370, 270, 260, 215, 200, 100, 80, and <80 Kb.

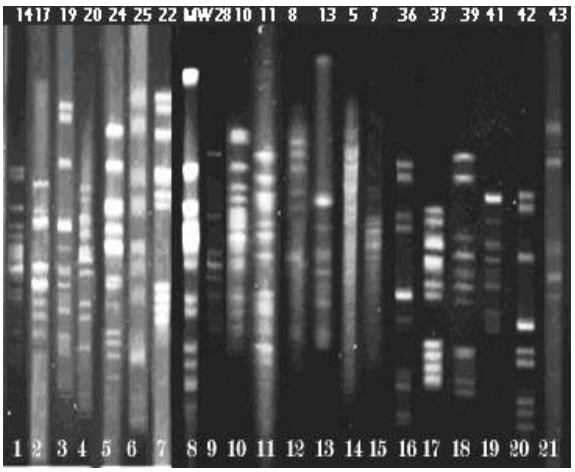

Fig. 1B: PFGE Profile comparing the *cagA*-positive isolates.

Numbers above of the fig represent the number of the strains according to Table 1. Lane 8: size marker *Staphylococcus aureus* NCTC 8325 strain (sizes of fragments: 674, 361, 324, 262, 257, 208, 175, 135, 80, and <80 Kb, respectively).

Discussion

Selection of unrelated children, favors comparison of genomic DNA among non-clonally related strains. To compare the patterns of PFGE among strains, several investigators have used *Not*I[19,31-32]. We found that *Xba* I restriction-endonuclease produced higher (5-14) number of fragments compared to *NotI* (4-8) which allowed a better comparison of various DNA fragments among strains. Comparison of PFGE patterns showed a marked genomic diversity among the strains (Fig. 1). This is in agreement with data reporting that diversity is more frequent in countries in which *H. pylori* infection is highly prevalent[33]. Most of the current information about genetic diversity of *H. pylori* has been obtained from genetic analysis of the sequence data obtained from *H. pylori* strains isolated in diverse geographical regions. In the present study, 44 *H. pylori* isolates from 44 different non-related individuals, were characterized in order to define the *H. pylori*

Table 2: Relationship between comparable shared fragments (bands) on PFGE gels and *vacA*/*cagA* genotype/status of the Strains

Genotype/Status	Number (%) of strains	Shared fragment(s) on PFGE gels
cagA-	16 (36)	no visible shared band
cagA+	28 (64)	no visible shared band
s2m2/cagA-	3 (7)	no shared band
s1m2/cagA-	5 (11)	no shared band
s2m1/cagA-	4 (9)	no shared band
s1m1/cagA-	4 (9)	one shared band (200 kb)
s2m2/cagA+	5 (11)	one shared band (130 kb)
s1m2/cagA+	11 (25)	one shared band (175 kb)
s2m1/cagA+	5 (11)	no shared band
s1m1/cagA+	7 (16)	two shared bands (340, 200 kb)

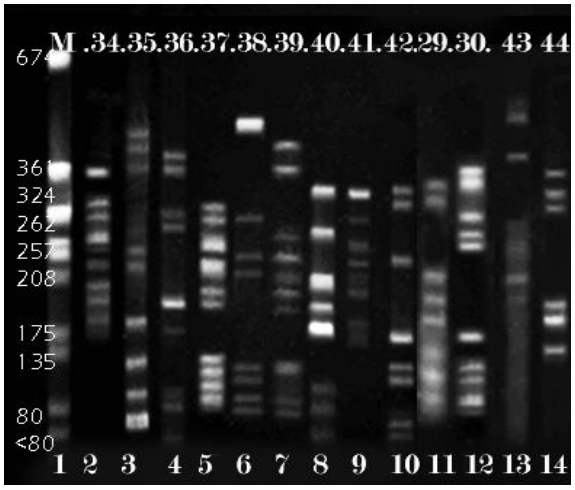

Fig. 2A: PFGE profile of isolates from group IV
Numbers above figures represent the number of strains according to Table 1. Lane 1: Size marker *Staphylococcus aureus* NCTC 8325 strain.

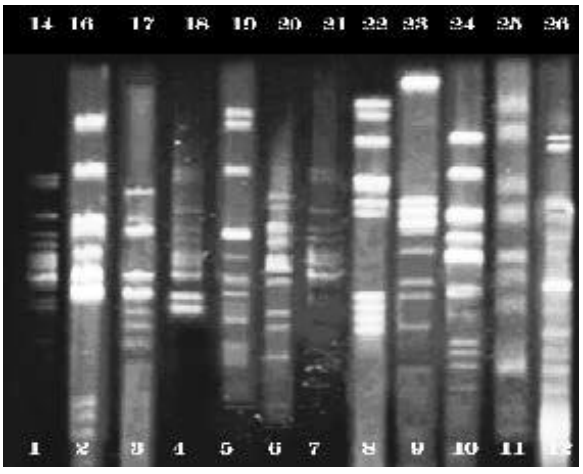

Fig. 2B: PFGE profile of isolates of group III.
Numbers above figures represent the number of strains according to Table 1.

population structure. It is a first work performed in this region demonstrating genetic diversity among *H. pylori* isolates from non-related patients.

Comparison of the PFGE patterns suggested that genomic diversity of the strains was not related to the genotype of *vacA*, status of *cagA* and the status of gastric inflammation in children (Fig. 1-2, Table 2). The fact that identical PFGE pattern did not occur in the strains with similar pathogenicity-associated markers such as *cagA* and *vacA* may suggest that the DNA fragments undergoing genetic changes did not encode proteins that are involved in virulence. Comparison of multiple *H. pylori* strains by different methods have shown that *H. pylori* genome has highly plastic gene content and nearly half of the strain-specific genes may be located in each region[34].

Among our isolates, more relationship was observed between the strains isolated in the close period (1997- 2009, 2001-2003, 2005-2007, and 2007-2009) and more difference was observed among those obtained in the distant periods (1997 and 2009) regardless of their *cagA*/*vacA* status/genotype. This may be due to adaptation of *H. pylori* strains to variable living conditions during transmission between various host individuals over time. Comparison of 44 unrelated strains suggested that the degree of genetic diversity occurred in this region is very high but this diversity is not related to virulence determinants of the strains, sex, age and pathological status of the children. As these genomic changes may be related to adaptation of *H. pylori* strains to variable living conditions during transmission between various hosts, it may

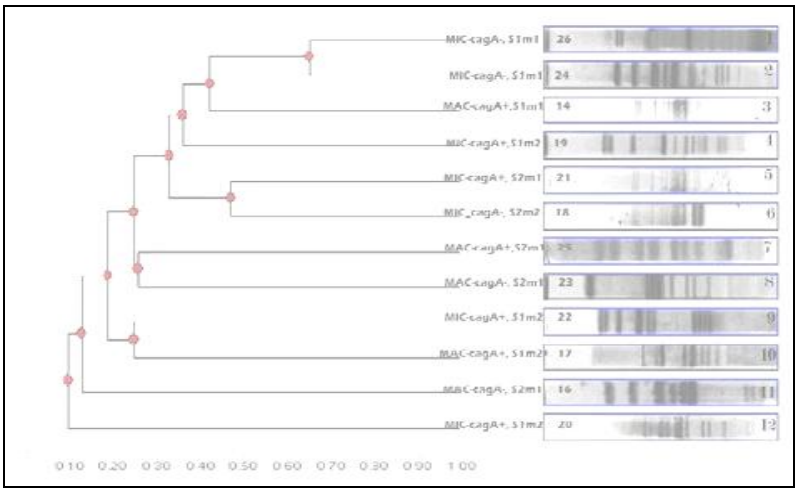

Fig. 3: Representative dendrogram for demonstrating relationship between PFGE patterns and *vacA*/*cagA* and pathological status among 12 isolates of group III performed by Total Lab and Phoenix software. Numbers in left represent the number of strains according to the Table

also increase during time in the regions with the high rate of infection such as Iran.

Conclusion

H. pylori strains isolated from children in Iran are extremely diverse and this diversity is not related to vacA /cagA genotype/status of the strains, as well as to sex, age and pathological status of the child patients. As more relationship existed between the strains isolated in the close period, this diversity may be related to adaptation of H. pylori strains to variable living conditions in various host, during time.

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Authors' Contribution

T. Falsafi, M.M Feizabadi: Design the study and write the manuscript also provide vital analytical tools
N. Sotoudeh: Performe the majority of experiments
F. Mahjoub: Performe the pathological examination and participate to critical reading of the manuscript.
All authors approved final version of the manuscript.

Conflict of Interest: None

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