Helicobacter pylori: Focus on CagA and VacA major virulence factors

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Abstract

After colonizing the human gastric mucosa, *Helicobacter pylori* can remain within the host for years and even decades, and is associated with several, highly significant gastric pathologies. In Mexico, the seroprevalence at 1 year of age is 20% and the estimated increment in seropositivity per year is 5% for children aged 1-10 years. More than 80% of adults are infected by the time they are 18-20 years old. Bacterial virulence factors have been proposed for *H. pylori*, such as urease, flagella, heat-shock protein, lipopolysaccharide, adhesions, vacuolating cytotoxin, *cag* pathogenicity island and the cytotoxin-associated protein, the latter being the most studied mechanism to date. The English version of this paper is available at: http://www.insp.mx/salud/46/eng

Key words: *Helicobacter pylori*; virulence factors; pathogenesis

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Resumen

Después de colonizar la mucosa gástrica humana, *Helicobacter pylori* puede permanecer por años e incluso décadas en el humano, y se asocia a varias patologías gástricas. En México, la seroprevalencia estimada es de 20% en niños de un año de edad, con una tasa de incremento en seropositividad de 5% anual durante los primeros 10 años de vida hasta alcanzar 80% en adultos jóvenes entre los 18 y 20 años de edad. Los factores bacterianos de virulencia propuestos para *H. pylori* son ureasa, flagelos, proteínas de choque térmico, lipopolisacárido, adhesinas, citotoxina vacuolizante, isla de patogenicidad y la proteína asociada a la citoxina; este último factor es el más estudiado hasta la fecha. El texto completo en inglés de este artículo está disponible en: http://www.insp.mx/salud/46/eng

Palabras clave: *Helicobacter pylori*; factores de virulencia; patogénesis

Helicobacter pylori (*H. pylori*), formerly known as *Campylobacter pyloridis* and then as *Campylobacter pylori*, was isolated for the first time by Barry Marshall and Robin Warren in 1982. This led to a new era in clinical microbiology, when bacteria were found to be a cause of peptic ulcers. *H. pylori* is at present recognized as an important pathogen.[1] *H. pylori* causes persistent infection in a high percentage of the population; particularly in countries with a high proportion of lower socioeconomic groups.[2] Once acquired, *H. pylori* in-

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fection persists in the majority of individuals for years, decades and in some cases for life. Infection causes gastric inflammation of the mucosa (superficial chronic gastritis) and polymorphonuclear infiltration (active chronic gastritis). However, the effect of infection varies from individual to individual with the majority becoming asymptomatic carriers, while others develop peptic ulcers. In some cases, individuals can develop chronic atrophic gastritis, a risk factor for the development of adenocarcinoma and gastric lymphoma.[3] The clinical outcome of *H. pylori* infection is determined by a complex interaction of environmental influences, and by factors related to the microbial virulence and host. *H. pylori* bacteria that carry the cagA and vacA genes have enhanced pathogenicity, and the coexistence of cagA and vacA with other bacterial virulence factors further worsens clinical outcomes.

Microbiological characteristics of *H. pylori*

H. pylori is a curved spiraled or S-shaped gram-negative organism, approximately 0.3 to 1.0 µm wide, 1.5 to 5.0 µm long, and microaerophilic (O_2 5%, CO_2 10% and N_2 85%). Its optimal temperature for growth is between 35 °C and 37 °C,[4] although some strains are still able to grow at 42 °C. Growth can also take place in 0.5% glycerol and in 0.04% triphenyltetrazolium chloride, and it is known that high humidity can be favorable. Incubation usually lasts between 3 and 5 days, although incubation of up to 7 days has occasionally been observed. Biochemically, *H. pylori* is catalase and oxidase positive and contains the potent enzyme urease, used in identifying the presence of *H. pylori*. The colonial morphology of *H. pylori* is described as small, gray, and slightly hemolytic colonies.[5]

Epidemiology

Epidemiological studies have shown that infection with *H. pylori* is a worldwide phenomenon.[6] However, infection rates between developed and developing countries differ significantly. For example, in the United States the annual incidence of infection is between 0.5% and 1% in children under 10 years of age, with infection rates increasing to 50% in adults around the age of 60 years.[7] Interestingly, certain ethnic groups, such as Afro-Americans, Hispanics, and Native Americans, are infected at an early age. Intra-familiar transmission, which is associated with low socioeconomic status, deficient healthcare practices and overcrowding, is high in these groups.[7] In developing countries, it seems that most of the population (80%) becomes infected at an average age of 10 years, which is probably also related to the aforementioned factors of high intra-familiar transmission.[8]

In 1997, Torres *et al* performed a seroepidemiological study using a bank of representative sera from populations throughout Mexico (11 605 sera), which included sera from individuals between 1 and 90 years of age.[8] Their results showed that 20% of one-year-old children had antibodies against *H. pylori*, with an increased seropositivity of up to 50% in children who were 10 years of age.[8]

Currently, there is no information that indicates the existence of a non-human reservoir, which suggests that infection is specific to humans. However, there are studies concerning the experimental infection of primates with *H. pylori*[9] that involve the development of gastritis and clinical ulceration very similar to those seen in humans. The specific transmission mechanisms of *H. pylori* remain unknown, but it is generally considered that infection is spread from individual to individual through an oral-oral and oral-fecal pathway. These methods of transmission are considered important in the wide distribution of *H. pylori*[6] infection although other methods are suspected.[10-12]

The understanding of *H. pylori* microbial ecology and vehicles of transmission remains unresolved.[13] Nevertheless, some epidemiological data suggests water as a possible vector. A few studies around the world have been carried out in aquatic environments focusing on a possible waterborne pathway for *H. pylori* infection. A correlation between water sources and *H. pylori* prevalence was described in Peru, highlighting municipal water sources an as important vector for *H. pylori* in addition to socioeconomic factors.[12,14,15] In Colombia, in addition to water itself, raw vegetables, especially lettuce, that are grown in fields irrigated with wastewater, are considered a potential source for infection.[16]

A population in Chile showed evidence of potential *H. pylori* infection from vegetables, consumed raw.[17] In a study in Sweden, the bacterium was detected in well water, municipal water, and treated wastewater. The presence of the bacteria in Swedish waters was remarkably high and not expected since the treatment plants were modern with effective treatment.[18] In the US, although *H. pylori* was found in surface water and shallow groundwater, no correlation was found between *H. pylori* and traditional indicator organisms.[13] In Mexico City, the *H. pylori* 16S rRNA gene was detected in five water systems.[11] In three of the water systems, microbiological samples yielded *Aeromonas hydrophila, Aeromonas caviae, Aeromonas veronii* and *Vibrio fluvialis*, and showed residual chlorine less than the inactivation dose, suggesting that conditions were

suitable for the potential survival of *H. pylori*, and other enteric pathogens in these environments.[12] A representative study carried out in the Mexico City Metropolitan Area showed the presence of *H. pylori* with CagA.[19] The viability of *H. pylori* in both water and vegetables needs to be confirmed, as does a relationship between human and environmental strains.

H. pylori is the etiologic agent in 70% - 80% of active chronic gastritis cases, the most common form of chronic gastric inflammation.[20] The disease affects the antrum and fundus, of the stomach, with histology frequently showing infiltration of lymphocytes, plasma cells and some eosinophils. It appears that the inflammatory process is more significant in the antrum than in other areas of the body.[21] *H. pylori* colonizes the gastric mucosa and can remain there for years or even decades with minimal symptoms in most cases. However, some cases have been reported describing significant morphological changes in the gastric mucosa, ranging from mild inflammation to ulceration. Others progress from chronic gastritis to cancer, through chronic atrophic gastritis, intestinal metaplasia, dysplasia and carcinoma.[22]

The high prevalence of infection *H. pylori* may be the cause of around 40% of all gastric cancer cases worldwide (47% in developing countries).[23] In some countries such as Mexico, there are zones of substantial risk. For example, in the highlands of Chiapas, there is a high incidence of *H. pylori*-associated gastric cancer among native Mexicans.[24] Based on seroepidemiological case-control studies, *H. pylori* was classified as a class I carcinogen by the International Agency for Research on Cancer in 1994. A prospective long-term study of 1 526 cases of distal gastric cancer and 280 controls revealed that, compared with *H. pylori*-negative individuals, those infected with *H. pylori* are 2.9-34.5 times more likely to develop distal gastric adenocarcinoma.[25] This depends on the grade of mucosal atrophy or the presence of intestinal metaplasia.[25] While this study shows the important role of *H. pylori* in the pathogenesis of gastric disease, it is still unclear which factors determine the subset of infected individuals developing life-threatening disease compared with those remaining as *H. pylori* carriers.

Virulence factors

Urease is an essential virulence factor, since it allows *H. pylori* to survive in the highly acidic and hostile environment of the lumen of the stomach before it reaches the mucus layer. Urease is a protein that is expressed in great quantities by *H. pylori*, representing 6% of its dry weight.[26] It is also the catalyst for urea hydrolysis, which in turn produces ammonium and carbon dioxide as by-products. The enzyme is made up of six subunits of UreA and six of UreB, organized in a double ring of 13 nm in diameter and a molecular size of 550 kDa.[27] The biosynthesis of urease is controlled by a group of seven contiguous genes (*ure*ABIEFGH), including the genes that code for urease (ureAB), and five accessory genes that are responsible for the reception and insertion of nickel ions (Ni2+) on the active site of the apoenzyme.[28]

Negative urease mutant strains, built by inserting resistant antibiotic cassettes in the ureA, ureB and ureG genes,[29] lost urease activity. However, alterations in the rate of growth were not studied, demonstrating that urease activity is not necessary for the viability of the microorganism in vitro. However, Tsuda *et al*.[30] demonstrated that a negative urease strain, created via the ureB gene, lacked the ability to colonize the nude mouse stomach. Also, the co-inoculation of a negative urease strain (*ure*G-) with a positive urease strain showed colonization by the positive urease strain in the stomach of gnotobiotic piglets,[31] suggesting that neutralization of the microenvironment is not the only function of urease.

Urease can contribute to cell toxicity by producing ammonium as a by-product, which characterizes urease as a chemotactic factor, activating polymorphonuclear leukocytes and monocytes for cytokine release. This process produces a localized inflammatory response leading to damage of the gastric epithelial tissue.[21,32] In the presence of ammonium chloride, vacuolating cytotoxin is highly active. The ammonium-induced cytotoxin generates more vacuoles compared with the toxin alone. This suggests that a substantial cooperation between urease and vacuolating cytotoxin causes cellular damage.[33]

Motility is also an essential factor in *H. pylori* colonization, based on the fact that aflagellate non-motile variants of *H. pylori* are able to infect gnotobiotic piglets.[34] *H. pylori* usually possesses two to six polar, sheathed flagella that are continuous with the outer membrane, with a similar composition in lipopolysaccharides, phospholipids and proteins.[35] However, the function of the flagellar sheath remains uncertain. Flagella are composed of two different flagellin subunits, encoded by the *flaA* and *flaB* genes.[36] Both flagellin subunits share a 50% identity in terms of amino acids and probably evolved from a common ancestor. The induced mutation of *flaA* and *flaB* genes showed that both genes are essential for motility[36] and for the colonization of gnotobiotic piglets.[37]

Binding of *H. pylori* to gastric epithelial cells is an important virulence factor for the colonization of the

human stomach. The microorganism adheres to the epithelial cells by means of adhesins and receptions.[38] Adhesins are bacterial proteins, glycoconjugates, or bacterial lipids involved in the initial stages of colonization by mediating an interaction between the bacterium and the receptors on the surface of epithelial cells, such as lipids, proteins, glycolipids or glycoproteins. Adherence of bacteria to host cell receptors triggers cellular changes that include signal transduction cascades, leading to infiltration of inflammatory cells (neutrophils and monocytes) and possibly to the persistence of the microorganism.[38] Once *H. pylori* reaches the epithelial layer, it adheres to the cells using BabA, SabA, AlpA, AlpB, HopZ, HpA, and other adhesins. The *H. pylori* BabA adhesin facilitates the binding of *H. pylori* to the fucosylated Lewis b histo-blood group antigen which is present on the surface of gastric epithelial cells. In animal models, Lewis b-dependent attachment of *H. pylori* to gastric epithelial cells is accompanied by increased severity of inflammation, development of parietal cell auto-antibodies, and parietal cell loss.[39] BabA has been investigated in the most detail. *H. pylori* strain CCUG 17 875 was used for the initial characterization of BabA and contains two copies of the *babA* gene (designated babA1 and babA2). In this strain, the *babA2* gene encodes a functional BabA outer membrane protein that binds to Lewis b; a 10-nucleotide segment containing the expected codon that initiates translation is deleted from the babA1 gene, and therefore this gene is not predicted to be translated. In contrast to *H. pylori* CCUG 17 875, each of two *H. pylori* strains for which entire genome sequences are available (strains 26 695 and J99) contain only one copy of *babA* (designated HP1 243 and jhp0833, respectively). Studies have been reported that detect a *babA2* genotype in 82 (71.9%) of 114 *H. pylori* isolates using PCR designed to amplify *babA2* but not *babA1* genes. Reverse transcription-PCR analyses have indicated that the *babA2* gene has been transcribed in each of the strains that contained a *babA2* gene. Moreover, 28 of 31 *babA2*-positive *H. pylori* strains were reported to bind to Lewis b compared to none of the 23 *babA2*-negative strains included in the study[40] Heterogeneity among *H. pylori* strains in expressing the BabA protein may be a factor in the variation of clinical outcomes among *H. pylori*-infected humans.[40]

Recently, *H. pylori* has been shown to express a second adhesin, SabA, that binds to sialylated and fucosylated glycoconjugates such as sialyl-dimeric-Lewis x. Such sialylated derivatives increase in the gastric epithelium as a consequence of inflammation, indicating that *H. pylori*-induced inflammation promotes colonization through the higher regulation of appropriate adhesion targets in host tissue. The *sabA* gene is subject to an on-off switch; hence, the gastric population of *H. pylori* is likely to contain bacteria that are either capable or uncapable of binding to inflamed tissue, a property that could contribute to bacterial persistence and shedding.[41]

Receptins[42] are microbial proteins with binding properties for mammalian proteins. However, this excludes the first step of adherence to the mammalian cell surface which is required for a microorganism to establish itself within a host. Instead, receptins relate to the later stages of adherence that allow the persistence of a long-lasting infectious process through the binding to a soluble serum protein (immunoglobulins and albumin) or via proteins of the extracellular matrix (heparin, heparin sulfate, fibronectin, fibrinogen, collagen, laminin, plasminogen, plasmin and vitronectin), of which many are also present in host serum. In a normal, healthy human, the soluble serum protein and extra cellular matrix proteins are not exposed and thus, are not accessible for interaction with bacteria; however, these molecules could become exposed after tissue trauma following a mechanical or chemical injury, or after an infection.[38] These events allow *H. pylori* to evade the host's immune defenses and to initiate the establishment of a persistent infection.[38]

Another virulence factor is the lipopolysaccharide (LPS) of *H. pylori* that expresses the Lewis "x" (Lex) and/or Lewis "y" (Ley) carbohydrates in its "O" antigen.[43] A recent study demonstrated that the LPS structure of the NCTC 11 637 strain of *H. pylori* is similar to the Lewis "x" antigen found in blood for type O and on gastric epithelial cells.[44] The MO19 strain of *H. pylori* has a Lewis "y" type LPS, whereas the P466 strain expresses both LeX and LeY.[45] The expression level of these Le antigens varies among strains, showing phase variation stimulated by the Lewis antigen expressed on the surface of epithelial cells.[46] Occasionally, *H. pylori* expresses other Lewis antigens[47] with a different number of fucose residues, Lea, Leb. The Lewis antigens of *H. pylori* show a dual participation in pathogenesis: (i) the induction of a molecular mimicry, possibly helping the microorganism to avoid the initiated immune response following stomach colonization and thus, favoring permanence of *H. pylori* for a long period within the gastric niche.[48] However, Takata *et al*[49] showed that LeX and LeY expression is not necessary for mouse gastric colonization or for *H. pylori* adherence to epithelial cells; (ii) the induction of an auto-immune response against Lewis antigens expressed by *H. pylori*, shared by the eukaryote and thus leading to direct or indirect damage.[50]

Another important virulence factor in *H. pylori* is the cytotoxin-associated protein (CagA), which was

identified as an immune dominant antigen, located on the surface of the bacterium. It varies in size from 120 to 130 kDa, and is present in approximately 60% of *H. pylori* strains.[51] Notably, the CagA protein is frequently co-expressed with vacuolating cytotoxin (VacA), which is the reason for designating it as a cytotoxin associated protein. However, these genes are separated by 300 kb and the expression of VacA is independent of the presence of the cagA gene; since null mutants in cagA, do not affect the vacuolating cytotoxin activity of *H. pylori*.[52,53] Also, the expression of the CagA protein for *H. pylori* strains is highly associated with peptic ulceration.[51]

The cagA gene encodes for a protein containing 1 147 amino acids with a theoretical mass of 128 012 Da. This protein is highly hydrophilic, shows an EFKNGKNDFSK and EPIYA sequence repetition, and an extension of six contiguous asparagines. In the 3 406 bp position of the gene sequence, a fragment of 102 bp is present, that is made up of three segments, which are present once in the 84-183 (ATCC 53 726) strain and three times in the G39 strain. The repetition of the 102 bp segment is what confers size variability on the CagA protein, without producing antigenic diversity. However, these repetitions are rich in proline and very hydrophilic; they also have a high probability of surface exposure and are highly immunogenic.[54]

In 1998, Evans *et al*[55] analyzed the variable region of the cagA gene, and found that there are two adjacent and variable regions, denoted as proximal and distal. The proximal-variable region of CagA extends from a motif of 4 to 6 asparagine residues to a 7 amino acid repeat sequence (KIDQLNQ); the distal-variable region is confined between KIDQLNQ and a well-conserved duplicate, KIDQLNQ. The variation in the size of CagA from one strain to another is a property of the distal variable region; however, it appears that the greatest diversity, in terms of nucleotide and amino acid sequence variations, occurs within the proximal variable region. This means that from 1 to 7 consensus amino acid residues may be present or absent immediately following the asparagine motif, because there are two different versions of the repeat sequence (EPIYA or PEEPIYA).

Additionally, in 1998, Yamaoka *et al* analyzed the number of repeat sequences in the variable region at end 3′ of the cagA gene, describing four types of primary structure, where the type C primary structure was associated with higher levels of anti-CagA antibody and more severe degrees of gastric atrophy.[56] In a later study, they found that *H. pylori* strains with more than three repeat regions at the 3′ region of the cagA gene were associated with enhanced histological injury and with reduced survival in acidic conditions.[57] Recently, Azuma *et al*.[58] analyzed the frequencies of the genotypes that contained more than four repetitions of the 5-amino acid sequence EPIYA at the 3′ region of the cagA gene and found that *H. pylori* infection with the cagA genotype with more than four EPIYA sequences may correlate with the pathogenesis of atrophic gastritis and gastric cancer.

Censini *et al*[59] analyzed the DNA regions flanking the cagA gene, which led to the discovery of a region of 40 kb, to which they designated the Cag Pathogenicity Island (cag-PAI). The Cag-PAI is a 40-kb locus that is inserted into the chromosomal glutamate racemase gene (glr). It contains 31 genes and flanks direct repetitions involved in the integration of the 40-kb locus in the genome. The presence of the 605 insertion sequence (IS605) and the low G + C content (35%), which is smaller than the rest of the *H. pylori* genome (39%), suggests that the cag-PAI was acquired from another microorganism by horizontal transfer. Additionally, the order of the genes in the cag-PAI is essentially conserved among the *H. pylori* strains.[59, 60]

Different studies revealed that the Cag-PAI can be present as a simple continuous unit or divided into two regions (cagI and cagII) for an IS605 element or for a segment of chromosomal DNA, and can also show a partial loss of the Pathogenicity Island.[59] This structural variation of the cag-PAI explains the existence of cag-PAI negative and cag-PAI positive strains. On the other hand, six of the genes in the cag-PAI are homologous to well-known genes present in a collinear arrangement in operons of *Bordetella pertussis*, *Agrobacter tumefaciens*, *Escherichia coli*, *Legionella pneumophilla*, *Rickettsia prowazekii* and *Brucella suis*. These operons encode for type IV secretion systems, specialized in the transfer of a variety of multimolecular complexes across the bacterial membrane to the extra cellular space or into other cells.[61]

A number of events occur in epithelial cells after contact with a bacterium that possesses the cag-PAI. Attachment of cag-positive *H. pylori* to human gastric cells results in the secretion of interleukin-8, an inflammatory mediator, by activating nuclear factor kappa beta complexes (NF-kB). It also induces the effacement of microvilli at the site of attachment and cytoskeleton rearrangement followed by the formation of a cup/pedestal directly beneath the bacterium,[62] due to the tyrosine phosphorylation of the CagA protein.[63] In addition, it activates transcription factor AP-1 and the ERK/MAP kinase cascade leading to expression of the proto-oncogenes *c-fos* and *c-Jun*. These result in ELK-1 phosphor-

ylation and increased c-fos transcription.[64] *H. pylori* mutated in the cag gene does not induce any of the above activities.

Two possible signaling mechanisms may occur in the epithelial cells after contact with *H. pylori*. The first involves an unknown factor, which is translocated into the cell by the type IV secretion system of cag-PAI that activates the transcription factor NF-kB to induce interleukin-8 expression. It is likely that this mechanism is involved in the activation of mitogen-activated protein (MAP) kinases and the activator protein (AP)-1 transcription factor. It is not yet certain whether this pathway needs a yet unknown effect, or whether it is simply activated by the type IV secretion system itself, which perturbs the cell membrane.[65] The second mechanism involves the CagA protein; this protein is translocated into the eukaryotic cells for the IV secretion system. Once inside the cells, the protein triggers signal transduction and acts as a growth factor, altering the integrity of the epithelium by interacting directly with host ZO-1 protein.[66] This is part of the apical junction complex, and the interaction disrupts the epithelium integrity. Inside the host cell, CagA may be phosphorylated in its tyrosine residues on the sequence EPIYA, located in the C-terminal region of the CagA protein[67] by the host-cell kinase c-Src.[68] After this process, the phosphorylated-CagA (CagA-P) is likely to bind to SHP-2 phosphatase and stimulate the phosphatase activity of SHP-2. This complex can activate multiple pathways: a) it may bind directly to the Neural Wiskott-Aldrich syndrome protein (N-WASP), inducing it to bind the Arp2/3 actin nucleator, thus stimulating actin polymerization with the consequent pedestal formation; b) the CagA-P-SHP-2 protein complex may activate the Rho family of the small, GTP-binding protein (Cdc42, Rac or Chp), which controls the organization of the actin cytoskeleton. This pathway could also cause actin polymerization and pedestal formation by activating N-WASP; or c) the CagA-P-SHP-2 protein complex may trigger a signaling cascade, possibly via the MAP pathway, which may induce transcription of nuclear genes. However, there is not enough evidence yet to support involvement of this pathway.[65]

Although the cagA gene is considered as a virulence marker, previous reports suggest the existence of allelic variations in *H. pylori* strains isolated from different ethnic populations.[69] These data have been confirmed by Arie Van der Ende *et al*[70] by comparing a 243 bp fragment of the cagA gene, isolated from Dutch and Chinese patients. However, the variable region at the 3′ end of the cagA gene is the region where there is more heterogeneity due to the number of 102 bp fragment repetitions, and where substantial nucleotide differences have been observed among the isolated occidental and East Asian strains.[57,58,71] The occidental strains present a specific sequence denoted as the "Western cagA-Specific Sequence" (FPLKRHDKVDDLSKVGRSVSPEPIYATIDDLGG P) whilst the specific sequence present in Asian strains has been termed the "East Asia cagA-Specific Sequence" (ESSAINRKIDRINKIASA GKGVGGFSGAGRSASPEPIYATIDFDEANQAG). Backert *et al*[67] demonstrated that the tyrosine phosphorylation sites in the EPIYA-containing repeat sequence of CagA from WSS and ESS sequences are essential for pedestal formation. Recently, Higashi *et al*[71] showed that the repeat sequence contains a tyrosine phosphorylation site, and that CagA proteins having more than one repeat sequence underwent greater tyrosine phosphorylation, exhibiting increased SHP-2 binding and inducing greater morphological changes. They also observed that the East Asia CagA-specific sequence conferred stronger SHP-2 binding and morphologically transforming activities than those related to the Western CagA-specific sequence. Such structural differences in the CagA protein could be implicated in the increased risk for developing gastric cancer in people infected with *H. pylori* strains that express the CagA protein with a high binding affinity to SHP-2.

Clearly, the ability to produce a vacuolating cytotoxin (VacA) is an important virulence factor of *H. pylori*. VacA is responsible for the in vivo formation of vacuoles in gastric epithelial cells,[72] as well as different in vitro cell lines.[73] It is possible that oral administration of cytotoxin to suckling mice produces a deterioration of the gastric mucosa.[74]

Vacuolating cytotoxin is coded for by the vacA gene located in a single copy within the *H. pylori* chromosome.[32,75] The vacA gene has approximately 3 864 bp and presents 5 open reading frames, only one of which is suitable for coding the vacuolating cytotoxin. Since 1994, the first complete sequence studies of the vacA gene[75,76] have been performed in *H. pylori* 60 190 tox+ and 87-203 tox- strains. Analysis of a fragment of the 1 541 bp of the vacA gene showed a 64.8% homology between the nucleotide sequence of the tox+ strain and tox- strain.[77] In addition, a region of approximately 567 bp was located upstream from the vacA gene, which corresponds to the tRNA gene of cystein-synthetase,[75] and is homologous to that found in *E. coli*. In 1995, the 0.73 kb region was analyzed in 10 strains of *H. pylori*, the position corresponding to the mid region of the vacA gene.[76] This mid region showed a nucleotide identity of 70.4% between tox+ and tox- strains, and an amino acid identity of 58.7%. Based on their sequence, strains were classified into two allele families: m1-type allele (for *H.*

pylori strains with cytotoxic activity) and m2-type allele (for *H. pylori* strains with no cytotoxic activity). In addition, the sequence of a 0.5-kb fragment was analyzed, which corresponded to a sequence-signal region of the vacA gene with important differences between tox+ and tox- strains. Based on these results, strains were classified into three further allele families: s1a-type, s1b-type, and s2-type alleles.

Atherton *et al*[76] performed the characterization of the vacA gene from[59] *H. pylori* strains isolated from patients with different pathologies (peptic ulcer, gastritis and asymptomatic patients). By using specific initiators and PCR, they amplified the signal sequence region (s1a, s1b, s2), and the mid region (m1 and m2) of each strain. They reported that there were patients who presented strains with s1a/m1, s1b/m2, s1b/m1, s1b/m2, and s2/m2 genotypes, but there were no patients who presented the s2/m1 genotype. Upon studying the cytotoxic activity in single layers of HeLa cells from supernatants of each strain isolated from their study population, they observed that the strains presented different cytotoxic activity, classifying them as: high, mid and null vacuolating activity.[73,76] In this study, a meaningful association between the vacA genotype and vacuolating activity was also observed, showing that strains with the s1a/m1 genotype presented the greatest cytotoxic activity. They also reported that strains with s1b/m1, s1a/m2, and s1b/m2 genotypes presented an activity from mid to low, whilst strains with the s2/m2 genotype presented no cytotoxic activity. In addition, they reported that s1a/m1 strains were isolated with greater frequency from patients with peptic ulcers, s1b/m1 strains were mainly found in samples from patients with gastritis, and s2/m2 strains were isolated in greater numbers from asymptomatic patients.[76]

Although this vacA genotyping system has been successfully used to study bacteria isolated from patients in the United States and some European countries, the m1/m2 dichotomy does not seem to be sufficient for classifying strains from Asian countries, and probably, from other regions of the world.[78] A study in Germany of 30 *H. pylori* strains isolated from patients with active chronic gastritis, peptic ulcers and gastric cancer, reported that only one strain could not be characterized by specific initiators in the mid region of the vacA gene.[76] Upon analyzing the mid region nucleotides of these strains, the study reported a nucleotide difference of 9.3% when compared with the mid region sequence of an m2-type strain (Tx30a). This new mid region was labeled as m3-type.[79] As in Germany, studies in Belgium[78] showed a new mid region for the vacA gene, designated as m1a, and another m1-m2 type mid region in strains isolated from Chinese patients. In addition, there are reports from China, Hong Kong, Japan, Thailand, France, Germany, Hungary, Italy, the Netherlands, Poland, Portugal, Rumania, Spain, Switzerland, United Kingdom, Brazil, Canada, Colombia, Costa Rica, Peru, United States, Australia and Egypt[78,79] where the characterization of the signal sequence region and/or vacA gene mid region could not be achieved using the proposed scheme. DNA sequencing and new specific primer designing was necessary for the characterization of "untypeable" strains. Nowadays, the vacA s1 region has been subtyped into s1a, s1b, and s1c; the m1 region was subtyped into m1a, m1b, and m1c; and the m2 region has been subtyped into m2a and m2b. Previous studies showed that the VacA s1a or s1b genotypes were predominant in strains from Western countries, whereas s1c is highly prevalent in strains from East Asia. The vacA m1a and m2a genotypes were predominant in strains from Western countries, the m1c genotype was predominant in strains from South Asia, and the m1b and m2b genotypes were predominant in strains from East Asia.[80] In Mexico, Morales-Espinosa *et al* showed vacA diversity was higher than that described in other populations, and that co-infection with multiple *H. pylori* strains with different VacA genotypes is common. In addition, this study found a previously undescribed vacA s2/m1 genotype.[81] The variation in the global distribution of the VacA genotypes may show a degree of congruence with reports linking vacA genotypes with certain clinical outcomes in different geographic regions.

The VacA cytotoxin is synthesized as a 140 kDa protoxin, which contains three functional domains: a) an N-terminal signal sequence of 33 amino acids (the leading peptide being 3 kDa), b) a mature 87 kDa cytotoxin, and c) a C-terminal domain of 50 kDa associated with an external membrane.[82] In order to be excreted, the protoxin breaks both its amino part end (signal peptide) and its carboxyl region end, remaining as a monomer of approximately 95 kDa to form a mature toxin composed of 6 or 7 monomers. Upon binding, a structural rearrangement takes place, the result resembling a 6- or 7-petal flower with a center (ring) of 30nm in diameter.[83] Each monomer presents a flexible exposed handle, which undergoes a proteolysis rupture dividing it into two sub-units: one of 37 kDa (P37) and the other of 58 kDa (P58).[82] However, it is unknown if the same toxin causes such a break or, if there are external proteases responsible for hydrolysis, which act on the bacterial surface. It is also unknown if the processing of two such sub-units is essential for toxic activity. It has been observed that

when the VacA is exposed to an acid pH or to weak bases such as ammonium chloride, there is separation of monomers, together with an increase in the vacuolating activity of single layers of HeLa cells.[72,82] Recent studies have shown that the sub-unit P58 carboxy-terminal domain is responsible for acknowledging receptors on the surface of epithelial cells, although interaction between both sub-units (P37 and P58) is necessary for the toxin to be internalized and actuated. In vitro studies[82] have proved that VacA is bound to a cell by interacting with specific receptors. This binding depends on both the cytotoxin concentration and the saturation of receptors on the cellular surface. It appears that cytotoxins enter the cytosol kinetically in a similar way that endocytosis is mediated by a receptor. It has been proposed that the receptor for the epidermal development factor (EGF) and a 140-kDa protein act as the receptors for VacA.[84,85]

Three mechanisms of action for vacuolating cytotoxin have been reported: (1) large vacuoles form in the cytoplasm of epithelial cells[72,73] originating at the perinuclear level, until they totally fill the cell cytosol, causing massive cellular vacuolation. Membrane vacuoles, induced by cytotoxin, contain a high level of the small binding GTP protein, Rab 7. This causes membrane vacuoles to be formed by a hybrid of late endosomes and membrane lysosomes.[86] The lumen of large vacuoles, induced by VacA in both the cytoplasm of culture cells and in gastric epithelial cells, is acidified by the activity of a proton pump via a vacuolar-type ATP-ase bound to the membrane of these vesicles.[87] ATP-ase V is present in various intra-cellular compartments of eukaryotic cells and participates in several processes in endocytic and exocytic pathways. This complex is bound to the membrane and hydrolyzes ATP for the proton pump inside the lumen of membrane limiting compartments. The VacA cytotoxin can change membrane traffic at the endosoma-prelysosome level. These changes affect the protein traffic, the bound traffic and the processing of antigens depending on bound changes, and produce deterioration in the proteolytic degradation within lysosomes, thereby bringing about lethal dysfunction of the cell. (2) A separate mechanism of action involved in VacA vacuole formation is the increase in permeability of in vitro polarized cells. This leads to an increase in the passage of low molecular weight molecules,[88] which seems to increase nutrient flow from inside the cell to the sub-mucosa, thereby favoring *H. pylori* survival. The adherence of VacA to polarized single layer cells causes a decrease in trans-epithelial resistance through the single layer. Evidence shows that the effect of VacA in decreasing trans-epithelial resistance is an increase in intra-cell permeability, since the active cytotoxin continues to be associated with the bacteria.[88] (3) The third mechanism involves the formation of channels through the lipidic layer of the cell membrane. Tombola *et al*[88] showed that the cytotoxin is activated by an acid pH, forming anion channels of selective low-conductivity, which are voltage-dependent. This activity increases permeability, which favors anions over cations, facilitating CI- and HCO_3- access and, although less efficiently, the entry of carboxylic acids, pyruvate and gluconate. The cytotoxin can interact with the lipidic double-layer, forming structures compatible with hexameral pores. It appears that the presence of both sub-units (P37 and P58) is essential to form the channels, with these two sub-units undergoing necessary changes in configuration to allow insertion of cytotoxin into the lipidic double-layer. The increase in the conductivity of ions in endosomal membranes activates the electrogenic V ATPase,[88] favoring the flow of H^+ and Cl^- from the cytosol to the endosome lumen. This causes osmotic changes leading to an increase in water access and therefore, a swelling of the endosomal compartment.

VacA has several specific effects that may contribute to *H. pylori* persistence in the gastric mucosa. It forms pores in epithelial cell membranes, allowing the egress of anions and urea. This is important since urea hydrolysis, catalyzed by *H. pylori* urease, protects against gastric acidity. VacA also induces the loosening of epithelial tight junctions, potentially allowing nutrients to cross the mucosal barrier to *H. pylori*'s gastric luminal niche. Recent in vitro work suggests that VacA may help *H. pylori* persistence by specific immune suppression, blocking phagosome maturation in macrophages, selectively inhibiting antigen presentation in T cells, blocking T cell proliferation, and downregulating Th1 effects by interacting with calcineurin to block signaling. In addition. VacA has direct cell-damaging effects in vitro, inducing cytoskeletal changes, apoptosis (in part by forming pores in mitochondrial membranes, allowing cytochrome C egress), suppression of epithelial cell proliferation and migration, as well as cell vacuolation.[89] Whether these effects are important in vivo is unknown, but cell damage could aid nutrient delivery from the gastric mucosa.

All of these findings indicate that the vacuolating cytotoxin is an important virulence factor in the pathogenesis of gastric disease in humans. *H. pylori* strains expressing the protein associated with the cytotoxin, and those containing producers of vacuolating cytotoxin with high biological activity, have been isolated more frequently from patients with peptic ulcers than from patients with chronic gastritis. This has been shown in various studies where patients with high

antibody levels against VacA and CagA proteins showed severe gastric pathologies,[7,20,76] and have a greater risk for developing adenocarcinoma and gastric lymphoma.[90]

Future considerations

Since *H. pylori* was first isolated and its role in peptic ulcer disease was demonstrated about 20 years ago, knowledge about the bacterium has increased enormously. However, many questions remain unanswered and ongoing studies using biochemistry, and molecular and cell biology will certainly offer additional information that will help in understanding the pathogenesis of *H. pylori* infection. However, it is likely that only studies using human and appropriate animal models will be able to provide definitive answers to the following questions: Why does the underlying inflammatory response not lead to clearance of *H. pylori* infection?; Is it possible that *H. pylori* has adapted itself to colonize inflamed mucosal surfaces?; In that case, would inflammation be a prerequisite for initial or prolonged colonization, or both?; Why do some subjects develop gastric ulcers, duodenal ulcers or gastric cancer, while the majority of colonized subjects remain free of clinical disease?; What is the definitive transmission route for *H. pylori*?

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