Original Article

Prevalence of *Helicobacter pylori cagA* and *vacA* genes in Cypriot patients

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Abstract

Introduction: The prevalence of *H. pylori* varies with geographic locations. To date there are no epidemiological data on its prevalence in Cyprus; therefore, we determined the prevalence and molecular characteristics of *H. pylori* infection in Cypriot patients.

Methodology: DNA extracted from 103 gastric biopsies was analyzed for the presence of *H. pylori* by PCR using primers for *ureA*. *H. pylori*-positive biopsies were characterized by PCR using specific primers for *cagA* and *vacA* genes. The presence of clarithromycin-associated resistant mutations such as A2143G, A2142G, A2142C in 23S rRNA gene of *H. pylori*-positive patients was determined using a real-time PCR allelic discrimination assay.

Results: *H. pylori* was detected in 41 (39.8%) biopsies and, out of these, 17 (41.5%) tested positive for the *cagA* gene. The *vacA* alleles m1, m2, s1a, s1b, and s2 were detected in 7 (17.1%), 34 (82.9%), 12 (29.3%), 2 (4.9%), and 22 (53.7%) isolates, respectively. One (2.4%) biopsy was *vacA* s1a and s2-positive while one (2.4%) was positive for *vacA* s1a, s1b, and s2. Three (7.3%) biopsies were untypable for *vacA* s1, s1b, and s2. The majority (35; 85.4%) of strains were susceptible to clarithromycin while two (4.9%) had the A2143G mutation. Three (7.3%) had a mixture of an A2143G point mutant and susceptible strains while one (2.4%) had a mixture of an A2142G point mutant and susceptible strains.

Conclusions: The distribution of the virulence factors *cagA* and *vacA* in the Cypriot strains resembled that of strains circulating in Middle Eastern countries geographically close to Cyprus.

Key words: H. pylori; ureA; cagA; vacA; clarithromycin

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Introduction

Helicobacter pylori (*H. pylori*), a spiral-shaped, microaerophilic bacterium that colonizes the human stomach, has been strongly associated with a range of gastroduodenal diseases including chronic gastritis, peptic ulceration, gastric cancer, and lymphomas [1-4]. Among the different genes involved in *H. pylori* virulence are the cytotoxin-associated gene (*cagA*), and the vacuolating cytotoxin gene (*vacA*) [5-9]. The *cagA* gene encodes for a high molecular weight protein shown to be a disease-associated virulence factor [5,10]. As indicated in different reports, *cagA*-positive *H. pylori* strains have been more frequently detected in patients with peptic ulcer disease than in patients with chronic gastritis [5-7,11]. In contrast to *cagA*, virtually all *H. pylori* strains harbor the *vacA* gene [12-14]. *vacA* has a mosaic structure, containing variable signal regions (type s1 or s2) and mid-regions (type m1 or m2) [14,15]. The s1 type is further divided into subtypes s1a, s1b, and s1c [14,16]. Subtypes m1a, m1b, m1c, and m1d have been identified in type m1, whereas the m2 type has subtypes m2a and m2b [14,15,17-19]. Various allele combinations have been associated with particular clinical outcomes. *H. pylori* strains positive for the *vacA* s1 region were found to be more commonly associated with peptic ulceration than were *vacA* s2-positive *H. pylori* strains [14,20]. Strains with a *vacA* m1 allele produced higher levels of cytotoxin activity *in vitro*, and more epithelial damage in the human stomach *in vivo*, than did strains with a *vacA* m2 allele [20].

Standard treatment for *H. pylori* infection involves a triple therapy with proton pump inhibitors, amoxicillin, and clarithromycin [21]. The prevalence

of clarithromycin resistance varies between 2% and 25% among different geographic areas but generally has been increasing worldwide [22]. More than 90% of clarithromycin-resistant cases have been attributed to the three main 23S rRNA point mutations (A2143G, A2142G, A2142C of domain V), with different prevalence rates of these mutations reported worldwide [23-26].

In Cyprus, there is no data regarding the prevalence and molecular characteristics of *H. pylori* in patients with gastrointestinal discomfort. This is due to the fact that research in Cyprus has just started to grow in the last ten years. The proposed study, which was the first of this type conducted in Cyprus, aimed to determine (a) the prevalence of *H. pylori* infection, (b) the *cagA* and *vacA* genotypes of *H. pylori* isolates, (c) the prevalence of clarithromycin-associated resistant mutations in Cypriot patients**,** and (d) the effect of diet on *H. pylori*-associated symptoms.

Methodology

Patients

The study included 103 adult patients who underwent routine upper gastrointestinal endoscopy due to dyspeptic complaint at Apollonion Hospital, Nicosia, Cyprus. At endoscopy, an antral/lower body biopsy was collected from each patient. Each gastric biopsy was subjected to PCR and real-time PCR analysis. None of the enrolled patients received antimicrobial therapy prior to collection of the gastric biopsy sample. This study was approved by the Cyprus national bioethics committee. All patients provided informed consent to participate in the study.

Diet regiment program

Following the collection of a gastric biopsy sample, all enrolled patients agreed by signing an informed consent form to participate in a diet regiment program aiming to evaluate the effect of diet in alleviating the gastrointestinal symptoms experienced on the day of gastrointestinal endoscopy. Initially patients underwent a full anthropometric assessment (weight, height, Body Mass Index, fat percentage) and filled out a specifically designed food frequency questionnaire. According to the results of the anthropometric assessment and the food frequency choices, each patient received a specific diet regiment. Briefly, each diet regiment suggested having meals at regular times each day and was divided into multiple small meals throughout the day. Specific recommendations such as which foods to avoid were also given at the beginning of the diet. A monthly follow-up session was scheduled and attended by all patients, during which their progress with regard to the symptoms and overall dietary status was assessed. This study was approved by the Cyprus national bioethics committee.

PCR detection of ureA*,* cagA*, and* vacA *in gastric biopsies*

DNA was isolated from fresh gastric biopsies (25mg) using a QIAamp DNA blood mini kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Isolated DNA was stored at -20°C until analysis. The DNA concentration was determined with a spectrophotometer and ranged from 19.5 ng/μL to 26.8 ng/μL. The genes *ureA*, *cagA*, *vacA* midregion (m1 and m2) and signal region (s1a, s1b, s2) were amplified by polymerase chain reaction (PCR). PCR was performed in a 25 μL reaction volume containing 1x PCR buffer supplemented with 1.5 mM $MgCl_2$ (Applied biosystems, Foster City, CA, USA), 200 μM of each deoxynucleotide triphosphate, sense and antisense primers at concentrations ranging from 0.3 μM-1 μM, 1.5 U of AmpliTaq Gold DNA polymerase (Applied biosystems, Foster City, CA, USA), and 1 μL aliquot of extracted DNA. The primers used and their details are shown in Table 1. The amplification reactions were performed using an MJR PTN-200 PCR machine (MJ Research Inc, Watertown, USA). The PCR conditions were as follows: (A) *ureA*: Initial denaturation step for 10 minutes at 95°C, followed by 37 cycles of 30 seconds at 95°C, 45 seconds at 45°C, and 72 °C for 30 seconds; (B) *cagA*: initial denaturation of 10 minutes at 95°C, followed by 37 cycles of 30 seconds at 95°C, 45 seconds at 55°C, and 72°C for 30 seconds; (C) *vacA* m1/m2/s1a/s1b/s2: initial denaturation of 10 minutes at 94°C, followed by 35 cycles of 45 seconds at 94°C, 45 seconds at 57°C, and 72°C for 45 seconds. PCR products were separated by electrophoresis on a 2% agarose gel and stained with ethidium bromide.

Real-time PCR detection of mutations associated with clarythromycin resistance

The A2142G, A2142C, A2143G point mutations in 23S rRNA gene were analyzed using a TaqMan real-time PCR allelic discrimination assay (Applied Biosystems, Foster city, USA) as previously described [27]. Assays were prepared to enable scoring of both genotypes in a single reaction (wild type and mutation for each of the three mutation sites). Probes and primers were designed to hybridize with wild type and mutant DNA. Because a previous study showed that

Table 1. Target gene, primer name, oligonucleotide primers and expected product size

Target	Primer	Sequence (5′-3′)	Product size (bp)	Reference
ureA	HPU1-F	GCCAATGGTAAATTAGTT	411	[53]
	HPU2-R	CTCCTTAATTGTTTTTAC		
cagA	B7628	AAGAAAGGCAAGAAGCAGAAAA	335	[54]
	B7629	ACACAGAAGACAGAGCGTTATT		
vacA m1	VAG-F	CAATCTGTCCAATCAAGCGAG	570	[55]
	VAG-R	GCGTCAAAATAATTCCAAGG		
vacA m2	VAG-F	CAATCTGTCCAATCAAGCGAG	645	[55]
	VAG-R	GCGTCAAAATAATTCCAAGG		
vacA s1a	SS1-F	GTCAGCATCACACCGCAAC	190	[55]
	VA1-R	CTGCTTGAATGCGCCAAAC		
vacA s1b	SS3-F	AGCGCCATACCGCAAGAG	187	[55]
	VA1-R	CTGCTTGAATGCGCCAAAC		
vacA s2	SS2F	GCTAACACGCCAAATGATCC	199	[55]
	VA1-R	CTGCTTGAATGCGCCAAAC		

the conjugation of minor groove binder (MGB) to oligonucleotides stabilizes nucleic acid duplexes, causing a dramatic increase in oligonucleotide melting temperature (Tm), we used attachment of the MGB which enables the use of shorter fluorogenic probes, thus resulting in improved mismatch discrimination [28]. The probes used were distinguished by being labeled with different fluorescent reporter dyes (FAM dye and VIC dye). A substantial increase in FAM or VIC fluorescence indicated homozygosity for the FAM- or VIC-specific allele, while an increase in both signals indicated heterozygosity [29]. Details of the primers and probes used are shown in Table 2. Briefly, the real-time PCR mixture was prepared in a 25-μL reaction volume containing Universal PCR master mix (Applied Biosystems, Foster City, CA, USA), 0.2 μM of each primer, 0.1 μM of VIC-labelled TaqMan MGB probe (for wild type), 0.1 μM of FAM-labelled TaqMan MGB probe (for mutant), and 1 μL aliquot of extracted DNA. Real-time PCR analysis was performed with an ABI 7500 instrument (Applied Biosystems, Foster City, CA, USA) and the conditions of the PCR amplification were as follows: 95°C for 10 minutes and 40 cycles of 92°C for 15 seconds and 60°C for 1 minute. All samples were detected in duplicate and positive and negative controls were included in each assay. Following a successful real-time PCR, data were analyzed with the use of Sequence Detector Software (SDS) (Applied Biosystems, Foster city, USA). Allelic discrimination results were analyzed and depicted in the form of scatter plots of wild type versus mutant allele. Scatter plots consisted of three major clusters that varied along the horizontal axis (wild type homozygous), vertical axis (mutated type homozygous), or diagonal axis (heterozygous).

Results

Detection of H. pylori *infection and characterization of* H. pylori *positive strains in terms of* cagA *and* vacA

Gastric biopsies from all 103 patients included in the study were initially assessed for the presence of *H. pylori* by PCR using *ureA*-specific primers encoding the urease gene. The 411-bp PCR product indicating the presence of *H. pylori* was detected in 41 patients (39.8%), whereas 62 patients (60.2%) were negative for the same PCR product. All *H. pylori*-positive biopsies were further analyzed to determine their *cagA* and *vacA* status. Seventeen biopsies (41.5%) were positive for the *cagA* gene, with the remaining 24 (58.5%) were classified as *cagA*-negative. In parallel, we characterized the *vacA* gene and allele types (Table 3). The primers used allowed amplification and differentiation of type s1a, s1b, and s2 *vacA* alleles and type m1 and m2 *vacA* alleles. The *vacA* mid-region was detected in all *H. pylori* strains studied. In detail, the *vacA* m1 allele was found in 7 (17.1%) strains and the *vacA* m2 allele in 34 strains (82.9%). For the signal-region, in strains where a single *vacA* allele was found, the majority (22; 53.7%) were

Table 2. Sequences of primers and probes for detection of mutations associated with clarythromycin resistance

Target	Primer/Probe	Sequence (5′-3′)	Reference
23S rRNA	23SA2142G-2142F[a]	TCAGTGAAATTGTAGTGGAGGTGAAAA	[27]
	23SA2142G-2142R[a]	CAGTGCTAAGTTGTAGTAAAGGTCCA	
	VIC[b]	AAGACGGAAAGACC for DNA WT	
	FAM[b]	AAGACGGGAAGACC for mutated DNA	
23S rRNA	23SA2143G-2142F[a]	TCAGTGAAATTGTAGTGGAGGTGAAAA	[27]
	23SA2143G-2142R[a]	CAGTGCTAAGTTGTAGTAAAGGTCCA	
	VIC[b]	AAGACGGAAAGACC for DNA WT	
	FAM[b]	CAAGACGGAGAGACC for mutated DNA	
23S rRNA	23SA2142C-2142F[a]	TCAGTGAAATTGTAGTGGAGGTGAAAA	[27]
	23SA2142C-2142R[a]	CAGTGCTAAGTTGTAGTAAAGGTCCA	
	VIC[b]	AAGACGGAAAGACC for DNA WT	
	FAM[b]	AGACGGCAAGACC for mutated DNA	

[a]: primer; [b]: probe; WT: wild type

positive for the *vacA* s2 allele, whereas 12 (29.3%) and 2 (4.9%) *H. pylori* strains were respectively positive for the *vacA* s1a and s1b alleles. In a minority of cases *H. pylori* positive biopsies were positive for more than one *vacA* signal region alleles, with one (2.4%) classified as positive for the *vacA* s1a/s2 genotype and one (2.4%) being positive for all three *vacA* signal region alleles tested. Three (7.3%) *H. pylori* strains were untypable for the the *vacA* signal region alleles tested. The most frequent *vacA* allelic combination in the *H. pylori* strains examined was s2/m2 (53.7%), followed by s1a/m2 (19.5%), s1a/m1 (9.8%), s1b/m1 (2.4%), and s1b/m2 (2.4%).

Prevalence of clarithromycin-resistant H. pylori *strains*

Clarithromycin resistance was assessed using a TaqMan real-time PCR allelic discrimination assay. Analysis included all biopsies obtained that tested positive for *H. pylori* before the patient underwent any treatment. The results related to clarithromycin-resistant *H. pylori* strains are shown in Table 4. Overall, 35 (85.4%) biopsies contained wild type *H. pylori* strains. The remaining 6 (14.6%) biopsies were determined to have either resistant or a mixture of resistant and wild type *H. pylori* strains. In detail, two (4.9%) biopsies were infected exclusively with resistant strains harboring the A2143G mutation, whereas the remaining four (9.7%) cases were characterized by a mixture of resistant and wild type strains. Three (7.3%) of these cases harbored wild type and resistant strains with the A2143G mutations, whereas one (2.4%) case harbored wild type and resistant strains with the A2142G mutation.

cagA *status,* vacA *alleles and clinical diagnosis of* H. pylori *positive patients*

According to the literature, virulent cytotoxin *vacA*- and *cagA*-producing strains are found more frequently among patients with peptic ulcer and gastric cancer [5-7,30]. In addition, in other studies, patients with various clinical symptoms including gastritis,

Table 3. Prevalence of *vacA* m1, m2, s1a, s1b, and s2 alleles in *H. pylori* positive gastric biopsies

Allele	No. of Samples	Prevalence (%)
vacA		
m1$^+$	7	17.1
m2$^+$	34	82.9
Total	**41**	**100**
vacA		
s1a$^+$	12	29.3
s1b$^+$	2	4.9
s2$^+$	22	53.7
s1a$^+$/s2$^+$	1	2.4
s1b$^+$/s1b$^+$/s2$^+$	1	2.4
s1b$^-$/s1b$^-$/s2$^-$	3	7.3
Total	**41**	**100**

duodenal and gastric ulcer, and esophagitis have been examined for *H. pylori* virulence factors [31-33]. In the present study, among the 41 *H. pylori*-positive patients, the majority suffered from one of the following: gastritis (29/41; 70.7%), gastroesophageal reflux disease (GERD) (6/41; 14.6%), duodenitis (6/41; 14.6%), peptic ulcer disease (4/41; 9.8%) or polyps (4/41; 9.8%). A minority of patients suffered from esophageal candidiasis (2/41; 4.9%), functional dyspepsia (1/41; 2.4%), esophageal diverticulitis (1/41; 2.4%), first-grade esophagitis (1/41; 2.4%), Barret's esophagus (1/41; 2.4%), or colelethiasis (1/41; 2.4%). Dual diagnosis was recorded for 15 (36.6%) of the *H. pylori*-positive patients. The distribution of *cagA* and *vacA* alleles in patient groups is shown in Table 5.

Effect of diet on H. pylori*-associated symptoms*

The effect of diet on *H-pylori*-associated symptoms was assessed two months following the initiation of a diet regiment program tailored to the needs of each patient. Results analysis revealed that most of the symptoms were minimized in all patients irrespective of their *H. pylori* status, especially the feeling of fullness in the upper abdomen, dyspepsia, nausea, epigastric pain, subcternal distress, and regurgitation. Patients who succeeded in losing weight (as instructed by the clinical dietician), as well as improving their diet, reported alleviation of abdominal pain and better intestinal function. When the anthropometric assessment determined that weight loss was indicated as part of the treatment, a 5% loss of weight was found to be sufficient to improve abdominal bloating and gastrointestinal discomfort. Also, eating dairy products in moderation (no more than three servings per day) and avoiding entirely particular fruits and vegetables reduced symptoms of gastritis. In conclusion, our results indicate that the implemented diets did not have a direct effect on *H. pylori* infection but nevertheless they contributed to the improvement of the overall health status of the patients.

Discussion

Several studies have highlighted that the prevalence of *H. pylori* infection as well as the incidence and/or severity of *H. pylori*-related gastroduodenal diseases may be different among geographic areas. This can be partly attributed to the different distribution of *H. pylori* virulence factors around the world [34]. Table 6 summarizes the distribution of the main *H. pylori* virulence factors worldwide. The present study is the first to determine the prevalence of *H. pylori* infection in Cypriot patients and molecularly characterize the two main *H. pylori* virulence-associated genes *vacA* and *cagA*.

The predominant single genotype in the 41 biopsies found positive for *H. pylori* by PCR was the *vacA* m genotype, with 34 (82.9%) being *vacA* m2 positive and 7 (17.1%) *vacA* m1 positive, followed by the *vacA* s genotype, with 24 (58.5%) and 16 (39%) being positive for the *vacA* s2 and *vacA* s1 alleles, respectively. The *cagA* gene was amplified in 17 (41.5%) of the biopsies. This result was different from previously reported prevalence of *cagA* (66-73%) in Europe but in agreement with the majority of studies conducted in Middle Eastern countries (*i.e.*, Turkey, Egypt, Israel and Jordan) where the *cagA* genotype was reported to vary between 26% and 44% [35-39]. In a similar manner, the prevalence rates of the *vacA* m2 (82.9%), m1 (17.1%), and s2 (58.5%) genotypes detected in Cyprus were in agreement with those reported in other Middle Eastern countries (Table 6) [35-39] rather than those reported for Europe [12,13,40-42]. The similarities in terms of the genotypes and their prevalence identified in the present study and what has been reported in Middle Eastern countries could be attributed to a geographic influence important in the adaptation of *H. pylori* to the environment and climate conditions. The close resemblance of strains in neighboring countries was also reported in Israel, Egypt, and Jordan [37-39], as well as in Germany, France, and Poland [42,43]. The prevalence of the *vacA* s1 allele has been reported to range between 46% and100% in the Middle East [35], 48% and 89% in Europe [12,13,40-42], and 57% and 68% in America [14,44,45]. In the present study, the *vacA* s1 allele was detected in 16 (39%) *H. pylori*-positive biopsies.

Clarithromycin resistance in *H. pylori* mainly results from point mutations in the peptidyltransferase loop region of the 23S rRNA gene [46]. In the present study, the most frequent point mutation was A2143G in 12.2% of Cypriot strains, followed by the A2142G mutation detected in 2.4% of cases. These results were in agreement with those of other studies in Europe, the Middle East, and America, showing that the A2143G mutation is the one most frequently detected in *H. pylori* strains, followed by the A2142G mutation [22,47-49]

Table 4. Distribution of point mutations (A2143G, A2142G, A2142C) in clarythromycin resistance

Type	No. of Samples	Prevalence (%)
Pure wild type	35	85.4
Pure mutant		
A2143G	2	4.9
A2143G	0	0
A2142C	0	0
Mixed		
Wild type + A2143G	3	7.3
Wild type + A2142G	1	2.4
Wild type + A2142C	0	0
Total	**41**	**100**

Table 5. Distribution of *cagA* and *vacA* alleles in patient groups

Alleles	No. (%) of isolates					
	Gastritis (n = 29)	GERD (n = 6)	Duodenitis (n = 6)	PUD (n = 4)	Polyps (n = 4)	Esophageal Candidiasis (n = 2)
cagA	9 (31%)	2 (33.3%)	3 (50%)	4 (100%)	0 (0%)	2 (100%)
vacA						
m1	4 (13.8%)	1 (16.7%)	1 (16.7%)	1 (25%)	0 (0%)	0 (0%)
m2	24 (82.8%)	5 (83.3%)	5 (83.3%)	3 (75%)	4 (100%)	2 (100%)
s1a	7 (24.1%)	0 (0%)	2 (33.3%)	4 (100%)	1 (25%)	2 (100%)
s1b	3 (10.3%)	0 (0%)	1 (16.7%)	0 (0%)	0 (0)	0 (0%)
s2	19 (65.5%)	5 (83.3%)	2 (33.3%)	1 (25%)	3 (75%)	0 (0%)

	Functional dyspepsia (n = 1)	Esophageal diverticulatis (n = 1)	1st grade esophagitis (n = 1)	Barret's esophagus (n = 1)	Colelethiasis (n = 1)
cagA	0 (0%)	1 (100%)	0 (0%)	0 (0%)	0 (0%)
vacA					
m1	1 (100%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
m2	0 (0%)	1 (100%)	1 (100%)	1 (100%)	1 (100%)
s1a	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
s1b	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
s2	0 (0%)	1 (100%)	1 (100%)	1 (100%)	1 (100%)

GERD: gastroesophageal reflux disease, PUD: peptic ulcer disease

Table 6. Distribution of main *H. pylori* virulence factors in Europe, Middle East, and America

Area	*vacA* alleles prevalence (%)				*cagA* prevalence (%)	Reference
	s1	**s2**	**m1**	**m2**		
Europe	48-89	11-51	22-37	50-63	66-73	[12,13,40-42]
Middle East	46-100	5-54	13-67	33-87	26-76	[35]
America	57-68	16-48	37-44	29-63	57-75	[14,44,45]

Evaluation of the molecular characteristics of *H. pylori* strains in conjunction with clinical diagnosis revealed that most gastritis cases were characterized by a *vacA* m2 genotype. The presence of the *cagA* and *vacA* s1a allele in 5/5 (100%) of the peptic ulcer disease cases is in agreement with results obtained in previous studies showing an association between these virulence factors and peptic ulcer development [5,6,12,35,50-52]. Furthermore, as previously reported [14,20], the *cagA* gene was mostly associated with the *vacA* s1a allele (10/17; 58.8%), compared to *vacA* s1b and s2 alleles that were found in 2/17 (11.8%) and 4/17 (23.5%) of *cagA*-positive strains.

As part of the study, all enrolled patients, *H. pylori* positive and *H. pylori* negative, were given diets tailored to their needs in an attempt to alleviate their symptoms. Overall, symptoms were improved in all patients, with symptom improvement being more evident in some patients compared to others. The results highlight the need for a comprehensive approach toward managing these groups of patients in Cyprus. The described study takes a first step in that direction by providing information on the molecular characteristics of infecting strains that can assist physicians in taking the appropriate steps for patient treatment and care.

Conclusion

The present study has provided information that was previously unavailable regarding the prevalence and molecular characteristics of *H. pylori* infection in Cypriot patients. It would be of interest to continue the described study on a larger group of patients and to evaluate in further studies the molecular epidemiology of *H. pylori* infection in the general population.

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