

## Characterization of the Vacuolating Cytotoxin in *Helicobacter pylori* Strains Isolated from Iran

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### Abstract

**Objective:** *Helicobacter pylori* (*H. pylori*) cytotoxin and its heterogeneity amongst strains has been closely linked to the varying infection-associated clinical outcomes. In order to determine the decisive role of the vacuolating cytotoxin (*vacA*) gene mosaicism in its corresponding gene expression and phenotype, we aimed to characterize *vacA* alleles of different *H. pylori* strains in addition to the resulting protein and its vacuolating activity in epithelial cell culture.

**Materials and Methods:** *vacA* gene polymorphism was determined for 80 *H. pylori* strains isolated from dyspeptic patients, using multiplex gene-specific polymerase chain reaction (PCR). VacA protein was detected by immuno-blotting assay using a polyclonal anti-VacA antibody. *In vitro* cytotoxicity assay was conducted on HeLa cells in order to evaluate the vacuolating cytotoxin activity.

**Results:** Genotyping revealed the following strain distribution: 26 (32.5%) s1m1, 35 (43.8%) s1m2, and 19 (23.8%) s2m2 subtypes. Infection with s1m1 type strain was significantly associated with gastric cancer as compared to non-ulcer dyspepsia ( $p=0.005$ ) and peptic ulcer disease ( $p=0.008$ ). A 95-kDa immuno-reactive band that represented the vacuolating toxin was demonstrated in SDS-PAGE analysis of concentrated culture filtrate (CCF) of *H. pylori* strains. *H. pylori* CCFs induced HeLa cell vacuolation which correlated with the strain genotype; s1m1 strains demonstrated higher levels of vacuolation as compared to s1m2 strains, whereas s2m2 strains showed no detectable cytotoxic activity.

**Conclusion:** The current study confirmed the relatively high cytotoxic activity of s1m1 type *H. pylori* strains which infect the majority of patients suffering from gastric cancer and may be partly responsible for the pathogenesis of this mortal disease.

**Keywords:** Vacuolation, Cytotoxins, Genotype, Virulence, *Helicobacter pylori*

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### Introduction

Colonization of the gastric mucosa by *Helicobacter pylori* (*H. pylori*) results in mucosal inflammation, peptic ulcer disease (PUD), gastric adenocarcinoma, and gastric lymphoma (1, 2). *H. pylori* strains secrete a cytotoxin known as VacA into the extracellular space (3). Increasing evidence indicates that cytotoxin contributes to the ability of *H. pylori* to colonize the stomach and plays a major role in the pathogenesis of peptic ulceration and gastric cancer (GC) (4). The most extensively characterized activity of the cytotoxin is its capacity to induce vacuolation in mammalian cells. In addition to inducing the formation of intracellular vacuoles, the cytotoxin causes multiple effects on target cells, including depolariza-

tion of the membrane potential, permeabilization of epithelial monolayers, and detachment of epithelial cells from the basement membrane (5).

The vacuolating cytotoxin gene (*vacA*) is one of the vastly studied virulence determinants of *H. pylori*. *vacA* gene has a mosaic structure comprising of two families of allelic variation; the signal sequence region (s1, s2) and the mid-region (m1, m2). The existence of various genotypes of the s and m regions allows for the formation of several genotypic combinations of *vacA* gene, even though the s2m1 genotype is rarely found. The different combinations of s and m regions determine the extent of cytotoxic activity (6, 7). It is believed that strains with the genotype s1m1 produce the highest levels of vacuolating cytotoxin

*in vitro*. On the other hand, strains with the s2 genotype produce an inactive toxin. Furthermore, strains with the m2 genotype differ in toxicity from that of m1 strains. Cytotoxic strains have been detected more frequently among patients with peptic ulcer disease as compared to those with chronic gastritis (8, 9).

The aim of this study was to characterize the *vacA* alleles of different *H. pylori* strains, as well as the cytotoxin protein and its vacuolating activity in epithelial cell culture.

## Materials and Methods

### Patients

The studied population consisted of 80 *H. pylori*-infected patients who underwent upper gastrointestinal endoscopy or gastric resection. The subjects were categorized based on endoscopic findings into the following groups: non-ulcer dyspepsia (NUD) [n=50], peptic ulcer disease (PUD) [n=12], and gastric cancer (GC) [n=18] patients. In the GC group, the presence of malignant lesions was confirmed by histopathology. Informed consent was obtained from every patient prior to sample collection which was performed according to standard protocols approved by the local Ethical Committee of the Pasteur Institute of Iran.

### Bacterial strains

*H. pylori* was cultured by smearing biopsy specimens on the surfaces of *H. pylori* specific peptone agar (HPSPA) medium, followed by incubation at 5% oxygen and 10% carbon dioxide for 72 hours at 37°C for up to five days. Typical colonies were identified as *H. pylori* by morphology, gram staining and biochemical tests which included both urease and catalase tests. Single colonies were subcultured for DNA extraction and liquid culture.

### DNA extraction

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

### Multiplex PCR assay

The *ureC* gene was primarily amplified to confirm the identity of isolated *H. pylori* strains (11). The *vacA* signal and mid region were typed by allelic type-specific multiplex PCR by using gene-specific primers (Table 1). Amplification of the target

genes was carried out in a total volume of 20 µl which contained 2 µl of 10× PCR buffer (Fermentas, Lithuania), 1.5 mM of MgCl<sub>2</sub>, 0.5 µl of each primer (25 pM/µl) (CinnaGen, Iran), 0.2 mM of deoxynucleotide, and 0.5 U of Taq Polymerase (Fermentas, Lithuania). Conditions for thermal cycling were 4 minutes of 94°C as primary denaturation followed by 35 cycles of 94°C for 60 seconds, 60°C for 60 seconds, 72°C for 60 seconds and a final extension step for 4 minutes at 72°C. Product sizes were differentiated on a 2% agarose gel and visualized by staining with ethidium bromide under UV light.

### Broth culture filtrates (BCFs)

*H. pylori* single colonies were grown in Brucella broth supplemented with 0.2% β-cyclodextrin (Fluka, USA) for 72 hours at 37°C under microaerophilic conditions and continuous shaking at 150 rpm. When bacterial suspensions reached an optical density of 0.5 units at 550 nm, bacteria were removed by centrifugation (3000 g for 15 minutes) and the supernatant sterilized by passage through 0.22 µm-pore-size membrane filters (Orange Scientific, Belgium). Brucella culture filtrates (BCFs) were then concentrated 30-fold, using centrifugal filter devices (Millipore, USA) and stored at -20°C as concentrated culture filtrates (CCFs).

### Detection of VacA by immuno-blotting

CCFs were loaded onto 8% SDS-polyacrylamide gels in the amounts corresponding to the identical initial culture volumes. Following transfer of the proteins to nitrocellulose membranes (Schleicher & Schuell, Germany), the membranes were blocked with 3% skim milk (Merck, Germany) in phosphate buffered saline (PBS) containing 0.05% Tween for 2 hours at room temperature. Cytotoxin was detected using polyclonal rabbit anti-VacA antiserum (Austral Biologicals, USA), which was further detected by peroxidase-conjugated anti-rabbit antibodies (Dako, Denmark) as the secondary antibody. Blots were developed using enhanced chemiluminescent (ECL) detection reagents (Amersham, England) (10).

### Cell vacuolation assay

HeLa cells (human cervical adenocarcinoma cell line, NCBI C115, National Cell Bank of Iran, Tehran, Iran) were cultured as monolayers in RPMI-1640 (Gibco Inc., UK) supplemented with 10% fetal calf serum (FCS) in 25cm<sup>2</sup> plastic flasks at 37°C and under 5% CO<sub>2</sub> atmospheric conditions. HeLa cells were detached from the flask using 0.25% trypsin, resuspended

Table 1: Primer sequences and amplicons size

Amplified region	Primer	Primer sequence	Size of amplified fragment (bp)	References
Signal region	VA1-F	5'-ATGGAAATACAACAAACACAC-3'	s1 :259 or	6
	VA1-R	5'-CTGCTTGAATGCGCCAAAC-3'	s2: 286	
Mid region	VAG-F	5'-CAATCTGTCCAATCAAGCGAG-3'	m1: 567 or	7
	VAG-R	5'-GCGTCAAATAATTCCAAGG-3'	m2: 642	

in RPMI1640 and counted by hemocytometer. Twenty-four hours prior to each vacuolation experiment,  $10^4$  cells /100  $\mu$ l were seeded into each well of 96-well plates. CCFs were then incubated with HeLa cells and the development of vacuoles was visually assessed via inverted microscope (Zeiss, Germany) for up to 24 hours of co-incubation. Un-inoculated broths were used as negative controls (12).

**Statistical analysis**

Data were analyzed using the SPSS (version 11.5) program. For univariate analysis, both the Chi-square and Fisher’s exact test were used. P values lower than 0.05 were considered as statistically significant.

**Results**

**Confirmation of *H. pylori* identity**

All of the 80 DNA samples were amplified with *ureC*-specific oligonucleotide primers which yielded 294-bp PCR products and thereby confirmed the identity of *H. pylori* (Fig 1).

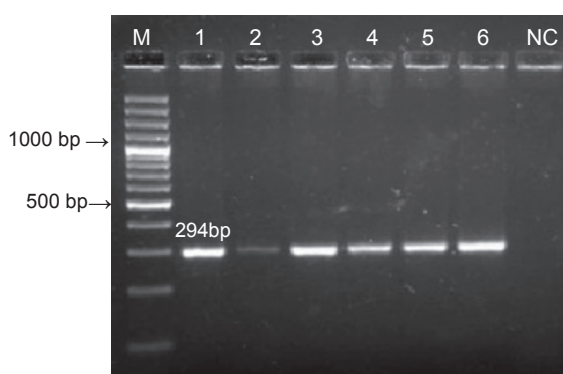


Fig 1: *ureC* gene amplification using specific oligonucleotide primers yielded a product of 294 bp product of representative of *H. pylori*. 1-6 represent *ureC*-positive strains. NC and M illustrate the negative control and 100 bp DNA marker, respectively.

***vacA* genotyping**

Genotyping of 80 clinical *H. pylori* strains was performed by multiplex PCR which generated product sizes pertaining to varying signal regions; s1 (259 bp) and s2 (286 bp), and mid-regions; m1 (567 bp) and m2 (642 bp) (Fig 2). Sixty one of the 80 iso-

lates (76.3%) were of the s1 genotype and 19 isolates (23.8%) were s2. With regard to the middle region, 26 strains (32.5%) were found to have the m1 allele type and 54 strains (67.5%) yielded the m2 allele genotype. Collectively, 26 (32.5%), 35 (43.8%) and 19 (23.8%) strains were s1m1, s1m2, and s2m2, respectively.

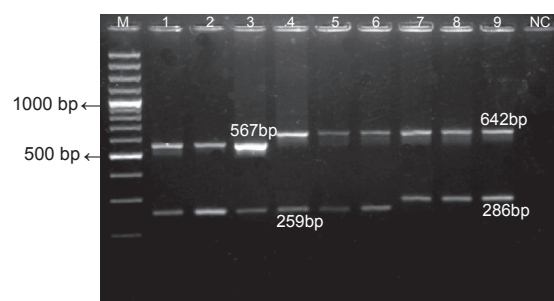


Fig 2: Multiplex-PCR amplification of signal and middle regions of *vacA* yielding: s1 (259 bp), s2 (286 bp), and m1 (567 bp), m2 (642 bp) amplicons. 1-3, 4-6, and 7-9 illustrate s1m1, s1m2, and s2m2 strains. NC and M illustrate negative control and 100 bp DNA marker, respectively.

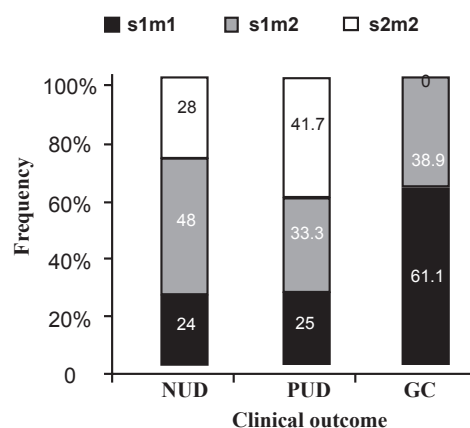


Fig 3: The distribution of *vacA* genotypes in patients who were categorized into non-ulcer dyspepsia (NUD), peptic ulcer diseases (PUD) and gastric cancer (GC).

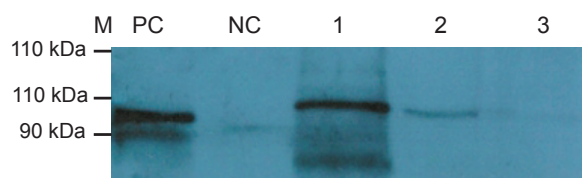
**Association of *vacA* genotypes with gastrointestinal disease**

All (100%) of the gastric cancer patients were infected with *vacA* s1 strains, in comparison to 36 of 50 strains (72%) from non-ulcer dyspeptic patients (p=0.008) and 7 of 12 (58.3%) strains from peptic ulcer patients (p=0.006). Analysis of the *vacA*

mid-region allele revealed that the *vacA* m1 genotype was found more frequently in strains from patients with GC (61.1%) than in strains from NUD patients (24%,  $p=0.006$ ) or those with PUD (25%,  $p=0.057$ ), but the difference for the latter group did not reach statistical significance. The distribution of the m2 genotype was slightly higher in non-ulcer dyspeptic patients (76%) than that of patients suffering from peptic ulcers (75%). The frequencies of *vacA* genotypes among patients with different clinical outcomes are demonstrated in figure 3. Possession of s1m1 genotype was significantly associated with gastric cancer when compared with NUD ( $p=0.005$ ) and PUD ( $p=0.008$ ) patient groups.

#### Characterization of VacA production

Analysis of CCFs in *H. pylori* strains with different *vacA* genotypes was carried out by immunoblotting using commercial anti-VacA antibody. This assay indicated that an immuno-reactive band of approximately 95-kDa was present in all of the concentrated culture filtrates of *H. pylori* strains. However, when standardized protein amounts of CCF protein from different strains were immunoblotted and compared, the VacA band in supernatants from the s1m1 strain as toxin-positive (Tox<sup>+</sup>) was more intense than that of the s2m2 strain as a toxin-negative (Tox<sup>-</sup>) supernatant (Fig 4).

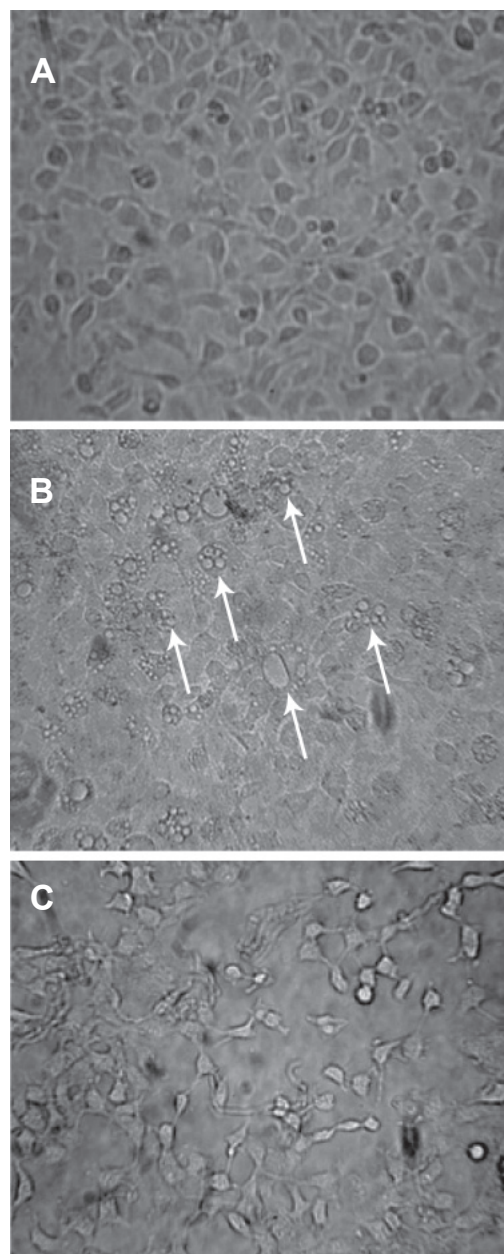


**Fig 4:** Western blotting analysis of native VacA detected in representative CCFs of: (1) s1m1 strain, (2) s1m2 strain, and (3) s2m2 strain. PC, NC, and M illustrate the positive control, negative control and protein molecular weight markers, respectively.

#### Assessment of vacuolating activity

*H. pylori* CCFs exhibited cytotoxic vacuolating activity when applied to cultured HeLa cell monolayers. The vacuoles were most prominent at 24 hours following co-incubation. The vacuolated cells were photographed under an inverted microscope and compared to cells treated identically with uninoculated broth media. Under vacuolating conditions, cultured epithelial cells retained their shape and adherence (Fig 5). The number of vacuoles per cell varied from one large vacuole to approximately ten vacuoles with size variability. Vacuolated cells usually comprised more than 50% of the total number of assayed cells.

This experiment indicated that s1m1 type protein produced prominent cell vacuolation whereas the s2m2 type lacked any detectable toxic activity (Fig 5).



**Fig 5:** Effect of toxic strains of *H. pylori* CCF on HeLa cell monolayers. (A) HeLa cells treated with un-inoculated *Brucella* broth, (B) HeLa cells treated with s1m1 strain, and (C) HeLa cells treated with s2m2 strain. Arrows indicate the vacuolated cells.

#### Discussion

*H. pylori* cytotoxin damages epithelial cells by inducing the formation of vacuoles and is frequently associated with the pathogenesis of duodenal ul-

cers and gastric adenocarcinoma. The presence of cytotoxic activity has been suggested as a marker of enhanced virulence acting either directly via cytotoxic action or indirectly via an enhanced inflammatory and immune response. The variable structure resulting from mosaicism of *vacA* alleles has been related to the differences in cytotoxin production and to distinct clinical outcomes of *H. pylori* infection (3, 5). Characterization of *vacA* gene mosaicism and the expressed cytotoxin determine whether distinct *H. pylori vacA* genotypes can predict the clinical outcomes of *H. pylori* infection. It may also be important to provide an understanding of the cytotoxin vacuolating activity of different *H. pylori* strains.

In the current study, a multiplex PCR-based assay for analysis of *vacA* allelic variation has demonstrated that the s1m2 genotype is the most prevalent polymorphic type of *vacA* in Iranian dyspeptic patients regardless of disease status. Morales et al. (13) and Ito et al. (14) from Mexico and Japan have reported that s1m1 is the dominant *vacA* gene subtype in their populations, whereas the most prevalent *vacA* subtype in China is s1m2 (15, 16). The frequency of the *vacA* s1m1 allelic type (32.5%) in this study was higher than reports from Hong Kong (26%) and Nigeria (24%), but lower than those of the Netherlands (36%), Ethiopia (48%), Korea (78%), and Brazil (80%) (17-21). This study demonstrated a significant association between s1m1 *vacA* genotype and gastric cancer which is in agreement with previous studies by Kidd et al. (22) and Miehlke et al. (23) who have found an association between s1m1 genotype and gastric adenocarcinoma. Atherton et al. (8) have demonstrated that s1m1 or s1m2 genotypes are more frequently associated with peptic ulceration as one of the consequences of *H. pylori* infection. Accordingly, the association between infection with s1m1 *vacA* isolates and PUD has also been demonstrated in the German (8), Dutch (19), North American (24) and Singaporean (25) populations. Other studies from East Asian countries such as China (16) and Japan (26) have reported no association between *vacA* genotype and PUD. Our results also revealed a predominance of the s2 genotype over s1 in our NUD patients whereas s1 was more frequent in GC patients. Therefore, the *vacA* s2 allele does not seem to contribute to PUD or GC development in our population.

Characterization of VacA in CCFs from *H. pylori* strains using polyclonal anti-VacA antibody indicated the presence of a 95-kDa band. The intensity of the s1m1 VacA band was higher than that in s2m2 strains. This finding has confirms the

fact that every *H. pylori* strain produces the VacA gene product, but the CCFs from s1m1 strains, as toxigenic strains, contain higher concentrations of VacA than non-toxigenic s2m2 strains. The heterogeneity among strains in the level of VacA may be due to the levels of *vacA* transcription or VacA secretion which is substantially related to amino acid sequence difference of these allelic subtypes. In this study, we have also demonstrated that s1m1 strains differed from s1m2 strains in the capacity to induce vacuolation when applied on HeLa cell monolayers. This experiment revealed that the s1m1 VacA protein produced prominent cell vacuolation, whereas the s2m2 VacA protein lacked any apparent vacuolating activity in this assay. It may be speculated that this differences may be due to higher concentrations of VacA in CCFs from s1m1 strains than s2m2 strains, but when the assayed samples were normalized for the concentrations of VacA, the enhanced effect was not diminished. Since the enhanced activity of the s1m1 cytotoxin may stimulate a greater degree of gastric inflammation, this *in vitro* finding is consistent with the high frequency of s1m1 strains isolated from GC patients.

Previous studies have mostly used HeLa cell lines to assay the classic activity of *H. pylori* cytotoxin. Although every *H. pylori* strain possess the *vacA* gene, only half of the *H. pylori* strains cause vacuolation of HeLa cells. This phenomena questions the potency of HeLa cells for evaluation of the true vacuolating activity of the tested strains. Previous studies have demonstrated that cytotoxin enters the cells by receptor-mediated endocytosis (27); hence the absence of specific receptor on HeLa cells may influence the accuracy of cytotoxin vacuolation on this cell line by a deficiency in the receptor-mediated entrance of cytotoxin into this epithelial cell line. Hence, the HeLa cell assay may not entirely reproduce the potential pathogenicity of clinical isolates of *H. pylori in vitro* and may underestimate the true frequency of cytotoxic activity of toxigenic strains.

## Conclusion

The significant mosaicism in *vacA* gene structure is reflected and can be detected in its functional properties using an appropriate cell culture assay. The functional toxicity analysis may determine the predictive value of *vacA* genotyping as a method of toxicity detection in *H. pylori* strains. On the other hand, the vast heterogeneity of the *vacA* gene, protein and function may partly explain the occurrence of different clinical outcomes following *H. pylori* infection.

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