Review Article

Vacuolating cytotoxin of *Helicobacter pylori*

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Abstract
Vacuolating cytotoxin (VacA) is one of the most important virulence factors of H. pylori (Hp), which is the only toxic protein that is secreted from Hp cell into the culture supernatant. The effects of VacA on eukaryotic systems is the subject of many previous and on going research studies. Intracellular targets for this toxin include: late endosomal and lysosomal compartments, mitochondria, cell-cell junctions and phospholipid bilayers. Its effects on these targets include vacuolation of late endosomal and lysosomal compartments, apoptosis and channel formation, which result in the increase of ion uptake specially anions. The aim of this review is to increase the perception on this toxin and its functions.
Keywords: *Helicobacter pylori, Vacuolating cytotoxin, vacA, Virulence, Pathogenesis.*

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Literature Review

Helicobacter pylori has been termed *Campylobacter pyloridis* for many years and was introduced as *Helicobacter pylori* in 1984 by Marshall and Warren (Warren and Marshall, 1983). Hp is a gram negative, spiral shaped bacterium that requires microaerophilic conditions for *in vitro* growth. It has flagella for movement and produces urease to convert urea to CO_2 and ammonia resulting in alkaline conditions in the gastric lumen and therefore creation of a tolerable environment (Cave, 1999).

This organism infects the gastric mucosa of about 60% of the world population and is involved in type B gastritis, gastric ulcers, duodenal ulcers (peptic ulcer disease, PUD) and gastric neoplasia (adenocarcinoma) (Schmitt and Hass, 1994; Covacci *et al.*, 1999). In developing countries, this bacterium affects more than 80% of the adult population whereas in the developed countries, infection is present in 20% of those under age of 30 and in 50% of population aged between 30 and 60 (Telford *et al.*, 1994b). Nevertheless, the majority of patients remain asymptomatic (van Doorn *et al.*, 2000).

The infection occurs at early ages (like other enteric diseases) and remains persistent for even as long as the life time of the host. Nevertheless, it remains non-invasive (Cave, 1999).

Immunological studies have shown that host immune responses to this bacterium are not protective. There are two types of cellular immune responses against Hp infection (Mohammadi *et al.*, 1996a; Mohammadi *et al.*, 1997; Nedrud *et al.*, 1998). One, which has a T helper (1) phenotype and is responsible for antral inflammation and the other with Th2 cells, which are responsible for humoral immune

responses through specific cytokine secretion, such as IL-4 (Mohammadi *et al*., 1996a).

Several studies suggest that Hp adheres to gastric epithelial cells without any significant destruction of cellular membranes (Hofman *et al*., 2000). This bacterium is capable of residing in the gastric pH of 2-7, pertaining to luminal surface to deep epithelial layers respectively (Cover *et al*., 1997). Hp infection is the second most common bacterial infection after *Streptococcus mutans* infection that is responsible for dental cavities (Telford *et al*., 1997).

There are some virulence factors, which are necessary for Hp pathogenesis. These factors include: **Urease**, which is required for neutralization of acidic pH of the gastric lumen and initial colonization of Hp cells (Labigne *et al*., 1991). This enzyme is active in acidic pH and hydrolyzes urea into CO_2 and ammonia which protects Hp cells by creating a neutral microenvironment (Dunn *et al*., 1997). **Flagellum** which is the major cause of bacterial movement in the gastric mucosal layer (Leying *et al*., 1992). **Cytotoxin-associated gene product (CagA)** which is present in about 60-70% of Hp strains and is associated with exacerbation of the associated gastric inflammation (Atherton, 1997). This effect may be due to the induction of IL-8 production (Atherton, 1997). *cagA* gene is a genetic marker for a major gene cluster which is called cagPAI and is about 40 kb and is suggested to be acquired from other bacterial species. Apparently Hp can infect more subjects than other Helicobacter species because of producing enzymes such as catalase, oxidase, protease and phospholipase (Ilver *et al*., 1998). **Vacuolating cytotoxin (VacA)** is one of the most important virulence factors of Hp that is extracellular and causes damage to the gastric epithelial layer as a result of vacuolation of late endosomal and lysosomal compartments of these cells.

All of the Hp strains possess the gene for vacA but only 50-60% of them manifest cytotoxic activity *in vivo*. This gene is composed of different regions beginning with the signal sequence (s region) followed by the mid region (m region) which display the following two genotypes respectively: s1 or s2 and m1 or m2. These genotypes are distributed differently in various parts of the world. The mosaic combination of these genotypes result in several genotypes, which include: s1m1, s1m2, s2m2 and the s2m1 genotype that has seldom been reported (Atherton *et al*., 1995; Letley *et al*., 1999; Mohammadi *et al*., 2003).

Pathological studies in western countries have shown that the majority of Hp strains that cause PUD are of the s1m1 or s1m2 genotype and the s2m2 genotype has little relation with PUD or gastric cancer. In our study and some eastern studies such as that from Taiwan, it was shown that s1m2 strains are more prevalent in PUD patients (Wang *et al*., 1998; Mohammadi *et al*., 2003).

VacA protein is composed of three parts: signal peptide (33 aa), mature protein (95 kDa), carboxy terminal region (45 kDa). The mature peptide has two parts with different roles in vacuolating activity of Hp. This protein is secreted from Hp cells into the gastric lumen and acidic pH of gastric juice causes activation of this protein and internalization of the protein into gastric epithelial cells. Vacuolation of these cells will occur as a result of changes in H^+-K^+ ATPase activity of late endosomal and lysosomal compartments.

1- *vacA* gene

As previously mentioned, almost all Hp strains have one copy of *vacA* gene but only 50-60% of them show cytotoxic activity (Cover *et al*., 1990; Han *et al*., 1998). *vacA* has an open reading frame (ORF) of about 3900 bp that is responsible for coding a protein with approximately 1296 aa with MW of about 140 kDa (Telford *et al*., 1994b). This ORF has an inverted repetitive sequence that is responsible for the stem loop conformation in mRNA (Cover *et al*., 1994). In the upstream of the *vacA* gene, AGGAA sequence (Shine Dalgarno) is located that is required for effective initiation of translation in prokaryotes (Phadnis *et al*., 1994). It should be noted that there are about 10 direct repeats in this coding region that most of them are translated in the same frame (Phadnis *et al*., 1994). Upstream, there is a region that includes promoter and other required signals for transcription and translation and -10 and -35 consensus motifs of promoter are located in this region that are separated with 15 bp. This promoter is located 100 bp upstream of coding region of *vacA* gene. Immediately following the coding region, there is a signal for Rho independent termination of transcription. This gene is located 224 bp downstream of the gene for cysteinyl-tRNA synthetase (Fig. 1) (Phadnis *et al*., 1994).

vacA gene has two different regions: signal sequence (s) and mid region (m) which are very heterogenic and vary in sequence in different strains from various locations in the world (Rudi *et al*., 1998).

The s region has two sub-types: s1 and s2. The s1

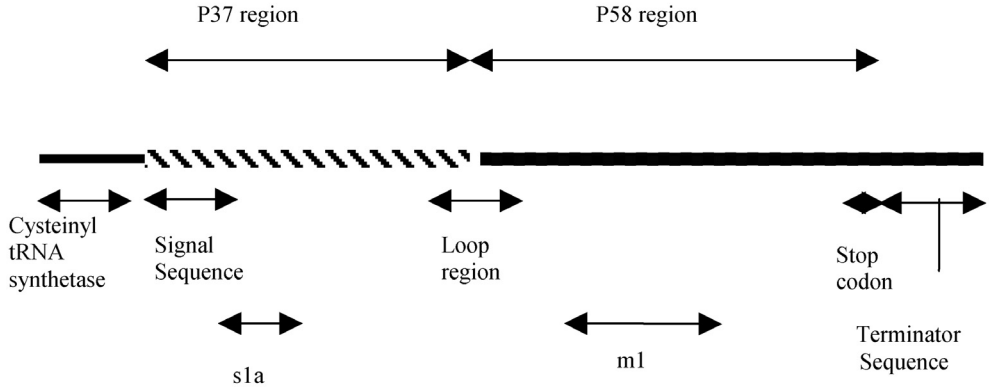

Figure 1. Schematic view of vacA gene and different parts of this ORF from an s1m1 strain of Hp.

type is associated with the presence of *cagA* gene, *in vitro* cytotoxic activity and gastric and duodenal ulcers (PUD & DUD) (Han *et al*., 1998; Rudi *et al*., 1998). The difference between these two types is related to small insertions of 24 bp in the s2 type. Thus it seems as the s2 type does not have effective secretion of cytotoxin through bacterial plasma membrane (Rudi *et al*., 1998). The s1 region has been divided into two types of alleles: s1a and s1b, of which s1a is more prevalent than s1b or s2 (Han *et al.,* 1998). In many pathogenesis studies, most of Hp isolates from patients with PUD history are s1 and most of them are *cagA*-positive and *iceA*-positive, that are stronger pathogens and have lower density than s2m2 Tox-negative strains (Atherton *et al*., 1995; Evans *et al*., 1998; Han *et al*., 1998; van Doorn *et al*., 1998, 2000). These studies were performed in western countries such as Germany, Portugal, Brazil and USA. Recently a new subtype has been found in eastern Asia, which is called s1c and is rarely found in other parts of the world (van Doorn *et al*., 2000).

The m region has two subtypes: m1 and m2. The m2 subtype has a 23 aa insert with a ATP/GTP binding motif which is not present in Tox+ (m1) strains (van Doorn *et al.,* 2000). But prevalence of m1 is commonly similar to m2 and little relation has been found between the m type and presence of *cagA* or occurrence of PUD (Han *et al.,* 1998; van Doorn *et al.,* 2000). However, we have reported that the majority of the Iranian Hp strains to be of the m2 genotype and this study showed that in our country, most of PUD samples are s1m2 instead of s1m1(Mohammadi *et al.,* 2003).

There are different combinations of s and m subtypes producing the following genotypes: s1m1, s1m2, s2m2 and the s2m1 type has yet to be detected in western countries (Atherton *et al.,* 1995). But we (Mohammadi *et al.,* 2003) and an African group (Letley *et al.,* 1999) have reported this genotype in some of the studied strains that is not represented in PUD group but is found in NUD group. The absence or low occurrence of s2m1 genotype may suggest that strains with this genotype have a selective disorder or lack viability (Rudi *et al.,* 1998).

2- VacA protein

VacA is the only toxic protein which is produced and released by Hp (Galmiche *et al.,* 2000) and it is an extracellular toxin (Phadnis *et al.,* 1994). The precursor of this protein has no homology to other reported proteins in the GenBank (Phadnis *et al.,* 1994) but it is similar to some exoproteins such as IgA protease types that are produced by *Neisseriae* and *Haemophilus* species (Schmitt and Hass, 1994).

VacA is produced as a 139 kDa precursor that releases a 90 kDa mature peptide out of the cell and a 45 kDa C-terminal fragment will be retained with bacterial cell wall that consists of two fragments with MW of 33 and 12 kDa (Telford *et al.,* 1994a) and it suggests that this fragment directs transport of the protein through the membrane (Telford *et al.,* 1994b).

The 90 kDa monomer forms oligomers with MW of up to 1000 kDa in bacterial culture media or *in vivo* conditions (Cover and Blaser, 1992; Pai *et al.,* 2000). Each oligomer consists of 6 or 7 copies of 90 kDa monomer (Lupetti *et al.,* 1996), and converts to monomeric forms under denaturing conditions, during SDS-PAGE (Lupetti *et al.,* 1996). In the bacterial supernatant the mature peptide (90 kDa) will be

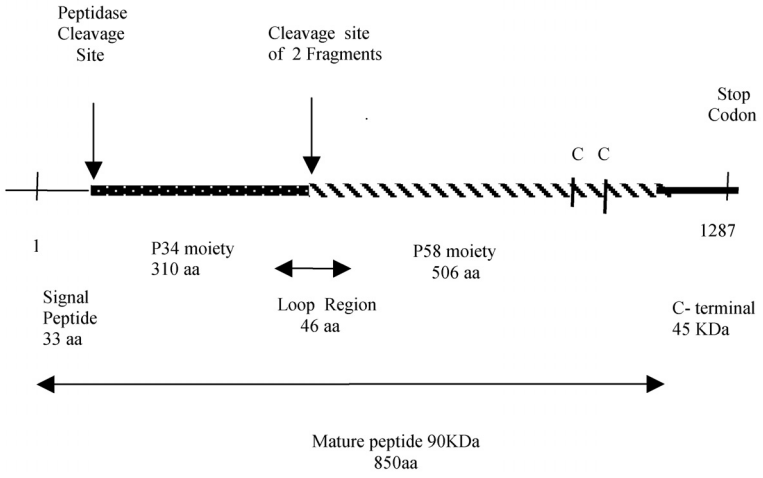

Figure 2. Schematic view of VacA protein, which represents its precursor and different fragments that are created during its passage through Hp plasma membrane.

processed mostly after alanine (at position 352) which is located in a hydrophilic region that forms a flexible loop in the protein, containing 46 amino acids (Telford *et al.,* 1994b; Manetti *et al.,* 1995) and results in the N-terminal 37 kDa and C-terminal 58 kDa fragments. Following cleavage, these two fragments remain associated with non-covalent interactions (Telford *et al.,* 1994b; Manetti *et al.,* 1995; Lupetti *et al.,* 1996; Galmiche *et al.,* 2000). According to Telford *et al.* (1994b) the mature protein has a terminal signal peptide with 33 amino acid residues that is most likely responsible for trans-membrane secretion of the protein (Fig. 2).

As mentioned above, each oligomer consists of 6 or 7 copies of 90 kDa monomers and nearly 70% of oligomeric, wild types of the toxin have heptameric radial symmetry and in mutant forms of VacA, hexameric forms are prevalent (Fig. 3) (Lupetti *et al.*, 1996). These hexameric molecules have a deletion in amino acids of loop region and this deletion causes the two subunits come closer and favors the formation of hexameric molecules.

Oligomerization is a transient state for hiding hydrophobic regions in a protein that are required for membrane interaction of the molecule (Molinari *et al.*, 1998). Mature peptide has about 850 aa but not all of the C-terminal amino acids of the p58 are required for vacuolation. Upon N and C terminal truncation it was found that C-terminally truncated molecule with 422 aa (amino acid 1 to 422) is the smallest fragment that can induce vacuolation in HeLa cells but non of the N-terminal truncated forms of VacA are able to induce vacuolation in the cytosol and more importantly deletion of 17 aa from the N-terminus of this protein (following signal peptide), destroys vacuolation activity. On the other hand, N-terminus of VacA protein is necessary for intracellular vacuolation (de Bernard *et al.,* 1998; Ye *et al.,* 1999).

In electronic micrographs, VacA oligomer appears flower-shaped and consists of two parts (Lupetti *et al.,* 1996; Cover *et al.,* 1997); large lobes like flower petals and ring structure toward the center of the molecule. In VacA protein, there are two cystein residues that likely have a role in secretion of *vacA* gene product, and are 7 or 11 aa apart (Cover *et al.,* 1994). Expected isoelectric pH (pI) of the 90 kDa mature VacA protein is 9.1, whereas the observed pI for this protein is 6.1 (Cover and Blaser, 1992). VacA protein is found in three forms (Molinari *et al.,* 1998):

1. Natural oligomeric structure that consists of 12 VacA monomers that are associated with non-covalent forces.

2. At pH lower than 5, oligomers dissociate to monomers and represent hydrophobic regions that are necessary for effective insertion into lipid bilayers.

3. Under neutralization, the structure of VacA changes by monomers forming into oligomers with different physical and structural properties.

Native VacA has low but detectable activity and with treatment at pH lower than 5, the activity may increase which is associated with conformational changes in the molecule (de Bernard *et al.,* 1995). This form of activation with acidic pH is related to the development of surface hydrophobicity that enables the toxin for

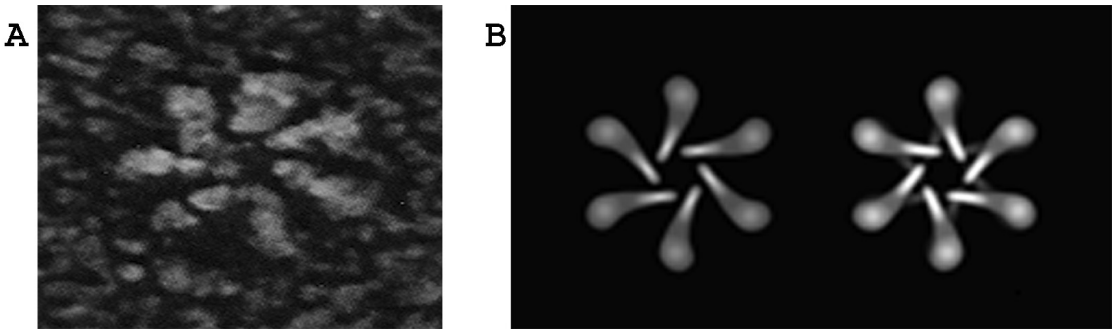

Figure 3. Electron micrographs from VacA heptamer (A) and hexameric (B) oligomers, (Lupetti *et al.*, 1996).

deep penetration into the lipid bilayers. Membrane insertion is a critical stage in the intoxication process of peptic toxins that have intracellular targets, because this involves in transport of their catalytic domains into the cytosol (Molinari *et al.,* 1998).

Incubation of VacA with epithelial cells at differing temperatures for 10 minutes has shown that the activity of this protein is stable in temperatures up to 55ºC but higher temperatures induce significant activity reduction (Yahiro *et al.,* 1997). N-terminal segment of the protein mostly consists of hydrophobic amino acids with no charge and a PI of about 5.83 (Cover and Blaser, 1992).

Unusual stability of Hp in low pH environment and the significant induction of activity upon treatment with acidic pH, are two specific characteristics of this protein that matches the acidic environment of the stomach (Molinari *et al.,* 1998).

This toxin has a slow rate of internalization requiring 2 to 3 hours, which has not yet been reported for other toxins (Garner and Cover, 1996) and this slow internalization is a temperature related process (Garner and Cover, 1996).

VacA protein purified from Hp culture supernatant is actually inactive, and treatment with acidic pH (below 5) causes conformational changes in the molecule that is associated with induction of vacuolating activity (de Bernard *et al.,* 1995).

3 - VacA function

3-1 Binding and vacuolation

Cleavage of oligomers at low pH does not inactivate the toxin but changes the conformation and will result in the exposure of hydrophobic regions, interaction with hydrocarbonic chains of membrane phospholipids. Hydrophilic regions can form channels by which the active moiety of VacA enters the cytosol (Lupetti *et al.,* 1996). Therefore, acidic pH enables the toxin for internalization into the cytosol. It seems that amino acid sequence of p58 is responsible in binding of VacA to different cell lines (Garner and Cover, 1996) but this binding is not sufficient for successful internalization of VacA, and it is proposed that amino-terminal p37 is also required for internalization (Reyrat *et al.,* 1999). On the other hand, it has been demonstrated that p37 domain plays an important role in channel formation (Ye *et al.,* 1999). Toxin can bind to a cell surface glycoprotein (250 kDa) that is called P250 and is a receptor for Tyrosin Phosphatase (RPTPß) (de Bernard *et al.,* 1995; Yahiro *et al.,* 1999). Subsequently it enters the cytosol of gastric epithelial cells through endocytosis and will be located in membrane of acidic compartments such as late endosomes and results in channel formation in endosomal membrane (Tombola *et al.,* 1999b). These are anion-specific trans-membrane channels (Tombola *et al.,* 1999a; de Bernard *et al.,* 2000). This increases penetration of anions such as HCO^{3-} and Cl^{-} from cytosol to endosomal lumen and results in depolarization of cell membrane and higher activity of Vacuolar-ATPase (influx of H^{+} and Cl^{-}) and accumulation of weak bases such as ammonium ions in the lumen of endosomes. This penetration causes increase of osmotic pressure in these compartments yielding influx of water and thus swelling of these organelles will take place (Garner and Cover, 1996; Tombola *et al.,* 1999a, 1999b) and large vacuoles will be formed. These vacuoles have Rab7 and V-H-ATPase that are characteristics of late endosomal compartments (Molinari *et al.,* 1997,

reviewed by Papini *et al.,* 2001). Thus, VacA interferes with the controlling processes of late endosomal structures and their cycling to lysosomes or golgi apparatus (Telford *et al.,* 1994a).

3-2 Induction of apoptosis

Intracellular expression of *vacA*, induces apoptosis and has been shown that p37 is the active moiety in this process (Galmiche *et al.,* 2000). The two known biochemical events in apoptosis procedure are release of cytochrome c from mitochondria into the cytosol and activation of Caspase 3. Cellular death has most likely a critical role in the appearance of atrophic gastritis, a pre-neoplastic condition. Therefore, VacA may play a role in carcinogenesis of Hp (Shirin and Moss, 1998; Galmiche *et al.,* 2000).

3-3 Blocking antigen presentation

VacA interferes with B-cell antigen presentation via inhibition of maturation and processing of antigens (Satin *et al.,* 1997). Furthermore, it seems that VacA inhibits T cell proliferation, reducing immune responses in gastric mucosal level and permitting the colonization of pathogenic strains of Hp (Molinari *et al.,* 1997).

3-4 Cellular damage

Several lines of evidence suggest that VacA causes cellular damage in gastric mucosal layers; intragastric administration of purified toxin to mice results in gastric epithelial damages and mucosal erosions (Telford *et al.,* 1994b); experimental infection of mice with Tox^+ strains of Hp result in peptic ulcers and epithelial vacuolation whereas infection with Tox^- strains does not (Marchetti *et al.,* 1995); peptic ulcer is commonly observed in patients infected with cytotoxic strains of Hp (Atherton *et al.,* 1995).

It is noteworthy to mention that VacA will be extremely activated at pH 1.5-6 without being affected by this range of pH. At pH 2 this protein is resistant to pepsin digestion. Some of VacA molecules that are released from Hp and are activated in the gastric juice can pass through pylorus and cause vacuolation of duodenal epithelial cells before digestion with intestinal proteases that protect other parts of intestinal lumen. Such processes can cause duodenal ulcers without actual presence of Hp (de Bernard *et al.,* 1995).

3-5 Transepithelial electrical resistance reduction

When VacA is added to the apical layer of gastric epithelial cells, it decreases the Transepithelial Electrical Resistance (TER) which indicates the degree of sealing and through this state, permeability to small molecules and ions such as pyruvate , Ni^{2+} and Fe^{3+} that are necessary for the survival of Hp will be increased.

Thus, one of the important roles of VacA is increasing permeability of mucusal layers that is seemingly required for Hp nutrition and maintenance in the stomach (Papini *et al.,* 1998).

4- Concluding Remarks

Several studies have been performed investigating various molecular and cellular aspects of VacA. Due to the critical roles of Hp cytotoxin in the pathogenesis of this bacterium, it is recommended as a strong vaccine candidate against Hp infection with applications in therapeutic and preventive approaches. Native VacA does not seem like an appropriate immunogen due to its pathological effects on host cells. However, a genetically detoxified or recombinant form of this protein can be used for this purpose. According to the studies about VacA binding and toxic activity, different parts of this protein have been identified and attempts are being made to use recombinant partial sequences or truncated forms for applications in therapeutic approaches such as a cocktail of Hp antigens as a complex vaccine, or in diagnostic ELISA or western blotting kits. Previous studies have shown that intragastric administration of Hp antigens, and particularly its cytotoxin, urease and the recombinant form of VacA into mice, induces protective immunity against reinfection (Mohammadi *et al.,* 1996b; Ghiara *et al.,* 1997; Lanzavecchia and Bellon, 1998). On the other hand, the vast heterogeneity present in *vacA* gene and protein sequences from Hp strains from various geographical locations, re-emphasize the need for using local strains as the source for the afore-mentioned recombinant proteins to match with the local infecting strains in the two aspects of “detection” and “prevention”. In accordance, the authors have amplified the *vacA* gene (related to mature peptide~90 kDa) from a local (Iranian) Hp strain, which has subsequently been cloned and sequenced. Complete sequencing confirmed the extreme heterogeneity for the *vacA* gene

between Iranian and western strains (GenBank accession number=AY232454). This protein is presently being expressed and purified for future diagnostic and vaccine trials.

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