Iranian J Publ Health, Vol. 38, No.2, 2009, pp.98-105

Original Article

Simultaneous Detection of *Caga* and *Cage* of *Helicobacter pylori* Strains Recovered from Iranian Patients with Different Gastroduodenal Diseases

*M Douraghi [1,2], M Mohammadi [2], *MH Shirazi[1], A Oghalaie [2], S Saberi Kashani [2], MA Mohagheghi[3], M Eshagh Hosseini [4], H Zeraati [5], M Esmaili [2], M Bababeik [2], N Mohajerani [2]*

[1]*Dept. of Pathobiology, School of Public Health, Tehran University of Medical Sciences, Iran*
[2]*Helicobacter pylori Research Group, Biotechnology Research Center, Pasteur Institute of Iran, Tehran, Iran*
[3] *Cancer Research Center, Tehran University of Medical Sciences, Iran*
[4]*Endoscopy Unit, Amiralam Hospital, Tehran University of Medical Sciences, Iran*
[5]*Dept. of Epidemiology and Biostatistics, School of Public Health, Tehran University of Medical Sciences, Iran*

(Received 11 Nov 2008; accepted 21 May 2009)

Abstract

Background: To asses the status of two representative genes of *cag* PAI i.e *cagA* and *cagE* of *Helicobacter pylori* strains infecting Iranian patients suffered from various clinical outcomes using one-step PCR.

Methods: A total of 120 *H. pylori* infected patients including non–ulcer dyspepsia, NUD (n=81), peptic ulcer disease, PUD (n=17), and gastric carcinoma, GC (n= 22) referred for endoscopy or gastric resection to AmirAlam Hospital or Cancer Institute from 2005 to 2008 were assessed. The status of *cagA* and *cagE* genes was determined by gene specific PCR.

Results: 84.2% and 90.8% of the tested strains were positive for *cagA* and *cage*, respectively. 81.7% strains were positive for both *cagA* and *cagE* genes, whereas 8 (6.7%) were found double negative. The prevalence of *cagA* in GC patients (100%) was slightly higher than PUD patients (94.1%). All of GC cases were infected with *cagA*-positive strains. The same distribution pattern was indicated for *cagE* gene in GC and PUD patients. The *cagA*-positive strains were significantly associated with GC as compared with NUD ($P< 0.05$) but this association did not gain statistical significance when *cagE* gene was assessed.

Conclusion: The concurrent detection of *cagA*/*cagE* genes allowed rapid and specific clarification of *cag* PAI status. The strains with *cagA*/*cagE* genotype are predominant in Iran regardless of clinical outcome and create a distinct cluster pattern from those in the West and similar to those of East Asian countries. The current study also demonstrated that *cagE* gene can be explored as a better indication of *cag*-PAI in Iranian *H. pylori* strains.

Keywords: Helicobacter pylori**,** Cag *PAI*, *Peptic ulcer, Gastric ulcer, Gastric carcinoma, Iran*

Introduction

Helicobacter pylori infect more than half the human population worldwide. Infection of gastric mucosa with *H. pylori* results in different disease outcomes including gastritis, peptic ulcer disease, and gastric adenocarcinoma (1-3).

Various studies have demonstrated that bacterial virulence factors, host genetic and environmental factors contribute to the development of disease (4). The most studied virulence marker of *H. pylori* is cytotoxin-associated gene pathogenicity-island (*cag* PAI) which is horizontally transferred from one strain to another. *Cag* PAI is a 40-kb gene locus in the chromosomal glutamate racemase gene with a 35% G+C content distinguishing it from of the rest of the bacterial genome with a 39% G+C content (5). *cag* PAI contains 31 genes, six of which encode type IV secretion system and are involved in *H. pylori*-host cell interactions and pathogenesis (6,7). *CagA* and *cagE* are the representative genes of *cag* PAI; *cagA* gene is located in the right half of the *cag* island affecting host cells following delivery into the gastric epithelial cells, whereas

***Corresponding author:** Tel: +98 21 66112379, E-mail: mhshirazi@sina.tums.ac.ir

the *cagE* gene is located upstream of *cagA,* and encodes a protein involved in the process of interleukin 8 expression and translocation of *cagA* (8, 9). Only 1/2 to 2/3 of Western isolates carry the *cag* PAI gene cluster. This is in contrast to East Asian strains, nearly all of which carry this gene cluster. The severity of *H. pylori*-related disease is correlated with the presence of the *cag* PAI. Infection with *cag* PAI-positive *H. pylori* is associated with duodenal ulcer, gastric mucosal atrophy and gastric cancer (10-12) Therefore, the presence and the integrity of the *cag* island appears to be a critical factor in the interaction between *H. pylori* and its host resulting in a variety of subsequent GI disorders.
In this study, we aimed to asses the status of two representative genes of *cag* PAI i.e *cagA* and *cagE* of *H. pylori* strains infecting Iranian patients suffering from various clinical outcomes using one-step PCR.

Material and Methods

Subjects

This cross-sectional study conducted for 120 *H. pylori* infected patients with upper gastrointestinal disorders referred for endoscopy to Amir Alam Hospital or gastric resection to Cancer Institute of Tehran University of Medical Sciences, Iran between 2005-2008. The subjects consisted of 81 non-ulcer dyspepsia (NUD), 17 peptic ulcer disease (PUD), and 22 gastric cancer (GC) patients. Informed consent was obtained from every patient prior to sample collection which was performed according to standard protocols approved by the local ethical committee.

Bacterial strains

H. pylori strains were primarily isolated from two antral biopsy specimens. Tissue samples were homogenized and plated onto *H. pylori* Specific Pepton Agar (HPSPA) medium (13). Incubation was performed at 37° C in microaerophilic atmosphere (10% CO2, 5% O2 and 85% N2) up to 5 d. Identification was based on morphology under Gram staining and biochemical tests including urease and catalase tests. One single colony was expanded and collected for further studies.

DNA extraction

Briefly, bacterial genomic DNA was extracted by incubating bacterial pellets of one single colonies in 50mM NaOH at 100° C for 20 min, followed by 10 min incubation in 1M Tris-HCl, pH 7.5. The supernatants containing genomic DNA were used as the template for PCR amplification (14).

PCR assays

The *ureC* gene was primarily amplified to confirm the identity of the isolated *H. pylori* strains in accordance with published reports (15).
Amplification of conserved regions of *cagA* and *cagE* genes were performed simultaneously using specific primers (Table 1). Every PCR was repeated to confirm the results. Amplification of the target genes was carried out in a total volume of 20 µl containing 2 µl of 10×PCR buffer (Fermentas, Lithuania), 1.5 mM of $MgCl_2$, 0.5 µl of each primer (25 pM/ µl), 0.2 mM of deoxynucleotide, 0.5 U of Taq Polymerase. The cycling programs, was preceded by 4 min at 94° C and consisted of 35 cycles of 94° C for 50 s, 54° C for 50 s and 72° C for 50 s, followed by a final extension step at 72° C for 4 min. PCR products were electrophoresed in 2.0% agarose gels and visualized by staining with ethidium bromide under UV light. *H. pylori* 26695 strain was used as a reference strain for every assay (14).

Statistical Analysis

Data were analyzed using SPSS (version 11.5) program. For univariate analysis, $\chi 2$ test and Fisher's exact test were used. A P value of ≤ 0.05 was considered statistically significant.

Results

Confirmation of H. pylori *identity*

All of the 120 DNA samples were amplified with the *ureC*-specific oligonucleotide primers and yielded a 294-bp PCR product confirming the identity of *H. pylori* thereby confirming presence of *H. pylori*.

Distribution of cagA and cagE genes

One hundred one (84.2%) of the 120 tested strains were amplified using gene-specific primers for conserved region of *cagA* and were thus considered *cagA*-positive. Only 19 (15.8%) of *H. pylori*-infected patients regardless of disease category were *cagA*-negative. The *cagE* gene was detected in 90.8% (109/120) of *H. pylori* strains. Eleven (9.2%) of the studied strains were not amplified using primers designed for detection of *cagE* and were thus reported as *cagE*-negative strains.

Coexistence of cagA and cagE

Ninety eight (81.7%) strains were positive for both *cagA* and *cagE* genes, whereas 8 (6.7%) were found double negative. The presence of the *cagA* and *cagE* genes was strongly linked ($P< 0.001$) with a trivial number of strains (2.5%) possessing *cagA* positive/*cagE* negative genotype. A *cagA* negative/*cagE* positive genotype was detected in eleven (9.2%) strains. The distribution of different *cagA*/*cagE* genotypes in relation to clinical outcome was as indicated in Table 2.

Association between cagA/cagE and clinical outcomes

Distribution of *H. pylori cagA/cagE* positive and negative strains in patients with different clinical diagnosis is as depicted in Fig. 3. The prevalence of *cagA* in GC patients was slightly higher than (100%) that in PUD patients (94.1%). All of GC cases were infected with *cagA*-positive strains. The same distribution pattern was indicated for *cagE* gene in GC and PUD patients. More than half of NUD cases (77.8%) also harbored *cagA*-positive strains. Accordingly, 87.7% of NUD cases were infected with *cagE*-positive *H. pylori* strains. *CagA-positive* strains were significantly associated with GC as compared with NUD ($P< 0.05$) but this association did not gain statistical significance with *cagE* gene.

Table 1: Primer sequences and DNA amplification conditions for each selected region

Amplified region	Primer	Primer sequence	Size of amplified fragment (bp)	Reference
UreC	HP-F HP-R	5'-GGA TAA GCT TTT AGG GGT GTT AGG GG-3' 5'-GCT TAC TTT CTA ACA CTA ACG CGC-3'	294	(15)
CagA	F1 B1	5'-GATAACAGGCAAGCTTTTGAGG-3' 5'-GCGTCAAAATAATTCCAAGG-3'	349	(16)
CagE	cagE-F cagE-R	5'-TTGAAAACTTCAAGGATAGGATAGAGC -3' 5'-GCCTAGCGTAATATCACCATTACCC -3'	508	(17)

Table 2: Coexistence of *cagA*/*cagE* in relation to clinical outcome

Clinical outcome	*cagA*/*cagE* status n (%)				
	Pos/Pos	Pos/Neg	Neg/Pos	Neg/Neg	Total
NUD	60 (74.1)	3 (3.7)	11 (13.6)	7 (8.6)	81 (100)
PUD	16 (94.1)	0 (0)	0 (0)	1 (5.9)	17 (100)
GC	22 (100)	0 (0)	0 (0)	0 (0)	22 (100)
Total	98 (81.7)	3 (2.5)	11 (9.2)	8 (6.7)	120 (100)

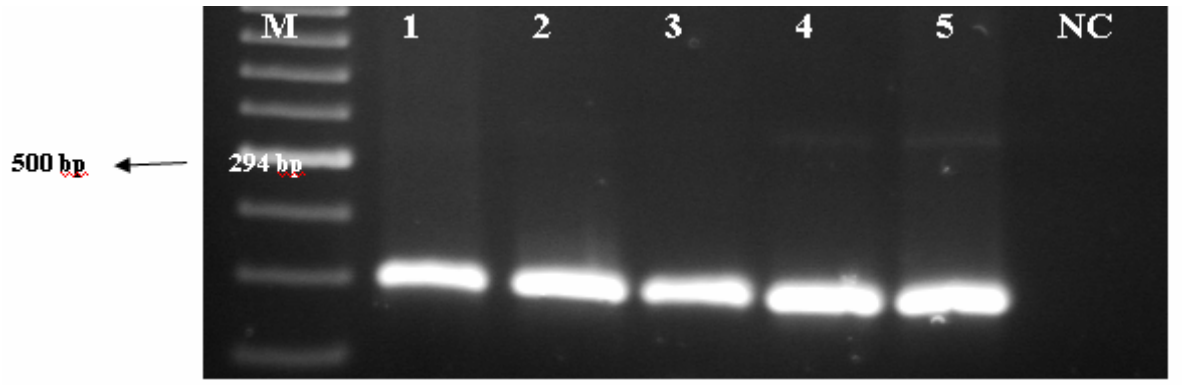

Fig. 1: The *ureC* gene amplification using specific oligonucleotide primers yielded a product of 294 -bp typical to *H. pylori* . NC and M illustrate the negative control and 100 bp DNA marker respectively

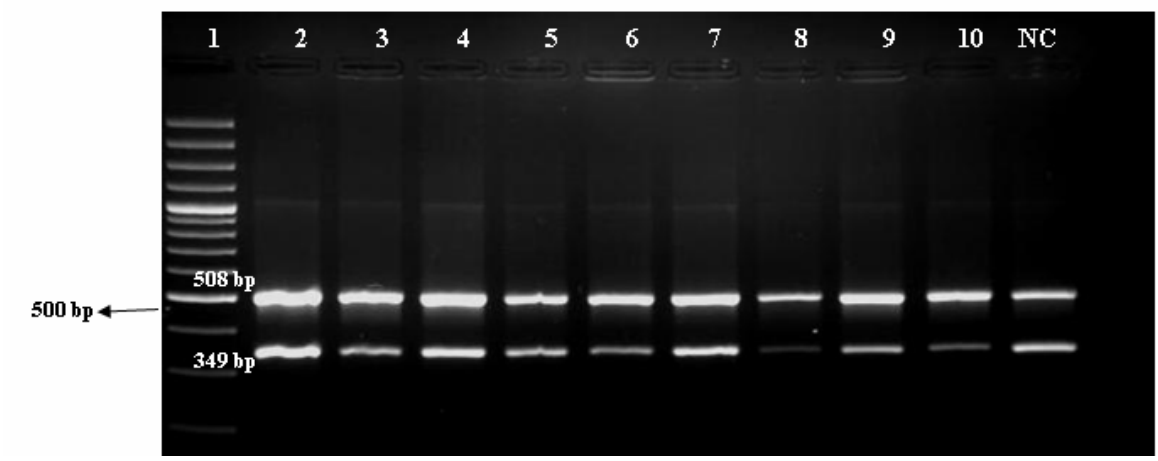

Fig. 2: The representative of simultaneous detection of *cagA* and *cagE* yielded 349 bp and 508 bp amplicon respectively. NC and M illustrate the negative control and 100 bp DNA marker respectively

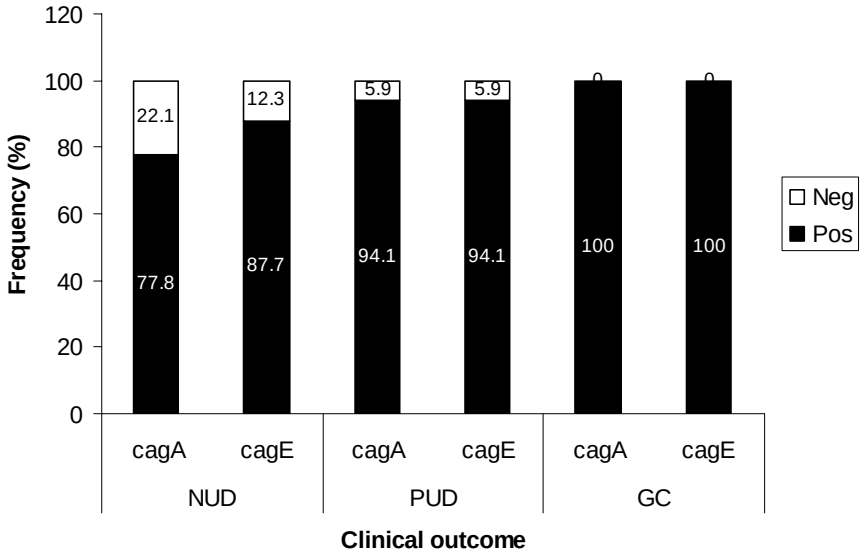

Fig. 3: Distribution of *cagA*/*cagE* in relation to clinical outcome

Discussion

H. pylori is associated with diverse diseases such as chronic gastritis, peptic ulcers, mucosa-associated lymphoid tissue (MALT) lymphoma, and gastric carcinoma (1-3). The occurrence of such diverse diseases is a multifactor process dependent on specific properties of the organism, host and environmental factors. Strain-specific diversity has been proposed to be involved in the organism's ability to cause different diseases. Furthermore, significant geographical differences have been demonstrated among *H. pylori* strains around the world.

The *cag* PAI is a major virulence determinant in *H. pylori* and strains lacking this island induce less pathogenicity that strains harboring *cag* PAI (8). Both *cagA* and *cagE* genes constitute major segments of the *cag* PAI, thus detection of the fomer genes may indicate the integrity of the latter gene.

This study carried out the simultaneous amplification of representative genes of *cag* PAI including *cagA* and *cagE* of Iranian *H. pylori* strains yielding distinguishable PCR products of distinct sizes. The concurrent detection of mentioned genes allowed rapid and specific clarification of *cag* PAI status. In addition, this one step PCR assay is cost and time efficient and recommended for routine laboratory use.

Analysis of *cagA* gene in patient with NUD, PUD, or GC revealed that it is present in 84.2% of examined strains regardless of the clinical status. The distribution of *cagA*-positive *H. pylori* strains in Iran is higher than Western countries such as the Netherlands (67%) and is closer to some geographical regions such as Ethiopia (79%), Brazil (94%), Korea (97%) and Japan (95%) (18-22). The variations in the prevalence of *cagA* gene in different countries may be due to distinct dyspeptic populations and geographical heterogeneity of *H. pylori* isolates. Although the possession of *cagA* is associated with GC when compared to NUD, due to the uniform distribution of *cagA* in all other disease categories detection of *cagA* alone can not be considered as a discriminative marker for a specific clinical outcome. Accordingly, in other Asian countries where the majority of *H. pylori*-infected individuals harbor *cagA*-positive strains, associations of *cagA* status and diseases are not observed (23, 24).

In the present study, 109 of 120 (90.8%) of strains possessed the *cagE* gene and its prevalence was higher among subjects with DU and GC as compared to NUD patients. *CagE* is more prevalent in Iranian *H. pylori* strains than that of Turkey (59.3%), United States (62%), Malaysia (59%) and is closer to Brazil (89.3%) and India (85.4%) (25-29). Although distribution of *cagE* was higher in GC and DU than NUD cases , the possession of *cagE* gene was not significantly associated with clinical pictures, a finding which is in contrast to previous study reporting a significant association between the *cagE* gene and DU (30).

The current study demonstrated that *cagE* gene can be explored as a better marker of *cag*-PAI in Iranian *H. pylori* strains. Previous studies in Japanese populations (22) and French isolates (31) also confirmed the application of *cagE* as a superior marker for *cag* PAI detection. This finding is in accordance with the observation of Ikenoue et al., who suggested that the *cagE* gene is a more precise marker of an intact *cag* island than are other *cag* genes (32). A study in Brazil also demonstrated that the frequency of *cagE* gene was higher than that of *cagA* and reported the former gene as a more accurate marker of *cag* PAI (33). Nevertheless, our study is in agreement with the study by Modena who showed no association between *cagE* and disease outcomes (34).

In conclusion, the majority of *H. pylori* strains in Iran are *cagA*/*cagE* double positive regardless of clinical outcome and construct a different pattern cluster from those in the West and similar to East Asian countries. Furthermore, this study introduces *cagE* as a more accurate marker of *cag* PAI gene cluster.

Acknowledgements

The authors declare that there is no conflict of interests.

References

1. McColl KE, El-Omar E, Gillen D (2000). *Helicobacter pylori* gastritis and gastric physiology. *Gastroenterol Clin North Am,* 29: 687-703.
2. Peek Jr RM, Blaser MJ (2002). *Helicobacter pylori* and gastrointestinal tract adenocarcinomas. *Nat Rev Can*, 2: 28-37.
3. Peterson WL (1991). *Helicobacter pylori* and peptic ulcer disease. *N Engl J Med*, 324: 1043-48.
4. Nguyen TN, Barkun AN, Fallone CA (1991). Host determinants of *Helicobacter pylori* infection and its clinical outcome. *Helicobacter,* 4: 185-97.
5. Censini S, Lange C, Xiang Z, Crabtree JE, Ghiara P., Borodovsky M, Rappuoli R, Covacci A (1996). *Cag*, a pathogenicity island of *Helicobacter pylori,* encodes type-I specific and disease-associated virulence factors. *Proc Natl Acad Sci U.S.A.,* 93: 14648-53.
6. Shibata W, Hirata Y, Maeda S, Ogura K, Ohmae T, Yanai A, Mitsuno Y, Yamaji Y, Okamoto M, Yoshida H, Kawabe T, Omata M (2006). CagA protein secreted by the intact type IV secretion system leads to gastric epithelial inflammation in the Mongolian gerbil model. *J Pathol,* 210: 306-14.7.
7. Odenbreit S, Puls J, Sedlmaier B, Gerland E, Fischer W, Haas R (2000). Translocation of *Helicobacter pylori* CagA into gastric epithelial cells by type IV secretion. *Science*, 287: 1497-500.
8. Proença Módena JL, Lopes Sales AI, Olszanski Acrani G, Russo R, Vilela Ribeiro MA, Fukuhara Y, da Silveira WD, Módena JL, de Oliveira RB, Brocchi M (2007). Association between *Helicobacter pylori* genotypes and gastric disorders in relation to the *cag* pathogenicity island. *Diagn Microbiol Infect Dis*, 59: 7-16.
9. Owen RJ, Sharp S, Lawson AJ, Durrani Z, Rijpkema S, Kidd M (2003). Investigation of the biological relevance of *Helicobacter pylori cagE* locus diversity, presence of CagA tyrosine phosphorylation motifs and vacuolating cytotoxin genotype on IL-8 induction in gastric epithelial cells. *FEMS Immunol Med Microbiol,* 36: 135-40.
10. Tan HJ, Rizal AM, Rosmadi MY, Goh KL (2006). Role of *Helicobacter pylori* virulence factor and genotypes in non-ulcer dyspepsia. *J Gastroenterol Hepatol*, 21: 110-15.
11. Sozzi M, Tomasini ML, Vindigni C, Zanussi S, Tedeschi R, Basaglia G, Figura N, De Paoli P (2005). Heterogeneity of *cag* genotypes and clinical outcome of *Helicobacter pylori* infection. *J Lab Clin Med*, 146: 262-70.
12. Graham DY, Yamaoka Y (1998). *H. pylori* and *cagA*: relationship with gastric cancer, duodenal ulcer, and reflux esophagitis and its complications. *Helicobacter,* 3: 145-51.
13. Stevenson TH, Castillo A, Lucia LM, Acuff GR (2000). Growth of *Helicobacter pylori* in various liquid and plating media. *Lett Appl Microbiol*, 30: 192-96.
14. Sambrook J, Russell D (2001). Molecular cloning. 3 rd ed. Cold Spring Harbor Lab Press. USA.
15. Labigne A, Cussac V, Courcoux P (1991). Shuttle cloning and nucleotide sequencing of *Helicobacter* genes responsible for urease activity. *J Bacteriol,* 173: 1920-31.
16. Tummuru MKR, Cover TL, Blaser MJ (1993). Cloning and expression of a high molecular weight major antigen of *Helicobacter pylori*. *Infect Immun*, 61: 1799-809.

17. Tomasini ML, Zanussi S, Sozzi M, Tedeschi R, Basaglia G, De Paoli P (2003). Heterogeneity of *cag* genotypes in *Helicobacter pylori* isolates from human biopsy specimens. *J Clin Microbiol*, 41: 976–980.
18. van Doorn LJ, Figueiredo C, Sanna R, Plaisier A, Schneeberger P, de Boer W, Quint W (1998). Clinical relevance of the *cagA*, *vacA*, and *iceA* status of *Helicobacter pylori*. *Gastroenterology*,115:58–66.
19. Asrat D, Nilsson I, Mengistu Y, Kassa E, Ashenafi S, Ayenew K, Wadström T, Abu-Al-Soud W (2004). Prevalence of *Helicobacter pylori vacA* and *cagA* genotypes in Ethiopian dyspeptic patients. *J Clin Microbiol,* 42: 2682-684.
20. Ashour AA, Magalhaes PP, Mendes EN, Collares GB, de Gusmão VR, Queiroz DM, Nogueira AM, Rocha GA, de Oliveira CA (2002). Distribution of *vacA* genotypes in *Helicobacter pylori* strains isolated from Brazilian adult patients with gastritis, duodenal ulcer, or gastric carcinoma. *FEMS Immunol Med Microbiol*, 33: 173-78.
21. Kim SY, Woo CW, Lee YM, Son BR, Kim JW, Chae HB, Youn SJ, Park SM (2001). Genotyping *cagA*, *vacA* subtype, *iceA1*, and *babA* of *Helicobacter pylori* isolates from Korean patients, and their association with gastroduodenal diseases. *J Korean Med Sci*, 16: 579-84.
22. Maeda S, Yoshida H, Ikenoue T, Ogura K, Kanai F, Kato N, Shiratori Y, Omata M (1999). Structure of *cag* pathogenicity island in Japanese *Helicobacter pylori* isolates. *Gut*, 44: 336-341.
23. Maeda S, Ogura K, Yoshida H, Kanai F, Ikenoue T, Kato N, Shiratori Y, Omata M (1998). Major virulence factors, VacA and CagA, are commonly positive in *Helicobacter pylori* isolates in Japan. *Gut*, 42: 338–43.
24. Yamaoka Y, Souchek J, Odenbreit S, Haas R, Arnqvist A, Borén T, Kodama T, Osato MS, Gutierrez O, Kim JG, Graham (2002). Discrimination between cases of duodenal ulcer and gastritis on the basis of putative virulence factors of *Helicobacter pylori*. *J Clin Microbiol*, 40: 2244-46.
25. Erzin Y, Koksal V, Altun S, Dobrucali A, Aslan M, Erdamar S, Dirican A, Kocazeybek B (2006). Prevalence of *Helicobacter pylori vacA*, *cagA*, *cagE*, *iceA*, *babA2* Genotypes and Correlation with Clinical Outcome in Turkish Patients with Dyspepsia. *Helicobacter*, 11: 574-580.
26. Podzorski RP, Podzorski DS, Wuerth A, Tolia V (2003). Analysis of the *vacA, cagA, cagE, iceA,* and *babA2* genes in *Helicobacter pylori* from sixty-one pediatric patients from the Midwestern United States. *Diagn Microbiol Infect Dis*, 46: 83-88.
27. Tan HJ, Rizal AB, Rosmadi MY, Goh KL (2005). Distribution of *cagA*, *cagE* and *vacA* in different ethnic groups in Kuala Lumpur, Malaysia. *J Gastroenterol Hepatol*, 20: 589-94.
28. Mattar R, Marques SB, do Socorro Monteiro M, dos Santos AF, Iriya K, Carrilho FJ (2007). *Helicobacter pylori cag* pathogenicity island genes: clinical relevance for peptic ulcer disease development in Brazil. *J Med Microbiol*, 56: 9-14.
29. Tiwari SK, Khan AA, Manoj G, Ahmed S, Abid Z, Habeeb A, Habibullah CM (2007). A simple multiplex PCR assay for diagnosing virulent *Helicobacter pylori* infection in human gastric biopsy specimens from subjects with gastric carcinoma and other gastro-duodenal diseases. *J Appl Microbiol*, 103: 2353-60.
30. Day SA, Jones NL, Lynett JT, Hilary A. Jennings HA, Fallone CA, Beech R, Sherman PM (2000). *CagE* is a virulence factor associated with *Helicobacter pylori*-

induced duodenal ulceration in children. *J Infect Dis* ,181:1370-75.

31. Audibert C, Burucoa C, Janvier B, Fauchere JL (2001). Implication of the structure of the *Helicobacter pylori cag* pathogenicity island in induction of interleukin-8 secretion. *Infect Immun*, 69: 1625-29.
32. Ikenoue T, Maeda S, Ogura K, Akanuma M, Mitsuno Y, Imai Y, Yoshida H, Shiratori Y, Omata M (2001). Determination of *Helicobacter pylori* virulence by simple gene analysis of the *cag* pathogenicity island. *Clin Diagn Lab Immunol*, 8: 181-86.
33. Ribeiro ML, Godoy AP, Benvengo YH, Mendonça S, Pedrazzoli J Jr (2003). Clinical relevance of the *cagA*, *vacA* and *iceA* genotypes of *Helicobacter pylori* in Brazilian clinical isolates, *FEMS Immunol Med Microbiol***,** 36: 181–185.
34. Proença Módena JL, Lopes Sales AI, Olszanski Acrani G, Russo R, Vilela Ribeiro MA, Fukuhara Y, da Silveira WD, Pimenta Módena JL, de Oliveira RB, Brocchi M (2007). Association between *Helicobacter pylori* genotypes and gastric disorders in relation to the *cag* pathogenicity island. *Diagn Microbiol Infect Dis*, 59: 7-16.