Eur J Clin Microbiol Infect Dis (2009) 28:437–446
DOI 10.1007/s10096-008-0644-x

ARTICLE

Prevalence of virulence-associated genotypes of *Helicobacter pylori* and correlation with severity of gastric pathology in patients from western Sicily, Italy

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Received: 27 March 2008 / Accepted: 3 October 2008 / Published online: 29 October 2008

Abstract In a bacterium like *Helicobacter pylori*, which is characterized by a recombinant population structure, the associated presence of genes encoding virulence factors might be considered an expression of a selective advantage conferred to strains with certain genotypes and, therefore, a potentially useful tool for predicting the clinical outcome of infections. However, differences in the geographical and ethnic prevalence of the *H. pylori* virulence-associated genotypes can affect their clinical predictive value and need to be considered in advance. In this study we carried out such an evaluation in a group of patients living in Sicily, the largest and most populous island in the Mediterranean Sea. *cagA*, *vacA*, *babA2*, *hopQ*, *oipA*, *sabA*, and *hopZ* were the *H. pylori* virulence-associated genes assayed; their presence, expression status or allelic homologs were detected in *H. pylori* DNA samples and/or isolated strains, obtained by gastric biopsy from 90 Sicilian patients with chronic gastritis, inactive (n=37), active (n=26), or active with peptic ulcer (n=27). Genotypes *cagA*$^+$, *vacA*s1, *vacA*m1, *babA2*$^+$, and *hopQ* I, I/II were identified in 51.8, 80.4, 35.2, 47.3, and 67.7% of the different samples respectively. Only these genotypes were associated with each other and with the active form of chronic gastritis, irrespective of the presence of a peptic ulcer. In our isolates their prevalence was more similar to values observed in the north of Italy and France than to those observed in Spain or other Mediterranean countries that are closer and climatically more similar to western Sicily.

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Introduction

Helicobacter pylori is a Gram-negative, micro-aerophilic, curved bacterium, characterized by its capacity to establish close contact with the gastric mucosal surface cells, exhibit prominent tropism for this type of cells, and persistently colonize the human stomach. In a few patients it is an established cause of chronic active gastritis and peptic ulcer disease and a major risk factor for precursor lesions and the development of gastric cancer [1, 2].

Choreographed interactions exist among the micro-organism, the host, and the environment, in particular if considered with respect to social economic status, sanitary conditions, food, dietary habits, and lifestyle [3, 4]. All these interactions, and the changing balance among them, are the probable cause of the different typology and the low

and in some cases geographically variable [5] incidence of the pathological events caused by *H. pylori*. They may also explain some of the conflicting bio-pathological peculiarities of the micro-organism, such as "the African enigma" [6], or the protective effects that it seems to produce, in children, against diarrheal diseases [7] and, in adults, in western countries, against gastro-esophageal reflux disease, Barrett's esophagus, and adenocarcinoma of the esophagus [8, 9].

Eradication of the organism has been associated with the histological improvement of gastritis, a lower relapse rate, less risk of bleeding from a duodenal ulcer [10], and reduced development of gastric cancer, particularly in individuals without precancerous lesions belonging to high-risk populations [11]. Nevertheless, the real advantage of the microbial eradication still needs to be confirmed, and a better assessment of the micro-organism's pathogenicity and of its interactions with the host and the environment is still required before global strategies can be launched to fight the micro-organism and its pathological effects.

It is now only possible to distinguish virulent *H. pylori* strains (i.e., those associated with duodenal ulceration or gastric carcinoma) from less virulent strains, and two candidate markers are generally considered useful for this purpose: polymorphism in vacuolating cytotoxin gene A (*vacA*) alleles and the presence or absence of the cytotoxin-associated gene A (*cagA*) [12, 13]. *vacA* is present in all strains and comprises two well-characterized variable parts: the s region, which exists as an s1 or s2 allele, and the m region, which occurs as an m1 or m2 allele. Strains carrying the s1m1 mosaic combination exhibit the highest levels of cytotoxic activity, while the s2m2 strains do not secrete the vacuolating cytotoxin [12]. A third recently identified *vacA* polymorphic site, the intermediate (I) region, has also been recognized to be present in the *H. pylori* strains as an i1 or i2 allele [14]; s1m1 and s2m2 strains are exclusively i1 and i2 respectively, whereas s1m2 strains are variable in their i-type; i1 confers toxicity and is defined as an independent marker of the VacA-associated pathogenicity. *cag A* encodes the high molecular weight, immunodominant antigen CagA, which is considered to be a marker for the presence of the *H. pylori cag* pathogenicity island (PAI); strains with genotype *vac*A s1/m1 are often CagA-positive, and strains *cag*A^{+}/*vac*A s1m1 are often associated with extensive damage in gastric mucosa [15].

Many other *H. pylori* virulence factors have been described and considered to be involved in determining the severity or the different typologies and evolutions of the bacterial pathological manifestations [1]. For some of them, in particular the adhesins BabA, SabA, and the outer membrane proteins (OMPs) OipA and HopQ, but not the OMP HopZ, evidence has also been obtained of an association of the presence, the functional state, or the virulence-related alleles of their encoding genes with the presence of the *cag* PAI and, very often, of the *vac*A s1 allele [16–21]. If confirmed, these associations, in a bacterium such as *H. pylori*, characterized by a recombinational population structure [22], might be considered an example of linkage disequilibrium and, presumably, might indicate a selective advantage in strains with certain virulence genotypes [21]. With the lack of reliable information regarding the pathogenic role of the different virulence factors, their association could provide the potentially useful tool required for predicting the clinical outcome of infections.

At present, however, there is no unanimous evidence that all the described associations represent a general and clinically significant phenomenon [23–26], nor it is known if the associations, or at least some of them, are conditioned by the well-known differences in the geographical and ethnic prevalence of the *H. pylori* virulence-associated genotypes [27–29].

As it has been suggested that an analysis should be made of *H. pylori* strains from multiple populations of patients and from various parts of the world [21], an investigation was undertaken in our laboratory to determine the prevalence of specific genotypes of the micro-organism, and their association with the severity of current gastric pathology, in patients from western Sicily, Italy. The investigation was also considered interesting because Sicily, the largest and most populous island in the Mediterranean sea, is strategically located midway between Africa and Italy, and its inhabitants, for historical and present-day reasons, constitute one of the most complex mixtures of different ethnic elements in Europe.

Materials and methods

Patients and samples

This study involved 90 patients, with a mean age of 51.7 years (median, 53.5 years; range 27–76 years), who underwent upper gastrointestinal endoscopy for a variety of indications at the Endoscopy Services of the Ospedali Civili Riuniti in Sciacca (Agrigento) and of the Gastroenterology, Internal Medicine and Elderly Care, and Emergency Surgery Units of the University Hospital A.O.U.P. Paolo Giaccone in Palermo, Italy. Informed consent was obtained from all patients. During gastroscopy, biopsy samples were taken from the antrum and the body of the stomach for rapid urease testing, histology, DNA extraction, and, in some cases, culture, when the proper conditions for sample transport were realizable. Biopsy samples for DNA extraction were sent to the microbiology laboratory in dry tubes; biopsy samples for *H. pylori* culture were placed in 0.5 ml

of Wilkins broth (Difco Laboratories, Detroit, MI, USA) containing 15% glycerol and transported in an ice bath to the microbiology laboratory where they were inoculated immediately onto culture medium or stored at −70°C. All patients were positive for *H. pylori* infection on rapid urease testing. Diagnoses, made on the basis of endoscopic and histological findings, were inactive chronic gastritis (CG), chronic gastritis, active (CGA), and chronic gastritis, active, with gastric or duodenal peptic ulcer (CGA+PU). Chronic inflammation was defined on the basis of the increase in lymphocytes and plasma cells in the lamina propria and activity when a neutrophilic infiltration of the lamina propria, pits or surface epithelium was found.

DNA isolation from gastric biopsies and *H. pylori* DNA detection by PCR methodology

In the microbiology laboratory, biopsy samples were immediately dissected and immersed in Eppendorf microtubes containing 300 μl of sterile extraction buffer (100 mM NaCl, 10 mM Tris-HCl pH 8, 25 mM EDTA, 0.5% SDS), and 5 μl of proteinase K(10 mg/ml), vortexed and incubated with moderate shaking for 18 h at 45°C. The DNA was extracted twice with an equal volume of a solution of phenol/chloroform/isoamylic alcohol (25:24:1) and precipitated at −20°C overnight by adding one-tenth volume of 3M sodium acetate (pH 5) and 2.5 volume of absolute cold ethanol. The extracted DNA was washed with 500 μl of 70% ethanol, air dried, and dissolved in 200 μl of sterile distilled water. The DNA (concentration) quality and quantity after each extraction was evaluated (spectrophotometrically) by horizontal electrophoresis in 1% agarose gel; the approximate quantity of DNA (for the purpose of PCR) was calculated according to a molecular weight standard (GeneRuler DNA ladder mix; MBI Fermentas, York, UK) always loaded on the gel together with the samples.

The presence of the conserved urease *ureA* gene, a marker of the presence of the *H. pylori* genome, was detected by a nested polymerase chain reaction in which, following Wang et al. [30] and Mravak-Stipetić et al. [31], the first two primer pairs shown in Table 1 were used.

In the first-round reaction, PCR was carried out in a 100-μl mixture containing 2–4 μl of each DNA solution, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM MgCl2, 200 μM dNTPs, 200 nM of each primer, and 2.5 U of Ampli Taq Gold polymerase (Applied Biosystems). The amplification was performed in a Perkin-Elmer ThermoCycler 2400 under the following conditions: at 95°C for 10 min, followed by 40 cycles at 94°C for 45 s, 56°C for 45 s, and 72°C for 45 s, and a final extension at 72°C for 5 min. In the second-round reaction, the same conditions were employed, except that 1 μl of the first PCR product was used as target DNA and that annealing was carried out at 55°C. Twenty microliters of each PCR product were analyzed by electrophoresis through a 2% (wt/vol) agarose gel containing 1 μg of ethidium bromide per millimeter, and the bands were visualized by excitation under UV light.

H. pylori culture

Biopsy samples in transport medium were vortexed vigorously for 2 min, and 100 μl of the media were placed on Columbia agar (Oxoid, Basingstoke, Hampshire, UK) with the addition of 7% horse blood and 0.4% of selective supplement. The plates were incubated at 37°C under microaerobic conditions (CampyGen; Oxoid) for 3–6 days. *H. pylori* bacteria were identified on the basis of characteristic colony morphology, appearance on Gram staining, rapid urea hydrolysis, positive catalase, oxidase production, and the API Campy Kit (bioMérieux). *H. pylori* cells that grew out of one biopsy on the primary culture plate were subcultured, collected as a pooled population after 2–3 days of incubation, and preserved in sterile brain heart infusion (BHI) broth (Difco) with 15% glycerol at −70°C. All such cultures were analyzed, but, when multiple *vacA* or *hopQ* alleles were observed, five isolated clones were also obtained from each culture and all the assays were repeated on these clones.

Extraction of target bacterial genomic DNA

Suspensions of each bacterial strain were prepared in sterile distilled water (200 μl) by inoculation from single agar plate cultures with a standard loop. Samples were washed twice in sterile distilled water, boiled for 10 min, and centrifuged for 5 min at 14,000 × g. Supernatants were aliquoted and stored at + 4°C. At the testing time, the DNA concentration was evaluated spectrophotometrically by measuring optical density at 260 nm, and aliquots of 50 ng of the genomic DNA were used for PCRs.

Detection of *cagA* and *babA2* and typing of *vacA* and *hopQ* homologs

A PCR-based methodology was applied to detect the presence, in some *H. pylori* strains, of the *cagA* and *bab2* genes and to identify, in all the strains, the different families of the *vacA* and *hopQ* alleles. According to Tummuru et al. [32], Atherton et al. [12], Gerhard et al. [16], Zambon et al. [20], and Cao and Cover [21], the sets of primers shown in Table 1 were employed to specifically amplify the desired genomic fragments. The annealing temperatures during the amplification cycles were: 53°C, for *cagA*; 50°C, for *vacA*s1 and *vacA*s2; 53°C, for *vacA*m1 and *vacA*m2; 53°C, for *bab2*; 40°C, for type I *hopQ*, and type II *hopQ*. With respect to all other conditions, PCR amplifications were carried out with

Table 1 Oligonucleotide primers used in this study[a]

Amplified region	Primer name	Primer sequence (5'–3')	Amplicon size (bp)	References
ureA	outer-F1[b]	GCCAATGGTAAATTAGTTCC	361	[30, 31]
	outer-R1[b]	CTCCTTAATTGTTTTTACAT		
	inner-F2[b]	AGTTCCTGGTGAGTTGTTCT		
	inner-R2[b]	AGCGCCATGAAAACCACGCT		
cagA	F1	GATAACAGGCAAGCTTTTGAGG	349	[12, 32]
	B1	CTGCAAAAGATTGTTTGGCAGA		
*vacAs*1	VA1-F[c]	ATGGAAATACAACAAACACAC	259	[12]
	VA1-R[c]	CTGCTTG AATGCGCCAAAC		
*vacA*s2	VA1-F[c]	ATGGAAATACAACAAACACAC	286	[12]
	VA1-R[c]	CTGCTTGAATGCGCCAAAC		
*vacA*m1	VA3-F	GGTCAAAATGCGGTCATGG	290	[12]
	VA3-R	CCATTGGTACCTGTAGAAAC		
*vacA*m2	VA4-F	GGAGCCCCAGGAAACATTG	352	[12]
	VA4-R	CATAACTAGCGCCTTGCAC		
babA2	BABA2-F	AATCCAAAAAGGAGAAAAAGTATGAAA	802[d] 607[e]	[16, 20]
	BABA2-R	TGTTAGTGATTTCGGTGTAGGACA		
	BABA2-R607	CTTTGAGCGCGGGTAAGC		
hopQ-I	OP5136	CAACGATAATGGCACAAACT	534	[21]
	OP4829	GTCGTATCAATAACAGAAGTTG		
hopQ-II	BA8363	TCCAATCCAGAAGCGATTAA	430	
	BA8364	GTTTTAATGGTTACTTCCACC		
oipA	OipA-F	CAAGCGCTTAACAGATAGGC	450[f]	[19]
	OipA-R	AAGGCGTTTTCTGCTGAAGC		
hopZ	HopZ-F	GCCTGATATGGGTGGCATGGG	450[f]	
	HopZ-R	ATTTGATAGCCCGCGCTGAT		
sabA	SabA-F1	AGCTATTGACCAGCTCAATG	480[f]	
	SabA-R1	TAGTTGGATTCGTTCTCATTA		

[a] *ureA*, *cagA*, *vacA*, and *babA2* regions were assayed on all biopsy DNA and *H. pylori* strains; *hopQ*, *oipA*, *hopZ*, and *sabA*, only on the bacterial isolated strains. The fidelity of all primers was assessed by amplicon sequencing and alignment analysis with existing sequences in databases
[b] Nested primers
[c] Identical sets of primers with amplicons differentiated on the basis of molecular size
[d] Amplicons obtained using the reverse primer BABA2-R
[e] Amplicons obtained using the reverse primer BABA2-R607
[f] Approximate size of our amplicons evaluated on the basis of their gel electrophoresis migration

the same procedure employed in the first-round reaction for *ureA* detection. The expected bp length of the PCR products after electrophoresis on a 2% agarose gel is shown in Table 1.

Evaluation of the functional status of *oipA*, *sabA*, and *hopZ*

The *oipA*, *sabA*, and *hopZ* genes are characterized by calcitonin (CT) dinucleotide repeats in their 5'-coding regions, whose length is thought to determine whether or not the complete open reading frames are in frame, and full-length proteins are translated [19, 33–34]. A PCR sequencing-based methodology was applied to detect the number of the CT repeats in all these genes and, therefore, to attempt assessment of their expression status. According to de Jonge et al. [19] the sets of primers shown in Table 1 were employed to amplify the regions of the *oipA*, *hopZ*, and at least some of the *sabA* genes, containing the CT repeats. The PCR amplifications were performed using the same method described above, at the annealing temperatures of 55°C for *oipA* and *hopZ*, and 50°C for *sabA*. The resulting PCR products were subjected to gel electrophoresis in a 2% agarose gel and subsequently purified for sequencing using a GFX™ PCR DNA and a Gel Band Purification Kit (Amersham Biosciences). Sequences of purified amplicons were directly determined, with the same forward and reverse primers employed as in the previous PCR reactions, using the ABI PRISM BigDye Cycle Sequencing Ready Reaction kit (Applied Biosystems), according to the instructions supplied by the manufacturer, and a model 310 ABI DNA sequencer (Applied Biosystems), for the analysis of products.

Statistical methods

The Chi-squared (χ^2) test with Yates' continuity correction or the two-tailed Fisher's exact test was used for statistical

analysis. A p value of less than 0.05 was considered statistically significant.

Results

cagA, *vacA*, and *babA2* genotypes in *H. pylori*-positive gastric biopsies

As revealed by the positivity on PCR urease amplification, *H. pylori* DNA was demonstrable in all 90 of the patients investigated: in both gastric body and antrum in 76 of them; in the body only in 11; in the antrum only in 3. All patients were affected by chronic gastritis, inactive (CG) in 37, active (CGA) in 26, and active and associated with a gastric or duodenal peptic ulcer (CGA+PU) in 6 and 21 respectively.

The *H. pylori cagA*, *vacA*, and *babA2* genotypes of *H. pylori* were defined in all the 166 positive samples, with no evidence of multiple infections in patients in whom more than one biopsy was positive, except for one belonging to the CGA+PU group, who showed the presence of two different genotypes in his stomach: a *cagA*$^+$/*vacA*s1m2/ *babA2*$^+$ genotype at the body level and a *cagA*$^-$/ *vacA*s2m2/ *babA2*$^-$ genotype in the antrum.

Considering all 91 different positive biopsies, the gene *cagA*, the alleles s1 and m1 of *vacA*, and the gene *babA2* were present in the 53.8%, 60.4%, 35.2%, and 47.3% of the samples respectively (Table 2). The genotype *cagA*$^+$ appeared to be significantly associated ($P < 0.005$) with the s1 and m1 alleles of *vacA* alleles and with the *babA2* gene; analogously, and always at high levels of significance ($P< 0.005$), the s1 and m1 alleles of *vacA* were associated with each other and with the genotype *babA2*$^+$ (data not shown). Mixtures of the s1-s2 or m1-m2 alleles of *vacA* were seen in 4.4% and 7.7% of samples respectively, and in one sample, which was positive for a genotype *cagA*$^+$/ *vacA*s1/ *babA2*$^+$, the m region of *vacA* was untyped.

Distribution and combined presence of virulence-associated *cagA*, *vacA*, and *babA2* genotypes in different gastric diseases

With respect to their distribution in the three groups of patients with different gastrointestinal diseases, the *cagA*$^+$, *vacA*s1, *vacA*m1, and *babA2*$^+$ genotypes of *H. pylori*, as defined in all the 91 PCR-positive samples (Table 2), were more frequently associated with a high level of significance, with chronic gastritis in an active (CGA) rather than in an inactive (CG) form. The frequencies of *cagA*$^+$ and *vacA*s1 genotypes did not vary significantly in patients with an active form of chronic gastritis whether a gastric or duodenal ulcer was also present (CGA+PU) or not (CGA); on the contrary, *vacA*m1 and *babA2*$^+$ genotypes were less frequently observed, with a low level of significance, in the CGA+PU than in the CGA group. The *vacA*m1 genotype was always more frequent in the CGA than in the CG group, but, unlike *cagA*$^+$, *vacA*s1, and *babA2*$^+$, only with a low level of significance.

Table 2 Virulence-associated *H. pylori* genotypes in patients with different gastrointestinal diseases (all cases)

Genotype	Number (%) of genotypes from patients with			Number (%) from all patients, n=91	χ^2 value	P value
	CG, n=37	CGA, n=26	CGA+PU, n=28			
cagA$^+$	4 (10.8)	23 (88.5)	22 (78.6)	49 (53.8)	34.49*	< 0.005
					27.73**	< 0.005
					0.37***	NS****
cagA$^-$	33 (89.2)		3 (11.5)	6 (21.4)	42 (46.2)	
*vacA*s1	8 (21.6)	24 (92.3)	23 (82.1)	55 (60.4)	27.76*	< 0.005
					21.04**	< 0.005
					0.50***	NS****
*vacA*s2	28 (75.7)	1 (3.8)	3 (10.7)	32 (35.2)		
*vacA*s mixed	1 (2.7)	1 (3.8)	2 (7.1)	4 (4.4)		
*vacA*m1	3 (8.1)	19 (73.1)	10 (35.7)	32 (35.2)	25.57*	< 0.005
					5.96**	< 0.025
					6.14***	< 0.025
*vacA*m2	33 (89.2)	4 (15.4)	14 (50.0)	51 (56.0)		
*vacA*m mixed	1 (2.7)	3 (11.5)	3 (10.7)	7 (7.7)		
*vacA*m negative	0 (0)	0 (0)	1 (3.6)	1 (1.1)		
babA2$^+$	6 (16.2)	22 (84.6)	15 (53.6)	43 (47.3)	26.23*	< 0.005
					8.53**	< 0.005
					4.67***	< 0.025
babA2$^-$	31 (83.8)	4 (15.4)	13 (46.4)	48 (52.7)		

*CGA vs CG; **CGA+PU vs CG; ***CGA+PU vs CGA; ****NS = not significant ($P \geq 0.05$)

When considered in their associated combinations (Table 3), the *vacA*m1 allele and/or the *babA2* gene, if present in addition to the *cagA* gene and the *vacA*s1 allele, did not allow a better differentiation of the CGA, and in particular of the CGA+PU group, from the group of the inactive forms of chronic gastritis (CG); therefore, they did not appear to increase the value of the infecting *H. pylori* genotype as a predictor of the worsening of the gastritis. Although present in a few samples, only genotypes including *cagA*$^+$ and *vacA*s1m2 combinations were more frequently observed in patients with a gastric or duodenal ulcer (CGA+PU group) than in patients of both the CG and CGA groups.

Genotypes of *H. pylori* strains isolated from patients with different gastric diseases

H. pylori isolation was carried out on the biopsy samples from 52 out of the 90 patients investigated, the only samples that were sent to the microbiology laboratory in a proper form for culture. Antrum and body biopsy samples from 32 patients were inoculated immediately and *H. pylori* growth was observed in 24 of the cultures; samples from 20 patients were inoculated after a freezing time of not more than 7 days and *H. pylori* growth was observed in 6 of the cultures. Isolates were obtained from both gastric antrum and body in 19 patients; from the body only in 5; from the antrum only in 6. Also, on genotyping these isolates, no evidence of multiple infections was observed, except for the same patient in the CGA+PU group, in whom the presence of two different genotypes had been already observed by analyses on biopsy DNA extracts. Concerning the 29 patients from whom one or only identical isolates were obtained, 12, 9, and 8 of them belonged to the CG, CGA, and CGA+PU groups respectively.

Genotyping of the 31 different *H. pylori* strains considered not only the *cagA*, *vacA*, and *babA2* genes, but also the allelic forms of *hopQ* and the functional status of *oipA*, *hopZ*, and *sabA*, which were not efficiently detectable in the biopsy DNA.

In all cases, even when mixtures of *vacA*s1-s2 or *vacA*m1-m2 alleles had been found, and their presence validated by the assays on the isolated clones, results concerning *cagA*, *vacA*, and *babA2* genes confirmed those previously obtained by the DNA analyses from biopsies, and analogously, in spite of the lower numbers, a confirmation was obtained (Table 4) of the significantly higher presence of the *cagA*$^+$, *vacA*s1, *vacA*m1, and *babA2*$^+$ genotypes in patients of the CGA and CGA+PU groups than in the CG group of patients.

The presence of *hopQ* in its homologous form "type I" is considered to be a virulence-associated characteristic of *H. pylori* [21] and, indeed, a significantly higher presence of type I *hopQ*, either alone or more frequently, in association with type II, also characterized the *H. pylori* strains isolated from our worst cases of gastritis (groups CGA and CGA+PU; Table 4).

All *cagA*$^+$, *vacA*s1, *vacA*m1, *babA2*$^+$, and type I *hopQ* genotypes were significantly correlated with each other (in all cases with $P \leq 0.01$).

The functional status of *oipA*, *hopZ*, and *sabA* in *H. pylori* isolates does not correlate with the severity of the gastric disease nor, in most cases, with the *cagA*, *vacA*, *babA2*, and hopQ genotypes

The *oipA*, *hopZ*, and *sabA* genes are described as other virulence-associated characteristics when they have "on" status, i.e., when they are expressing their whole coding potentialities. Unlike the *cagA*, *vacA*, *babA2*, and *hopQ* genotypes, the "on" status of all these three genes, evaluated on the basis of the sequencing detection of the CT repeats in the PCR-amplified DNAs, was observed in our *H. pylori* strains with no significant difference with

Table 3 Prevalence of combined *H. pylori cagA*, *vacA*, and *babA* genotypes in the different disease groups

Genotype	Number (%) of genotypes from patients with			χ^2 value	*P* value
	CG, *n*=37	CGA, *n*=26	CGA+PU, *n*=28		
cagA$^+$/ /*vacA*s1	3 (8.1)	22 (84.6)	20 (71.4)	34.21*	< 0.005
				25.25**	< 0.005
cagA$^+$/ *vacA*s1m1	2 (5.4)	18 (69.2)	10 (35.7)	25.83*	< 0.005
				7.81**	< 0.01
cagA$^+$/ *vacA*s1m2	1 (2.7)	2 (7.7)	9 (32.1)		0.04***#
cagA$^+$/ /*vacA*s1/*babA2*$^+$	2 (5.4)	20 (76.9)	14 (50.0)	31.29*	< 0.005
				14.76*	< 0.005
cagA$^+$/*vacA*s1m1/*babA2*$^+$	2 (5.4)	17 (65.4)	6 (21.4)	23.31*	< 0.005
				2.45*	NS#
cagA$^+$/*vacA*s1m2/*babA2*$^+$	0 (0)	2 (7.7)	6 (21.4)		NS***#§

*CGA vs CG; **CGA+PU vs CG; ***CGA+PU vs CGA; #*P* value determined by Fisher's exact test; §NS = not significant ($P \geq 0.05$)

Table 4 Virulence-associated *H. pylori* genotypes in patients with different gastrointestinal diseases (cases of bacterial isolation)

Genotype	Number (%) of genotypes from patients with			Number (%) from all patients, n=31	P value* CGA vs CG	P value* CGA+PU vs CG
	CG, n=12	CGA, n=9	CGA+PU, n=10			
cagA$^+$	2 (16.7)	8 (89.9)	9 (90)	19 (61.3)	0.0019	0.0009
cagA$^-$	10 (83.3)	1 (11.1)	1 (10)	12 (38.7)		
*VacA*s1	4 (33.3)	9 (100)	7 (70)	20 (64.5)	0.0045	0.011
*VacA*s mixed[a]	0 (0)	0 (0)	2 (20)	2 (6.5)		
*VacA*s2	8 (66.7)	0 (0)	1 (10)	9 (29.0)		
*VacA*m1	1 (8.3)	7 (77.8)	3 (30)	11 (35.5)	0.0003	0.046
*VacA*m mixed[a]	0 (0)	1 (11.1)	2 (20)	3 (9.7)		
*VacA*m2	11 (91.7)	1 (11.1)	4 (40)	16 (51.6)		
*VacA*m negative	0 (0)	0 (0)	1 (10)	1 (3.2)		
babA2$^+$	2 (16.7)	8 (88.9)	8 (80)	18 (58,1)	0.0019	0.0082
babA2$^-$	10 (83.3)	1 (11.1)	2 (20)	13 (41.9)		
hopQ I	3 (25.0)	5 (55.6)	1 (10)	9 (29.0)	0.024	0.011
hopQ mixed[a]	1 (8.3)	3 (33.3)	8 (80)	12 (38.7)		
hopQ II	8 (66.7)	1 (11.1)	1 (10)	10 (32.3)		
oipA on	8 (66.7)	9 (100)	7 (70)	24 (77.4)	NS**	NS**
oipA off	4 (33.3)	0 (0)	3 (30)	7 (22.6)		
sabA on	2 (16.7)	4 (44.4)	5 (50)	11 (35.5)	NS**	NS**
sabA off	6 (50.0)	4 (44.4)	1 (10)	11 (35.5)		
sabA negative	4 (33.3)	1 (11.1)	4 (40)	9 (29.0)		
hopZ on	5 (41.7)	5 (55.6)	4 (40)	14 (45.2)	NS**	NS**
hopZ off	5 (41.7)	4 (44.4)	5 (50)	14 (45.2)		
hopZ negative	2 (16.7)	0 (0)	1 (10)	3 (9.7)		

* P value determined by Fisher's exact test; strains with unidentified genotypes were excluded by the evaluations; mixed and virulence-associated genotypes were considered as a whole

**NS = not significant ($P \geq 0.05$)

[a] Mixed genotypes were in all cases confirmed by the assays on the isolated *H. pylori* clones

respect to their clinical provenience (Table 4). In addition, no significant relationship could be demonstrated regarding the functional status of the three *oipA*, *hopZ*, and *sabA* genes, nor between the *cagA*, *vacA*, *babA2*, and *hopQ* genotypes and the functional status of *hopZ* and *sabA*; with regard to *oipA*, a correlation was only observed with the *babA2* (P= 0.007) and *hopQ* (P= 0.022) genotypes.

Discussion

Considerable geographical variations have been described with respect to the distribution, among the *Helicobacter pylori* strains, of many virulence-associated genes, as well as to the effective role of the virulence-associated *H. pylori* genotypes in the outcome, in the patient populations, of gastric histopathology and diseases such as chronic gastritis, peptic ulceration, gastric adenocarcinoma, and gastric mucosa-associated lymphoid tissue (MALT) lymphoma. In particular, for instance, considering comparable groups of patients with or without different gastric pathologies, the *cagA*$^+$ status was observed, in the isolated strains, in percentages of more than 90% in China [35], Hong Kong [36], and Colombia [37], of 73–75% in Turkey [38] and Brazil [39], of 62.9% in Padua, Italy [20], of 50–60% in France [40] and Portugal [37], and of 48 and 22%, in Kuwait, from patients of Middle Eastern or African origin respectively [41]. The *vacA*s1 allele was observed, in a study by van Doorn et al. [5] that considered strains from Asia, Central and South America, Europe, North America, Australia, and North Africa (Egypt), in percentages of 95, 71, 67, 62, 57, and 36% respectively. The *vacA*m1 allele is usually observed in percentages lower than those of the s1 allele [20, 35, 38, 40], except in the Iberian peninsula and in Central and South America [5]. The *babA2* genotype was present in all the isolates in Taiwan [42], in 56% and 86% of gastric biopsy specimens from routine gastroscopy or from gastric cancer patients respectively, in Korea [43], in 69% of the strains in Brazil [39], in 54% in France [40] and Turkey [38], and in 34% in Padua, Italy [20]. The presence of mixed combinations of *vac*A s1-s2 or m1-m2 alleles is often observed and also in this case with considerable geographical variations (i.e., 10% of the isolates in Kuwait [41], 37% in Portugal [37], and 45 and 52% in two different areas of China [35]); as each *H. pylori* strain contains only one *vac*A s and *vac*A m region [44] both the mixed s1-s2

and m1-m2 combinations must be considered to be a strong indication of the presence of multiple strains. Multiple infections are more frequent in areas of high *H. pylori* prevalence and may reflect the adaptation of multiple bacterial genotypes to different, non-overlapping micro-niches in the same stomach [45].

The *cagA*$^+$, *vacA*s1, *vacA*m1, and *babA2* genotypes are usually significantly associated with each other and are involved in the establishment of different types of histo-pathological lesions and gastric pathologies. However, as indicated at the beginning of this discussion, in the evaluation of the clinical involvement of the *H. pylori* virulence-associated genotypes, geographical differences must also be considered. This mainly concerns areas where the putative virulence factors are predominant and where their role in the clinical outcome cannot be demonstrated and cannot be helpful in predicting the patient's disease status [46]; in addition, if western and East Asian groups of H. pylori are differentiated on the basis of the vacA and cagA sequencing, only the western group has been reported to be associated with peptic ulcer disease [47].

Our results, obtained by analysis of all the specimens collected, showed that *cagA*$^+$, *vacA*s1, *vacA*m1, and *babA*2 genotypes were present in percentages of 53.8, 60.4, 35.2, and 47.3% respectively. Two different *H. pylori* strains were isolated by only one patient and mixed infections, revealed by a combined presence, in a single strain, of *vacA* s1-s2 or *vac*A m1-m2 alleles, were observed in 12.1% of the patients. On the basis of these results, and with respect to the *cagA*, *vacA*, and *babA* genotypes, the *H. pylori* strains circulating in western Sicily, southern Italy, although exhibiting, as expected, the general characteristics of the strains present in the European and Mediterranean areas, appeared to be more similar to those circulating in northern Italy [20] and France [40] than in Spain [5], owing to the lower prevalence in Sicily of the *vacA*m1 allele, or in Portugal [37], owing to the lower number of mixed infections, or in Turkey [38] or North Africa [5, 41], owing to the generally lower or higher prevalence respectively, again in Sicily, of the virulence-associated genotypes.

According to the more frequent observations in western countries, and again considering all the specimens we collected, *vacA*s1 and *vacA*m1 alleles and the *babA2* genotype were significantly associated with the *cagA*$^+$ status, and all *cagA*$^+$, *vacA*s1, *vacA*m1, and *babA2*$^+$ genotypes were significantly more prevalent in specimens collected from patients with chronic gastritis in an active form or with peptic ulcer disease than in those from patients with an inactive form of gastritis. On the contrary, no combination of well-characterized virulence-associated genotypes allowed better differentiations among the different groups of pathologies, nor did a single virulence-associated genotype characterize, with a higher prevalence, the pathological condition of the peptic ulcer disease. Although in contrast with some data in the literature [20, 21, 48] these last observations would appear to support the conclusions of Backert et al. [26] who say, at least with respect to the *cag*PAI functions, that they are important for the pathogenicity of *H. pylori*, but not sufficiently important to explain the development of the different clinical outcomes, the determination of which is highly complex and involves multiple bacterial and/or host factors. A new determinant of the *H. pylori* vacuolating cytotoxin, the type 1 of the *vacA* i region, has been recently described [14] and assessed to be associated with the risk of peptic ulcer [49]. Its possible presence in the *vacA*s1m2 strains, if confirmed, could give pathogenic significance to the higher number of *cagA*$^+$*vacA*s1m2 genotypes observed in our patients with peptic ulcer than in those without. Anyway, even if the *cagA*$^+$*vacA*s1m2 genotype was considered, no differences were observed between biopsy-extracted or microbial *H. pylori* DNA obtained from patients with gastric or duodenal peptic ulcers, and peptic ulcers were considered as a whole.

The other *hopQ*, *oipA*, *hopZ*, and *sabA* virulence-associated genotypes were assayed only on the 31 *H. pylori* strains isolated from 30 of the 90 patients.

With respect to the OMP 27 encoding *hopQ* locus, 29.0% of our strains exhibited only type I allele and 38.7% both type I and type II alleles. The latter percentage was somewhat higher than the 23.33% originally observed by Cao and Cover [21], but in any case, as they reported, the presence of type I allele, either alone or in a mixed combination with type II allele, was significantly associated with the *cagA*$^+$ status and with both active chronic gastritis and peptic ulcer disease.

On the contrary, with respect to the *oipA*, *hopZ*, and *sabA* genotypes, no significant association was observed between their status and the clinical outcome in patients from whom the related strains had been isolated and, in most cases, between their "on" or "off" statuses and the presence of any other virulence-associated genotype. The results in these cases conflict with data in the literature [1], mainly concerning the *oipA* gene, encoding an OMP usually considered to be involved in some crucial aspects of *H. pylori* pathogenicity and useful as a marker to discriminate duodenal ulcer from gastritis [50]. Because we evaluated the *oipA* functional switch status using DNA sequencing, on the basis of the number of the CT dinucleotide repeats in the 5' region of the gene [28], the effective production of the OipA protein needs to be tested [51] and the possibility has to be excluded that in our strains mutations in the promoter region prevented the switch effect of the CT repeats on the expression of a functional open reading frame.

In conclusion, although the need for these latter evaluations remains, it was possible to define the prevalence of

many virulence-associated genotypes in our Sicilian *H. pylori* isolates and their association with the clinical outcome in the infected patients. It is interesting to note that the prevalence values of the genotypes assayed were more similar in our isolates to values observed in the north of Italy and France than to values observed in Spain or other Mediterranean countries that are nearer and climatically more similar to western Sicily.

Acknowledgements The authors thank Dr. Salvatore Distefano for technical assistance.

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