Cent. Eur. J. Med. • 3(1) • 2008 • 41-46
DOI: 10.2478/s11536-007-0062-y

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Central European Journal of **Medicine**

Helicobacter pylori isolation, serology and *cag*A, *cag*E and *vir*B11 detection in patients with non-ulcer dyspepsia from Turkey: Correlation with histopathologic findings

Research article

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Received 5 September 2007; Accepted 28 September 2007

Abstract: Colonization with *Helicobacter pylori* (HP) may have major clinical consequences and HP virulence factors are associated with more severe gastroduodenal pathologies. In this study, prevalence of HP in patients with Non-Ulcer Dyspepsia (NUD) was determined by rapid urease test and culture and correlations of histopathologic changes with bacterial virulence factors and serologic profiles were investigated. Gastric biopsies from sixty-nine patients admitted to Haydarpasa Training Hospital Department of Gastroenterology were evaluated for rapid urease, HP isolation and examined histopathologically. PCR was employed for HP confirmation and detection of HP *cag*A, *cag*E and *vir*B11 genes. For each patient, IgG and IgA antibodies and anti-*cag*A antibodies were also determined by ELISA tests. HP was isolated and confirmed by PCR in 74% (51/69) of the patients. Anti-HP IgG and IgA were detected in 96% (49/51) and 53% (27/51), respectively. Anti- *cag*A were present in 51% (26/51). *cag*A, *cag*E and *vir*B11 were positive in 56.8% (29/51), 60.7% (31/51) and 58.8% (30/51) of the patients, respectively. Statistically significant correlation was observed between *cag*A PCR and inflammation/activity scores. Detection of *cag*A by molecular assays can be an alternative test that can be employed for individual patient assessment.

Keywords: *Helicobacter pylori • Serology • Pathogenesis • Non-ulcer dyspepsia • CagA*

1. Intorduction

Helicobacter pylori (HP) is a gram-negative spiral-shaped bacteria that colonize the human stomach. HP belongs to the genus *Helicobacter* of the ε subdivision of the *Proteobacteria*, order *Campylobacterales*, family *Helicobacteraceae* [1]. Members of the genus *Helicobacter* are all microaerophilic organisms and in most cases are catalase, oxidase and urease positive. HP strains are also motile via unipolar flagella [2,3]. It is firmly established that gastric colonization with HP can lead to variety of upper gastrointestinal disorders, such as chronic gastritis, peptic ulcer disease, gastric mucosa-associated lymphoid tissue (MALT) lymphoma, and gastric cancer [4,5]. Thus, HP infection has a major clinical impact with regard to the diagnosis of

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Table 1. Primer sequences and expected length of PCR amplicons in the study.

Target	Sequence (5'-3')	Product Lenght
*glm*M	GGATAAGCTTTTAGGGGTGTTAGGGG (forward) GCTTGCTTTCTAACACTAACGCGC (reverse)	293 bp
*cag*A	ATAATGCTAAATTAGACAACTTGAGCGA (forward) AGAAACAAAAGCAATACGATCATTC (reverse)	128 bp
*cag*E	TTGAAAACTTCAAGGATAGGATAGAGC (forward) GCCTAGCGTAATATCACCAATTACCC (reverse)	508 bp
*vir*B11	TTAAATCCTCTAAGGCATGCTAC (forward) GATATAAGTCGTTTTACCGCTTC(reverse)	491 bp

these diseases. For the correct management of peptic ulcer disease and gastric MALT lymphoma, as well as obtaining information on a wide range of diseases associated with HP infection, effective diagnostic methods are mandatory. Certain important issues, like the transmission route of HP, are still poorly understood [5]. Although the prevalence of HP in the Western hemisphere is decreasing, gastric colonization by HP remains widespread in the developing countries [6]. Infection with HP can be diagnosed by a variety of tests and can often be successfully treated with antibiotics. Unfortunately, the increase in antibiotic resistance is starting to affect the efficacy of treatment, and, in spite of the impact of HP, preventive vaccination strategies still do not exist [7]. Certain virulance factors are defined for HP and are thought to play a role not only in the survival of the bacteria in the microenvironment of the stomach but also in pathogenesis of the HP-induced diseases. Among several bacterial factors that have been implicated in the pathogenicity of HP, the immuno-dominant protein CagA, encoded by the *cag*A gene, which is a marker for the presence of the *cag* pathogenicity island (*cag*PAI) is the most-thoroughly studied. HP strains that harbor *cag*A are associated with more severe clinical outcome [5]. In this study, prevalence of HP infection in a group of patients with Non-Ulcer Dyspepsia (NUD) was determined by rapid urease test and culture, and correlations of histopathologic changes with *cag*A and two other cagPAI-encoded genes, *cag*E (also called *pic*B) and *vir*B11 genes were investigated along with serologic profiles.

2. Material and Methods

2.1. Study Population and Samples

Sixty-nine adult patients that had admitted to Gulhane Military Medical Academy Haydarpasa Training Hospital Department of Gastroenterology with dyspeptic complaints, clinically diagnosed as NUD and had undergone eusophagogastroduodenoscopy between July 2005 and May 2006 were included in the study. Informed consent from patients and approval from local ethics committee was taken previously. Rapid urease test reactivity and histopathologic findings suggesting HP infection were determined as the selection criteria. Patients that had been using antibiotics, bismuth containing drugs, proton pump inhibitors, H_2 receptor blockers for the last 30 days and antiacids for the last 2 days were excluded from the study.

2.2. Endoscopic Examination

Upper gastroscopic examination and sampling were performed by expert gastroenterologists using a videogastroscope (Fujinon EG 200 FP®, Japan). Endoscope and biopsy forcipes were disinfected by 2% gluteraldehyde solution for 15 minutes. Three antral samples were obtained from each patient, one being used for rapid urease detection, another sent for histopathologic examination in 10% formol solution and the last one for bacterial isolation in Brain-Hearth Infusion Broth (BHIB).

2.3. Histopathologic Examination

Biopsy specimens were evaluated and reported according to Sydney criteria as described previously by an expert pathologist after staining with hematoxylene / eosin and toluidine blue (8).

2.4. Rapid Urease Test

CLO-Test® (Kimberly-Clarke,USA) was used to detect urease-producing bacteria in the biopsy specimens according to the manufacturer's instructions. Samples were kept at room temperature or incubated at 37°C for initial evaluation. Incubation were prolonged to 24 hours for unreactive samples before discarding as negative.

2.5. Cultivation of H. pylori

Biopsy samples transported in BHIB were inoculated on Helicobacter Agar® (Salubris, Turkey) as a selective media for HP and incubated in a microaerophillic environment at 37°C for 4-7 days. Appearance of small, circular, smooth colonies after 3 to 4 days on the

Table 2. Results of PCR for *cag*A, *cag*E and *vir*B11 regions and serological tests from patients with HP isolation.

	Serological Tests			Molecular Tests		
	HP IgG	*HP* IgA	*cag*AIgG	*cag*A	*cag*E	*vir*B11
Positive	49 (95.9%)	27 (52.9%)	26 (50.9%)	29 (56.8%)	31 (60.7%)	30 (58.8%)
Negative	2 (4.1%)	24 (47.1%)	25 (49.1%)	22 (43.2%)	20 (39.3%)	21 (41.2%)
Total	51 (100%)			51 (100%)		

selective media, observation of spiral-shaped, motile bacteria and cytochrome oxidase, catalase and urease positivity led to the presumptive identification of HP.

2.6. Serological Tests

Serological tests for detecting HP IgG, IgA and *cag*A were performed for patients from which HP was isolated. After separation, sera were stored in -20°C until studied. For the detection of specific IgG and IgA antibodies against HP, Captia *H.pylori* IgG ELISA® (Trinity Biotech, USA) and Captia *H.pylori* IgA ELISA® (Trinity Biotech, USA) tests were used according to the manufacturer's instructions. IgG ELISA test had a reported sensitivity of 98.5% and specificity of 97.1% whereas IgA ELISA had sensitivity and specificity of 88.9% and 97.1%, respectively. Antibodies against *cagA* were evaluated by HP *cagA* IgG ELISA® (Dia Pro Diagnostic Probes Srl, Italy) as directed by the manufacturer. Sensitivity and specificity of this test was 98% as reported.

2.7. Molecular Tests

For each isolated strain, HP-specific *glm*M and pathogenicity-associated *cag*A, *cag*E and *vir*B11 PCR were performed with minor modifications as described before (9). Fresh bacteria from on selective solid media were resuspended in DEPC-treated sterile distilled water and heat-extracted in a boiling water bath for 5 minutes. PCR mix composed of 10X Buffer (Fermentas, Lituania), 2mM $MgCl_2$, 200 µM of each deoxyribonucleotide, 10 pm of primers and 1U Taq Polymerase (Fermentas, Lituania). Primer sequences employed in the study were provided in Table 1. For glm*M* PCR, thermocycling program that included an initial denaturation at 95°C for 3 minutes, followed by 35 cycles of denaturation at 94°C for 60 seconds, primer binding at 52°C for 60 seconds, elongation at 72°C for 60 seconds and a last synthesis step for 3 minutes at 72°C was employed. For *cag*A, *cag*E and *vir*B11 PCR, thermocycling program included a denaturation step at 95°C for 3 minutes, denaturation at 94°C for 60 seconds, primer binding for 45 seconds, elongation at 72°C for 45 seconds for 50 cycles and 72°C for 3 minutes. Primer binding temperatures for cag*A*, cag*E* and vir*B11* PCR were 48°C, 53°C and 49°C, respectively. All amplifications were performed in a iCycler IQ® Thermocycler (Bio-Rad, USA). Amplicons of 293 bp for glm*M* PCR, 128 bp for cag*A* PCR, 491 bp for vir*B11* PCR and 508 bp for cag*E* PCR were separated by electrophoresis in 1.5 % agarose gel and visualized under ultraviolet light after staining with ethidium bromide. Previously-identified HP strains and purified DNA from a normal gastric endoscopic biopsy sample that were processed as above were used as positive and negative controls. DNA extraction, PCR and electrophoresis were performed in separate laboratories in order to avoid contamination.

2.8. Statistical analysis

Chi-square test was applied for linear trends where appropriate. P values <0.05 were assumed as statistically significant. Data analyses were performed by SPSS ® Version 12.0.

3. Results

3.1. HP isolation

A total of 69 patients with CLO-Test® reactivity and histopathologic changes suggesting HP infection were evaluated. Male to female ratio and mean age were noted as 38 (55%) to 31 (45%) and 44, respectively. HP was isolated in 51 of 69 (74%) of the patients. No statistically significant difference was observed between age or gender and HP isolation (p> 0.05).

3.2. Serological Tests

HP-specific IgG was detected in 49 of the patients from which HP was isolated (49/51, 96%) whereas IgA antibodies were detected in 27 out of 51 (53%). Anti-*cag*A antibodies were detected in 26 (51%) of the study population. Serological test results are summarized in Table 2. No significant difference was observed between age or gender and presence of any anti-HP antibody (all p values >0.05).

Table 3. Histopathologic scores (according to the Sydney criteria, reference 8) and *cag*A PCR detection results of HP positive and negative patients.

Histopathologic Evaluation		HP (+)		HP (-)
		cagA (+)	cagA (-)	
	Score	n:29	n:22	n:18
Inflammation	1	0	0	5
	2	6	12	7
	3	14	6	5
	4	9	4	1
Activity	1	3	10	15
	2	9	7	2
	3	14	5	1
	4	3	0	0
Atrophy	1	21	20	17
	2	6	2	1
	3	2	0	0
	4	0	0	0
Intestinal Metaplasia	1	29	22	15
	2	2	0	3
	3	0	0	0
	4	0	0	0
HP Amount	1	0	0	18
	2	15	15	0
	3	7	7	0
	4	0	0	0

3.3. Molecular Tests

All presumptively identified strains were positive by *glm*M PCR, thus confirmed as HP. Presence of *cag*A was detected in 29 out of 51 (57%) patients. No significant difference was observed between age or gender and any *cag*PAI gene detection. Inflammation and activity scores for *cag*A patients were observed to be significantly higher than *cag*A negative and HP uninfected patients (p values >0.05). No significant difference was noted between atrophy, intestinal metaplasia and HP intensity scores and *cag*A positivity. Histopathologic scores of the patients according to HP infection and *cag*A detection are given in Table 3.

All HP strains that revealed *cag*A positivity also displayed positive results for *cag*E and *vir*B11 PCR. One HP strain was found to have *cag*E and *vir*B11 but not *cag*A. Another strain was observed to possess *cag*E only. Histopathologic scores for these patients for inflammation, activity, atrophy, intestinal metaplasia and HP intensity were noted as 2-1-0-0-1 and 1-1-0-0-1 respectively. PCR detection rates are given in Table 2.

4. Discussion

H. pylori is widespread throughout the world and is present in about 50% of the global human population [10,11]. The prevalence of HP displays considerable geographical variation. In various developing countries, more than 80% of the population is infected, even at young ages [6]. The prevalence in industrialized countries generally remains under 40% and is lower in children and adolescents than in adults and elderly people [12]. Within geographical areas, the prevalence of HP inversely correlates with socioeconomic status, in particular in relation to living conditions during childhood [13]. Data from multicentral studies from Turkey indicate that HP infection prevalence is observed as 60-81% in patients with dyspeptic complaints. The detection rate increases to 88-96 % when the presence of gastric and/or duodenal ulcer is observed in endoscopic examination [14,15]. In our study with patients with a preliminary diagnosis of HP infection by rapid urease detection, HP isolation was successful in 74% (51/69) of the study population. All strains were further confirmed as HP by *glm*M PCR.

H. pylori persistently colonizes the human stomach despite development of a humoral and cellular immune response. A humoral immune response to HP is elicited in nearly all infected humans [16]. Local secretory IgA response can also be observed in the gastric mucosa of HP-infected persons [17,18]. In our study, HP-specific IgG and IgA antibodies are detected in 96% and 53% study population respectively. Thomasini et al. reported seropositivity rates of 88.8% for IgG and 50% for IgA; and in another study detection rates for IgG and IgA were 84.5% and 51.3% respectively [9,19]. Thus, our results are concordant with previous studies and similar seroprevalence rates are observed in Turkish patients with HP infection.

Colonization with the *cag*A positive HP strains has been associated with higher grades of gastric mucosal inflammation as well as severe atrophic gastritis and has been suggested to play an important role in the development of gastric carcinoma [20–22]. It is known that *cag*A proteins induce morphological alterations of the gastric epithelial cell, while other genes of the *cag*PAI lead to production of interleukin 8 [23,24]. The *cag*E gene, also within the pathogenicity island, has shown to stimulate production of several cytokines from infected epithelial cells [25]. The chemokine production is recognized as being responsible for an increased gastric inflammation and subsequent disease development. Numerous studies have shown that strains harboring the *cag*PAI are associated with

more severe diseases, especially peptic ulcer disease and gastric adenocarcinoma, as well as precancerous lesions and extradigestive diseases [5]. However, these associations have not been found everywhere. Fifty-seven percent (29/51) of our HP isolates from Turkish patients were observed to harbor *cag*A gene by PCR and anti- *cag*A antibodies were detected by ELISA in 51% (26/51) of the population. Presence of *cag*A gene was correlated with the degree of inflammation and the inflammatory activity in the stomach as determined by the histopathologic scoring. Although it has been suggested that pathogenic association with *cag*A cannot be used in case control studies due to high level of *cag* PAI positivity detected in most HP strains from Asia, our results reveal that this may not be the case for Turkey; for statistically-significant relation was revealed with the disease activity due to HP infection and *cag*A gene [26,27]. For anti-*cag*A seroprevalence in Turkey, positivity rates of 70.5 – 82 % was reported from NUD patients previously, which indicate probable regional differences [28-30]. Thus, detection of *cag*A by molecular methods may be employed as test for individual assessment for eradication treatment or other consequences of HP infection. Another point of interest is that most *cag*A positive strains are reported to contain a complete and contiguous *cag*PAI, but a significant proportion (about 10%) carry an incomplete, and thus not fully functional *cag*PAI [31,32]. The effect of this on disease outcome is still unclear (5). In our study, detection of *cag*E and *vir*B11 in all *cag*A positive strains might indicate a contiguous *cag*PAI, which may also be associated to the histopathologic correlation of disease severity with *cag*PAI in this study.

As a result, HP was isolated and confirmed by PCR in 51 out of 69 NUD patients (74%). Twenty-nine patients (56.8%) carried *cag*A gene which correlated with the results of the histopathologic evaluation.

Acknowledgement

Primers used in this study were kindly provided by Iontek Biotechnoloy Inc. (Istanbul, Turkey). We are also grateful to Mustafa Tamay Ozer, molecular microbiology laboratory technician, for the assistance in nucleic acid extraction and PCR.

Part of this study was presented as a poster (57-032) at the XIIth International Congress on Infectious Diseases, Lisbon, Portugal, June 15-18, 2006.

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