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

Tahereh Falsafi¹ PhD; Nazli Sotoudeh¹ Msc; Mohammad-Mehdi Feizabadi² PhD; Fatemeh Mahjoub³ MD

¹Department of Microbiology, Faculty of Biology, Alzahra University, Tehran, ²Department of Genetics, ³Department of Pathology, Tehran University of Medical Sciences, Tehran, Iran

Received: Jan 02, 2014; Accepted: Nov 11, 2014; First Online Available: Nov 28, 2014

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.

Iranian Journal of Pediatrics, Volume 24 (Number 6), December 2014, Pages: 703-709

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...
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 PFG E[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.

| 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.

**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 NotI[19,31,32]. We found that XbaI 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-      | 5 (9)                 | no shared band                          |
| s1m1/cagA-      | 4 (11)                | one shared band (200 kb)                |
| s2m2/cagA+      | 5 (11)                | one shared band (130 kb)                |
| s1m2/cagA+      | 5 (11)                | one shared band (175 kb)                |
| s2m1/cagA+      | 11 (25)               | 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
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.

**Acknowledgment**

We thank all members of Children’s Medical Center, Tehran (Department of Gastroenterology & Pathology) for their help in data collection. This work was supported by a grant from Vice Chancellor for Research, Alzahra University, Iran. It was also supported by Dr. M.M. Feizabadi, Tehran University of Medical Sciences, Tehran, Iran.

**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

**References**

1. Del Giudice G, Malfertheiner P, Rappuoli R. Development of vaccines against Helicobacter pylori. *Expert Rev Vaccines* 2009; 8(8):1037-49.

2. Fischback W, Chan AO, and Wang BC. *Helicobacter pylori* and gastric malignancy. *Helicobacter* 2005; 10(Suppl 1):34-9.

3. Achtman M, Azuma T, Berg DE, et al. Recombination and clonal groupings within *Helicobacter pylori* from different geographical regions. *Mol Microbiol* 1999; 32(3):459-70.

4. Salaun L, Linz B, Suerbaum S, et al. The diversity within an expanded and redefined repertoire of phase-variable genes in *Helicobacter pylori*. *Microbiol* 2004; 150(Pt 4):817-30.

5. Saunders NJ, Boonmee P, Peden JF, et al. 2005. Inter-species horizontal transfer resulting in core-genome and niche-adaptive variation within *Helicobacter pylori*. *BMC Genomics* 2005; 6:9.

6. Linz B, Schuster SC. Genomic diversity in *Helicobacter* and related organisms. *Res Microbiol* 2007; 158(10):737-44.

7. Raymond J, Thibierge JM, Chevalier C, et al. Genetic and transmission analysis of *Helicobacter pylori* strains within a family. *Emerging Infect Dis* 2004; 10(10):1816-21.
8. Solca NM, Bernasconi MV, Valsangiacomo C, et al. Population genetics of Helicobacter pylori in the southern part of Switzerland analyzed by sequencing of four housekeeping genes (atpD, glmA, scoB and recA) and by vacA, cagA, iceA and IS605 genotyping. Microbiology 2001; 147(Pt 6):1693-707.

9. Falush D, Wirth T, Linz B, et al. Traces of human migrations in Helicobacter pylori populations. Science 2003; 292(5612):1582-5.

10. Kersulyte D, Mulhopadhyay AK, Velapatinio B, et al. Differences in genotypes of Helicobacter pylori from different human populations. J Bacteriol 2000; 182(11):3210-8.

11. Van der Ende A, Pan ZJ, Bart A, et al. cagA-Helicobacter pylori populations in China and the Netherlands are distinct. Infect Immun 1998; 66(5):1822-6.

12. Zhang Y, Liu H, Zhou H. Lack of correlation of vacA genotype, cagA gene of Helicobacter pylori and their expression products with various gastroduodenal diseases. Chin Med J (Engl) 2001; 114(7):703-6.

13. Israel DA, Salama N, Krishna U, et al. Helicobacter pylori genetic diversity within the gastric niche of a single human host. Proc Natl Acad Sci U S A 2001; 98(25):14625-30.

14. Kraft C, Stack A, Josenhans C, et al. Genomic changes during chronic Helicobacter pylori infection. J Bacteriol 2006; 188(1):249-54.

15. Park CY, Kwak M, Gutierrez O, et al. Comparison of genotyping Helicobacter pylori directly from biopsy specimens and genotyping from bacterial cultures. J Clin Microbiol 2003; 41(7):3336-8.

16. Ribot EM, Fitzgerald C, Kubota K, et al. Rapid pulsed-field gel electrophoresis protocol for subtyping of Campylobacter jejuni. J Clin Microbiol 2001; 39(5):1889-94.

17. Owen RJ, Xerry J. Tracing donality of Helicobacter pylori infecting family members from analysis of DNA sequences of three housekeeping genes (urel, atpA and aphC), deduced amino acid sequences, and pathogenicity-associated markers (cagA and vacA). J Med Microbiol 2003; 52:515-24.

18. Roma-Giannikou E, Karameris A, Balatsos B, et al. Intrafamilial spread of Helicobacter pylori: a genetic analysis. Helicobacter 2003; 8(1):15-20.

19. Smith SI, Luck PC, Bayerdorfer E, et al. 2003. Genotyping of Nigerian, Helicobacter pylori isolates by pulsed-field gel electrophoresis. J Med Microbiol 2003; 52(Pt 10):931.

20. Taylor DE, Eaton M, Chang N. Construction of a Helicobacter pylori demonstration of diversity at the genome level. J Bacteriol 1992; 174(21):6800-6.

21. Tee W, Lambert J, Smallwood R, et al. Ribotyping of Helicobacter pylori from clinical specimens. J Clin Microbiol 1992; 30(6):1562-7.

22. Kiru JN, Saidi SM, Goddeersis BM, et al. Molecular characterization of Vibrio cholerae O1 strains carrying a SXT/R391-like element from cholera outbreaks in Kenya: 1994-2007. BMC Microbiol 2009; 9:275.

23. Raymond J, Thibierge JM, Chevalier C, et al. Genetic and Transmission Analysis of Helicobacter pylori strains within a family. Emerg Infect Dis 2004; 10(10):1816-21.

24. Falsafi T, Mobasheri F, Nariman F, et al. Susceptibilities to different antibiotics of Helicobacter pylori strains isolated from patients at the Pediatric Medical Center of Tehran, Iran. 2004. J Clin Microbiol 2004; 42(1):387-9.

25. Dixon MF, Genta RM, Yardley JH, et al. Classification and grading of gastritis. The updated Sydney System. International Workshop on the Histopathology of Gastritis. Houston 1994. Am J Surg Pathol 1996; 20(10):1161-81.

26. Falsafi T, Valizadeh N, Najafi M, et al. Culture of Helicobacter pylori from Stool Sample in Children. Can J Microbiol 2007; 53(3):411-6.

27. Falsafi T, Favaedi R, Mahjoub F, et al. Application of Stool-PCR test for diagnosis of Helicobacter pylori infection in children. World J Gastroenterol 2009; 15(4):484-8.

28. Chang N, Taylor DE. Use of pulsed-field gel electrophoresis to size genomes of Campylobacter species and to construct a SalImap of Campylobacterjejuni UA580. J Bacteriol 1990; 172(9):5211-7.

29. Taylor DE, Eaton M, Yan W, et al. Genome maps of Campylobacterjejuni and Campylobacter coli. J Bacteriol 1992; 174(7):2332-7.

30. Yeh YC, Chang KC, Yang JC, et al. Association of metronidazole resistance and natural competence in Helicobacter pylori. Antimicrob Agents Chemother 2002; 46(5):1564-7.

31. Salama S, Jiang Q, Chang N. Characterization of Chromosomal DNA Profiles from Helicobacter pylori strains isolated from sequential gastric biopsy specimens. J Clin Microbiol 1995; 33:2496-7.

32. Hosaka Y, Irihoda K, Nakano R, et al. Use of restriction enzyme EcoRI for pulsed-field gel electrophoretic analysis of Helicobacter pylori. J Clin Microbiol 2005; 43(2):931-2.

33. Hussein NR. Helicobacter pylori and gastric cancer in the Middle East: A new enigma. World J Gastroenterol 2010; 16(26):3226-34.

34. Yamaoka Y. Roles of the plasticity regions of Helicobacter pylori in gastroduodenal pathogenesis. J Med Microbiol 2008; 57(Pt 5), 545-53.