

# *Helicobacter pylori*: Determinant and markers of virulence

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**Abstract.** In this review, we examine the main virulence determinants in *Helicobacter pylori* (*Hp*) strains and the correlation between these and the different diseases following *Hp* infections. We also discuss the host response to *Hp* and the implications and advantages of the development of non-invasive pre-endoscopy screening of molecular and serological markers associated with *Hp* virulence and disease progression putting an emphasis on new screening techniques such as the Luminex – X multi analytes profiling technology and the multiplex PCR.

**Keywords:** Virulence determinants, *Helicobacter pylori*-associated infections, immune response and immunopathology, serological markers, multiplex PCR

## 1. Introduction

*Helicobacter pylori* (*Hp*) was first isolated in culture media by Warren and Marshall in 1983. It is a Gram-negative, spiral-shaped bacterium, with positive findings for urease, oxidase and catalase. The bacterium colonizes the human gastric epithelium. *Hp* is the main cause of chronic active gastritis, peptic ulcer disease, gastric carcinoma and mucosa-associated lymphoid tissue (MALT) lymphoma. Further, epidemiological and eradication studies have demonstrated a casual relationship between *Hp* infections and endothelial dysfunction, leading to vascular diseases [6,31,63]. Generally, the colonization occurs primarily during childhood especially in the developing areas, usually in the same family for a cohort effect [51]. This colonization is widely asymptomatic even if a long-lasting infection can be established in some subjects. Infection is virtually lifelong in the absence of treatment, implying that evasion of the host response is efficient. The infection outcome mainly depends on three factors: strain virulence, host response and environmental factors. Environmental factor, such as a cigarette smoking, is a major

risk factor for duodenal ulceration among *Hp*-infected persons [10]. Other important factors include stress, childhood living conditions, diet, alcohol and NSAIDs (non-steroidal anti-inflammatory drugs) use [99,114]. Here we discuss determinants of virulence affecting the *Hp* related diseases, the immune response deriving from the infection with different *Hp* strains and the implications and advantages of non-invasive pre-endoscopy screening of body fluids through multiplex PCR and Luminex – X -(map) multi analytes profiling technology.

## 2. Determinant of virulence

Gastric colonization is a prerequisite for *Hp*-associated disease and this is mediated by both flagella and urease: mutant strains lacking these features cannot establish infection. Adhesion by adhesins to epithelial gastric cells is important for the beginning of infection and for the enhanced inflammatory response. Following colonization, *Hp* must acquire nutrients from the gastric mucosa of which the acquisition of iron from the host is particularly important [10]. *Hp* has been reported to be genetically extremely variable (Table 1) and this heterogeneity is proposed to be involved in the ability of *Hp* to cause different diseases [9,31], detrimental and non-detrimental chronic infections [81].

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Table 1  
Genetic heterogeneity in *H. pylori*

Type of diversity	Evidence
Macrodiversity	Gene map differences identified by pulse-field gel electrophoresis
Microdiversity	Individual nucleotide changes observed from various strains
Allelic variation	Microdiversity measured using DNA-based techniques and by multilocus enzyme electrophoresis
Strain-specific gene	Some strains lack the <i>cag</i> pathogenicity island demonstrated by subtractive hybridization
Mosaicism	Shown with the vacuolating cytotoxin ( <i>vacA</i> ) gene

Modified from R.A. Alm et al. (1999) [1].

*Hp* is well adapted to the human host as evidenced by its chronic persistence in the gastric niche and by the finding that the bacterial surface carries structures (antigens) which are identical to those found on human cells [7,92].

The low incidence of severe disease associated with infection has suggested that there may be “beneficial” *Hp* organisms in addition to those that cause disease. In fact, since humans have co-evolved with *Hp*, they might derive some benefits from them [10]. Certainly man adapts physiologically to infection during his lifetime and treatment can lead to problems such as reflux oesophagitis. In view of these real problems, it would seem preferable to screen for and treat only strains that are known to cause disease. In *Hp* a lot of virulence determinants have been detected affecting the infection course.

### 2.1. Vacuolating cytotoxin (*VacA*) and *vacA* gene (vacuolating cytotoxin gene A)

In people with ulcer, a protein in culture supernatant that induces vacuolation in a variety of cultured epithelial cell lines, was isolated [9,62,112,116]. The expression of *Hp vacA* gene leads to the production of a vacuolating cytotoxic protein VacA, (present only in about 40% of isolates), which is responsible for inducing the formation of acidic vacuoles [10,28]. The toxin causes epithelial damage but appears to have little effect on inflammatory cell infiltration [10]. This secreted protein toxin is responsible for the gastric epithelial erosion observed in infected hosts [120]. Vacuolating cytotoxin VacA induces cytochrome C release from mitochondria leading to apoptosis more than cytoplasmic vacuolation in gastric epithelial cells [46]. Moreover the protein was shown to cause gastroduodenal damage in a mouse model [84] and to increase the risk for gastric ulcer in *Hp*-infected Mongolian gerbils [97].

The *vacA* gene contains two variable regions: the s- (signal) region encoding part of the signal peptide with the N-terminus of the mature protein (hydrophilic part) and the m- (middle) region encoding C-terminal portion of the final processed polypeptide (hydrophobic part within the p58 domain). These regions are both cleaved upon secretion to yield a mature toxin monomer of 87–95 kilodaltons [63] (Fig. 1).

A further region of the *vacA* gene (i- (intermediate) region) has been reported in literature to be associated with gastric cancer [110]. We will discuss this in the next session.

The s-region exists as s1 or s2 allelic types. Among type s1, further subtypes known as s1a, s1b and s1c have also been identified [9,126]. The m-region occurs as a m1 or m2 allele. Each *vacA* allele can consist of any combination of signal sequence and mid region except s2/m1 [8]. This implies that some alleles have arisen by recombination between strains *in vivo*. The combinations between the s and m region determine the strains virulence and are correlated with the disease. Simple polymerase chain reaction (PCR)-based methodology has led to many studies [8,11,98,133,123] correlating *vacA* allelic type and gastrointestinal disease. In many populations where *vacA* polymorphism has been found, the combination of s1m1 alleles was the most toxigenic and has been shown to be associated with duodenal and gastric ulceration [10,62]. In detail, s1-type strains are associated with vacuolating activity *in vitro* whereas s2-type is non-vacuolating because of the presence of a hydrophilic N-terminus extension [75,87]. Mid region polymorphisms determine the specificity of cell vacuolating because they affect the toxin binding to epithelial cells [110]. In brief, the final structure of a *VacA* allele is very important because it provides a potential mechanism for spread of favourable characteristics, such as virulence and antibiotic resistance determinant. This structure influences the toxin activity: strains with *vacA* m1 alleles are

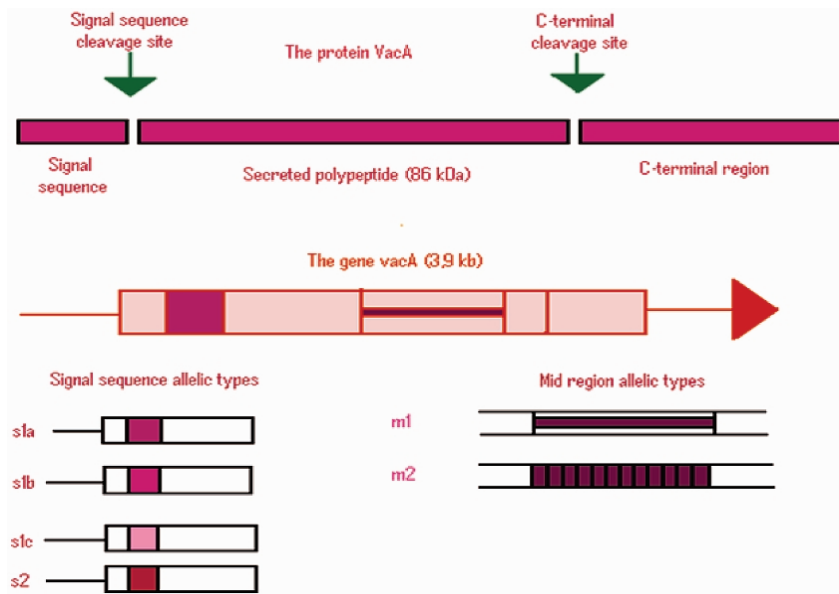


Fig. 1. Schematic structure of VacA (adapted from J.C. Atherton (1998) [10]).

more toxigenic than strains with *vacA* m2 alleles and within the first group, the *vacA* s1m1 allele is more correlated with cytotoxin activity and peptic ulceration than s1m2 allele being the subtype s1a m1 the most virulent. This type is commonly involved in patients with ulcers, 90% of patients with s1a strains has past or present ulcer [62] whereas strains with *vacA* s2 allele are uncommonly isolated from ulcer patients. It is thought that the *vacA* signal sequence type is a marker for the level of cytotoxin production [10]. Kaklikkaya et al. [62] in a histological study done on Turkish patients showed that gastritis were independent on both *vacA* m types and *vacA* s types of *Hp* isolates. This is probably due to the fact that generally other studies [8, 95,99] considered *Hp* strains isolated from peptic ulcer disease rather than only from gastritis.

## 2.2. Intermediate region (i) of *vacA*

In addition to the main principal sites of *vacA*, some Authors [110] have characterized a new *vacA* polymorphic site, called intermediate (i) region. As we have already reported, *vacA* type s1m1 strains have been shown to be associated with ulceration (gastric and duodenal) making this allelic form an important determinant of disease-associated *Hp* strains. Through a simple PCR-based typing system [110], it has been demonstrated the presence of one different region called intermediate region (i) that in *Hp* strains has been associated with the occurrence of gastric carcinoma (GC),

being consequently considered a better predictor of *Hp* strain carcinogenic potential than signal region (s) or mid-region (m).

The i-region, located between the signal region and mid-region within the p37 domain, can be divided in 2 types, i1 and i2, both common among clinical *Hp* isolates. The s1m1 type always includes the i1 region and is vacuolating whereas s2m2-type allele involves the i2 region and is invariably non-vacuolating. Differently, the s1m2 type can include either i1 region or i2 region. The s1i1m2 type induces vacuolation while s1i2m2 strains does not. These data can explain why, in s1m2 types, some strains result pathogenic whereas others do not: that depends on the presence of either i1 or i2 region. The differences of these two regions concern the amino acid substitutions in 3 clusters named A, B and C [30,48].

## 2.3. *CagA* protein and the *cag* pathogenicity island

The *cagA*-positive strain increases the risk of development of atrophic gastritis and mucosal inflammation [62]. The *cagA* gene is a marker for the *cag* pathogenicity island (*cag* PAI) (Fig. 2), seven genes of this island are known to code for the components of a type IV secretion system. Among these seven genes, *cagT* and *cageE* have been reported to play prominent roles by forming a syringe and a needle apparatus that delivers CagA protein (a protein of about 1200 amino acids whose size varies between strains) into the host

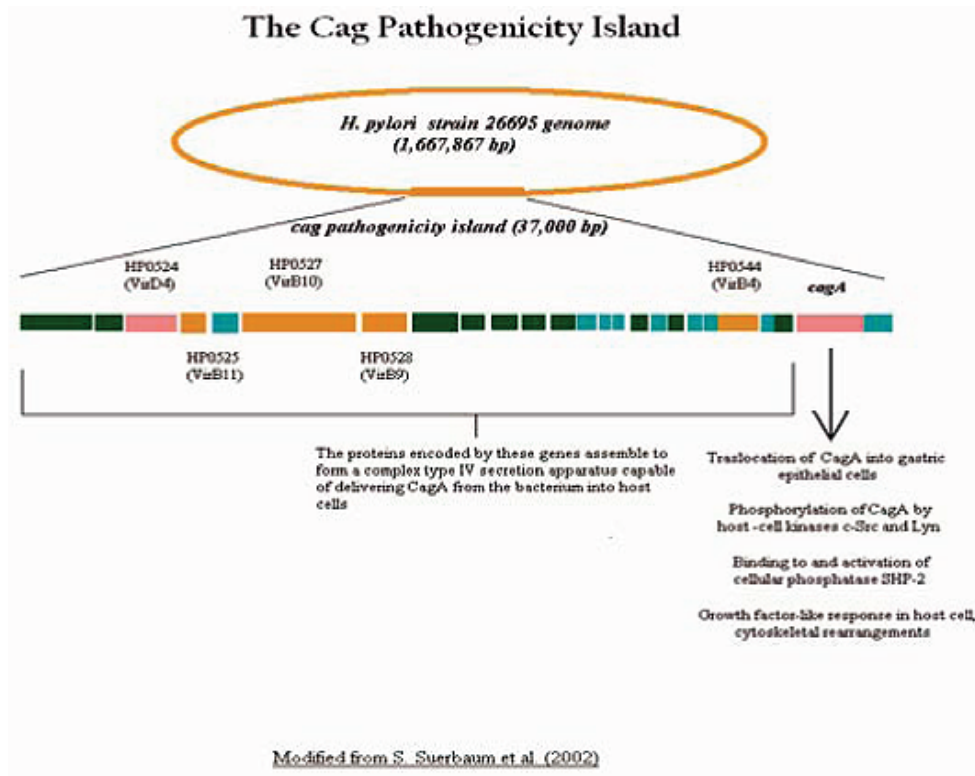


Fig. 2. *cag* pathogenicity island, a chromosomal region with about 37,000 bp and 29 genes. Four genes (marked in bright gray) are similar to components of the type IV secretion system. Proteins encoded by the island are involved in two major processes, the induction of interleukin-8 production by gastric epithelial cells and the translocation of CagA from the bacterium into the host cell. The genes with solid lines are essential for the induction of interleukin-8; light gray lines indicate genes required for translocation of CagA.

cells where it becomes tyrosine phosphorylated and stimulates epithelial cell to produce IL-8 [99] (Fig. 3). The *cagA* gene (3–6 kb) that has been reported to have functional implication in disease causation, is at one end of a collection of about 31 genes which are usually present as a group [22,123]. If some of these genes are disrupted, a limited induction of IL-8 production by epithelial cells infected is noticed. The *cag* region of *Hp* is similar to pathogenicity islands in other bacteria but it results different in nucleotide composition to other *Hp* genes and is genetically unstable. Following these points, it is probable that this region has been acquired relatively late in evolution from an external source, perhaps a bacteriophage or plasmid. The presence of CagA protein is strongly correlated with duodenal ulcer, neutrophil infiltration and gastric adenocarcinoma [15,101] Studies [8,99] report the detection of *cagA* highly associated with the presence of ulcer. This correlation is seen both in children and in adults and its presence in the second group may be the most predictable factor for ulcer risk even if environmental elements such as the smoking and non-steroidal anti-

inflammation drugs (NSAIDs) consumption [114] can play a more important role in the occurrence of ulcer in adult population.

The *cagA* status is reported to be unique in Turkish subjects [62] and it is considered a strong marker for atrophy and gastritis activity differently from *vacA* gene.

#### 2.4. *Hsp60* superficial protein

Hsps (heat shock proteins), called also stress proteins, are families of highly conserved proteins serving as a strong antigenic target for the immune response linked with pathology. *Hp* produces two Hsps: a groEs-like HspsA (size 13 kD) and groEL-like HspsB (size 54–60 kD). Hsp60 is in particular shared by *H. pylori* and eukaryotic cells [74]. The homologies between bacteria and human Hsp60 antigens leads to an antibody response directed to both the bacteria and to human tissues that express Hsps including vascular endothelial cells (autoimmune response).

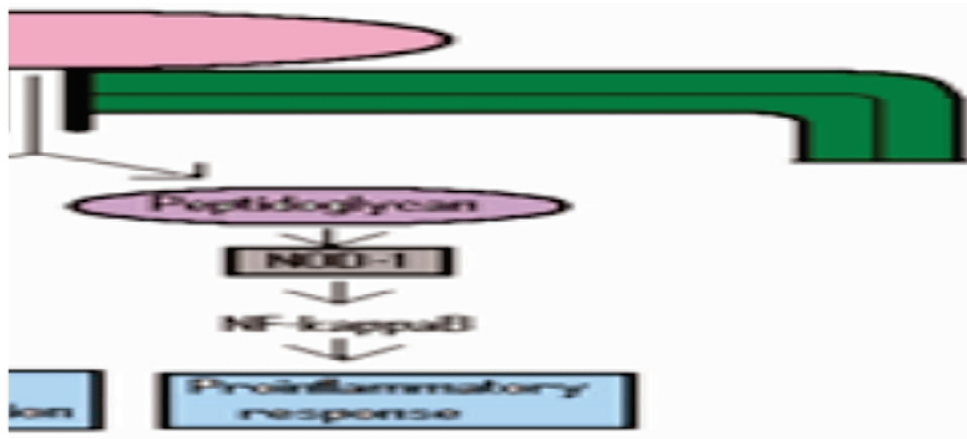


Fig. 3. Different roles of CagA protein, Cag typeIV secretion system and peptidoglycan in pro-inflammatory response, cell proliferation and morphological changes (adapted from J.G. Kusters et al. (2006) [72]).

### 2.5. *homA* and *homB* determinant

A putative ulcer-associated outer membrane protein (OMP) was recently identified by means of subtractive hybridization in *Hp* strain isolated from a young child with duodenal ulcer [97]. *homB* is similar to another gene belonging to OMPs called *homA*. The clinical importance of *homB* for peptic ulcer disease (PUD) in children has been shown [99]. In young children, the PUD occurs immediately after the *Hp* infection accounting for the involvement of more pathogenic strains. *homB* correlates with a number of other virulence determinants such as *cagA* and *vacAs1* as well as *babA2* that is a gene which modulates the attachment of bacteria to epithelial cells and other less known virulence markers such as *hopQI* and *oipA* ( $p < 0.001$ ). In one study [101], the genotype of strains isolated from children with PUD and NUD (non-ulcer-dyspepsia) in comparison with that detected from adults is reported. In children, Parsonnet et al. [101] notice a strong correlation between the presence of *homB* (but not *homA*) and other virulence markers in PUD (see above). On the contrary genotypes *homA* and the *sabA* “on” were strongly associated with NUD. In strains isolated from adults, only *cagA* is significantly associated with PUD indicating that *cag* PAI is the major independent predictor of disease in this population in which other situations can play a crucial role (smoking, diet, stress, etc.). However, the fact that *homB* was an important marker for PUD in children suggests that disease severity in young population is more related to the virulence of infecting strains. From these results, it is possible to emphasize *cagA* (*cag* PAI), as predictor of PUD in both children and adults.

The gene *homA*, which has a 90% of homology with *homB*, is correlated with less virulent *Hp* strains generally involved in NUD (non-ulcer disease). Moreover, the presence of two copies of *homB* in a single strain accounts for more strong ulcerogenic strains whereas one copy of *homA* is a marker for non-ulcerogenic isolates.

### 2.6. *BabA* adhesions

The presence of bacterial adhesins devoted to the attachment to human gastric epithelium, is essential. *babA* gene codes for the blood group antigen-binding adhesin *BabA* whereas the *babB* product is associated with a non-binding phenotype [60]. *BabA* major adhesin is directed to the fucosylated Lewis b blood group antigen not present in all kinds of gastric cells. In fact the AGS cells express variable levels of Lewis b blood group antigen [117] whereas the gastric epithelial Kato III cells are deficient in Lewis b epitope. For these cells, *Hp* adherence is independent on *BabA*-Lewis b binding.

### 2.7. *HP-NAP* and *napA* gene

Most of *Hp* virulent strains are known to activate neutrophils directly *in vitro*: 50% of strains induce a rapid, strong neutrophil oxidative burst and the remaining 50% a slower and weaker burst [10]. The first group is more often correlated with patients showing peptic ulcer disease in which a stronger vacuolating cytotoxin activity is seen. The *Hp* neutrophil-activating protein (*HP-NAP*) is a virulence determinant of *Hp* that stimulates in neutrophils high production of oxygen radicals

and adhesion to endothelial cells [2]. *HP*-NAP, highly conserved protein, is a member of a broad family of ferritin-like proteins, being able to activate neutrophils in a dose-dependent manner. The gene for this protein, similar to other genes encoding other cytoplasmic proteins not involved in neutrophils activation, is *nap A*, which has been cloned and sequenced [39].

### 2.8. Urease virulence determinant

Urease can play a broad role in the pathogenesis associated with infection of *Hp*. Although urease is known for its enzymatic activity, it is also correlated with the dual function of adhesivity and immunogenicity [14]. This is not surprising since bacteria contain genes involved in a wide range of physiological processes probably for adapting themselves to different growth conditions.

*H. pylori* urease, deriving from a pool of bacterial surface proteins (OMPs), is involved in the attachment to gastric epithelial cells and exactly to CD74 receptors [14]. The interaction between urease B and CD74 has been demonstrated [96]. It can be suggested that urease is specifically important for the attachment of *Hp* and for the pro-inflammatory immune response initiated by the bacterium. The binding of the subunit urease B to CD74 expressed on gastric cells may therefore contribute to increase the bacterial virulence during infection.

### 2.9. *H. pylori* restriction endonuclease-replacing gene A (*hrgA*)

Another class of virulence factor that is responsible to produce severe disease pathologies is *H. pylori* restriction endonuclease-replacing gene A (*hrgA*) [123]. This gene is considered as an important virulence determinant in *Hp*-associated gastric diseases such as carcinoma [4,5]. The strains possessing *hrgA* are most prevalent in Asian patients with gastric carcinoma probably having a direct effect on differential induction of IL-8 release or apoptosis in gastric epithelial cells [2]. Strains harbouring the *vacA* including the intermediate region (i) and *hrgA* are associated with increased risk of developing form of gastric disorders and carcinoma.

## 3. *Helicobacter pylori*: Immunology, immunopathology and host susceptibility

### 3.1. *Helicobacter pylori*: Immunology and immunopathology

Chronic *Hp* infection is characterized by gastric inflammation and mucosal infiltration of the full range of inflammatory cell types with a predominant amount of lymphocytes. However, and this is uncommon when considering chronic infections, there is a consistent neutrophilic component [111].

The rate of inflammation and the consequent development of gastric disease is known to be multifactorial: indeed it involves the virulence of different strains of *Hp*, environmental co-factors and the host immune response. Particularly, the principal determinant of the *Hp* associated pathology is the interaction between the host immune response and the bacterial virulence factors [72].

*Hp* infection strongly induces and maintains both innate and acquired immune response. However, rarely the immune response to *Hp* results in clearance of the infection [72]. This is due to the ability of *Hp* of evading the host's immune system [18], because of the particular localization of *Hp* after infection.

#### 3.1.1. Innate immune response

The innate response to different bacterial components is the first line of defence to *Hp* infection; it involves several mechanisms, especially the pattern recognition receptors (PRRs) and the intracellular receptors for Gram-negative bacteria known as Nod family. The best studied PRRs for *Hp* infections are Toll-like receptors (TLRs), which are expressed on epithelial cell surfaces and recognize many bacterial products, such as LPS (TLR4), peptidoglycan (TLR2) and flagella (TLR5). It has been shown that *Hp* LPS is a weak inducer of a TLR-dependent response, comparing with LPS of other Gram-negative bacteria [72]. *Hp* antigens predominantly utilize TLR2 and TLR5 rather than TLR4. TLR-dependent response induces NF- $\kappa$ B activation and consequently the expression of genes encoding pro-inflammatory cytokines, such as IL-8. However, during chronic *Hp* infection, TLRs receptors seem to play a marginal role when considering the induction of innate response: in fact *Hp*-antigens activate NF- $\kappa$ B in a TLRs-independent way [127]. The principal TLRs-independent way is mediated by the recently discovered Nod family of intracellular (Nod1) and extracellular (Nod2) receptors for components of Gram-negative

bacteria. *Hp* peptidoglycan, which enters the epithelial cells by cag PAI-mediated contact between bacteria and cell (Fig. 3), interacts with Nod1: in this way, cag PAI-positive *Hp* strains activate the innate immune response inducing pro-inflammatory cytokine production by Nod1-dependent signal. As well as TLRs mediated response, the way dependent on Nod1 family involves the activation of NF- $\kappa$ B with the consequent expression of IL-8, which is known to induce recruitment of lymphocytes and neutrophils and IL-12, which is crucial for the activation of monocyte-macrophage and the development of Th1 acquired immune response [127].

The presence of gastric infiltrating inflammatory cells, which depends on the production of cytokine and chemokines (such as MIP-3 $\alpha$ ) from the *Hp*-infected epithelial cells, enhances the amount of gastric inflammation and induces further pro-inflammatory gene expression; activated dendritic cells (DCs) secrete IL-6, IL-8, IL-10, IL-12, IL-1 $\beta$  and TNF $\alpha$ , in this way polarizing the immune response to Th1 pattern. The way *Hp* interacts with DCs seems to involve the DC- SIGN receptor (receptor C-type lectin ICAM-3-grabbing non integrin) [13].

Gastric infection with *Hp* is characterized by a high immune response, but it rarely results in complete bacterial clearance. Recent data show that *Hp* can survive in phagocytic cells for up to 24–48 hours [18]: bacterial virulence factors as urease [90] and catalase [128] are involved in *Hp* intracellular survival, respectively by increasing intraphagosomal pH and by neutralizing the effects of reactive oxygen species produced by the macrophage. Recently, it has been demonstrated that a crucial role in *Hp* intracellular survival is dependent on *Hp* arginase (encoded by the gene rocF), which competes with macrophage inducible nitric oxide synthase (iNOS) for the common substrate L-arginine [50].

### 3.1.2. Acquired immune response

When a pathogen contacts with immune system, macrophage and dendritic cells are able to express bacterial antigens bound to major histocompatibility complex (MHC) proteins on cell surface and to serve as antigen presenting cells (APCs): this is crucial for the activation of the acquired immune system. *Hp* gastric infection evokes a vigorous local and systemic acquired humoral and cell-mediated response.

In the humoral response, B lymphocytes are activated by APCs (activated DCs or T lymphocytes) and produce mucosal and systemic IgA and IgG antibodies, but it has been shown that the humoral response

is not essential for *Hp* infection protection [111]. In fact, the development of *Hp* related gastritis depends predominantly on T-cell mediated immunity [12]. In the cell-mediated arm, T lymphocytes are activated by APCs; the mononuclear cells produce IL-12 and induce the differentiation of naïve T cell into T-helper1 (Th1). Th1 cells secrete IL-2, IFN $\gamma$  and IL-12, in this way enhancing the ability of macrophages to express pro-inflammatory cytokines and to increase the level of gastric inflammation. At the same time, there is a strong reduction in Th-2 cytokines pattern production, as IL-4, IL-5, IL-10, which supports the humoral system.

Several *Hp* virulence factors have been associated with the promotion of Th-1 polarized response, including the cagPAI. Furthermore, recent data demonstrated that the *Hp* neutrophil-activating protein (*HP*-NAP) plays an essential role in promoting Th-1 immune response [2].

### 3.1.3. Regulatory T-cells (Tregs) involvement

Regulatory T- cells (Treg), whose action is to down-regulate immunity, have been extensively studied in the pathogenesis of allergy, autoimmunity and inflammatory bowel diseases; recently, many researches have been focused on their role during *Hp* infection [94, 111]. Natural Treg cells, expressing CD4+CD25+ and the transcription factor FOXP3 (forkhead box P3), are selected in the thymus and move to the periphery. In the periphery, CD4+ T cells can be induced to become Treg (called adaptative Treg cells) and hence secrete IL-10 or TGF- $\beta$  or both. High levels of IL-2 induce the proliferation of Tregs and also contribute to their efficiency and fitness. Furthermore, it is known that bacterial heat-shock protein 60 (Hsp60) and flagellin enhance the suppressive functions of CD4+CD25+ T cells through the production of IL-10 and TGF- $\beta$ . Treg cells inhibit the function of both Th1 and Th2 CD4+T cells and their production of cytokines; additionally, their role is to suppress the CD8+ T cell activity. In this way, they reduce the amount of gastric inflammation but contribute to bacterial persistence and colonization of the gastric mucosa [94].

Previous studies on animal model showed that concurrent intestinal helminths infections reduced the amount of *Hp*-related gastric inflammation and atrophy. These findings can be explained considering that the occurrence of parasitic infections evokes a Th2 polarized response, which can shift the *Hp* immune response from Th1 to Th2 [43]. Conversely, mouse co-infection with *Toxoplasma gondii*, known to be a strong Th1 response inducer, enhances the degree of gastric inflammation and carcinogenesis [76].

### 3.1.4. Virulence factors and pathogenesis

*Hp* is known to be a pathogen with a significant genetic diversity, mainly due to two major mechanisms: macroheterogeneity and microheterogeneity. The first is characterized by a genetical variability in large chromosomal regions, the latter is characterized by an individual genes feature sequence diversity. This wide genetic diversity is responsible for the different virulence among various *Hp* strains [1]. Here we describe the effects on the host immune response of the principal determinant of virulence.

#### 3.1.4.1. The *cag* pathogenicity island (PAI)

The CagA protein is highly immunogenic and CagA+ *Hp* strains induce a higher inflammatory response comparing with CagA- strains. The action of this protein, which has been widely described previously [10,62,123], allows *Hp* peptidoglycan to enter the host epithelial cells and, through the interaction with NF- $\kappa$ B, to induce the expression of pro-inflammatory cytokines, such as IL-8 and chemokines as MIP-3 $\alpha$  [29]. *In vivo* CagA+ strains elicit a high expression of IL-8 from gastric cells [102]. The activation of this transcription factor has been associated with the enhanced expression of the promoter region of Fas ligand (FasL) gene on T cells, implying that *Hp* induces apoptosis in Fas-bearing T cells and modulates the acquired immune response [70]. It's interesting that the induction of apoptosis in gastric epithelial cells is different from that in T cell: in fact, viable *Hp* strains, both *cagA* PAI positive and negative, induce apoptosis in epithelial cells, whereas only the *cagA* PAI positive strains are able to cause FasL dependent apoptosis in T cells [129].

#### 3.1.4.2. The *VacA* (vacuolating cytotoxin)

The VacA protein has immunosuppressive effects, because it inhibits T cell proliferation and production of IL-2, due to the interference with the T cell receptor/IL-2 signaling pathway at the level of the Ca<sup>2+</sup>-calmodulindependent phosphatase calcineurin [47]. Moreover, the activation of NFAT, a nuclear transcription factor, which is needed for the expression of the genes involved in the T-cell dependent acquired immune response, is inhibited by *cagA* bearing strains [17].

Recent studies [115] identified that the *vacAs1m1* binds specifically to the  $\beta$ 2 (CD18) integrin receptor subunit of activated T lymphocytes and that its internalization is necessary for the immunomodulant effects of the VacA protein. It has been demonstrated that Va-

cA also suppresses the proliferation of primary human CD8+ T cells, CD4+ T cells and B cells [115]. VacA, as well as other *Hp* virulence factors, influences the mononuclear cells intracellular killing of the bacterium: in fact, it can induce the formation of vacuoles in eukaryotic cells and avoid the phagosome maturation [77]. Clinical and epidemiological studies support a causal relationship between *Hp* infection and endothelial dysfunction leading to vascular diseases. The VacA – dependent nitric oxide reduction is involved in a significant pro-atherogenic effect on a range of vascular endothelial dysfunction markers [52,93,124]. The VacA-mediated local immune suppression, together with its evasion from intracellular killing, can explain the chronicity of *Hp* infection and the severity of gastric pathology of the *vacAs1m1 Hp* strains.

#### 3.1.4.3. *Helicobacter pylori* Neutrophil Activating Protein (HP-NAP)

It's known that the degree of mucosal damage is correlated with local neutrophils infiltration: recent studies identified HP-NAP as one of the principal *Hp* pro-inflammatory protein involved in the recruitment of inflammatory cells [91].

In fact, its ability to cross the endothelia promotes polymorphonuclear cells (PMNs) adhesion through an acquisition of the active form of  $\beta$ 2 integrin which is responsible for the arrest of rolling cells on the endothelium and for the consequent inflammatory cells recruitment [103]. Moreover, human PMNs are directly stimulated by HP-NAP in a TLR2-dependent way and produce several chemokines including CCL3, CCL4, CXCL8 whose principal role is to enhance and maintain the amount of local inflammation [103].

It has been reported that HP-NAP, acting as TLR2 agonist, is able to induce the expression of IL-12 by neutrophils and monocytes and the expression of IL-23 by monocytes. These findings, together with the MHC class II up-regulation in DCs, underline the importance of HP-NAP in the promotion of a Th1 immune response [2]: in fact, IL-12 induces the differentiation of naïve Th-cells into Th1 phenotype, and IL-23 promotes the differentiation of dendritic cells into mature and activated DCs.

#### 3.1.4.4. *Helicobacter pylori* Heat shock protein (Hsp60)

A strong relationship between coronary heart disease (CHD) and chronic bacterial infection has been shown [74] suggesting an essential role of inflammatory diseases in the pathogenesis of vascular cardiac



Table 2  
Genetic polymorphisms and *H. pylori* infection

Polymorphism site	Effect	Association with <i>H. pylori</i> infection
Immune mediators		
IL-1 gene cluster	Higher IL-1B expression polymorphisms	Higher expression of IL-1B results in a proinflammatory response associated to hypochlorhydria, pangastritis and increased risk of atrophic gastritis and gastric cancer [45,59,106]
IL-10 (ATA/GCC Haplotypes)	GCC haplotype results in increased expression of IL-10	Colonization by more virulent <i>H.pylori</i> strains ( <i>cagA</i> +, <i>vacAs1</i> + and <i>babA2</i> +); ATA haplotype is associated with increased risk of gastric cancer [53,55]
TNF-A gene	TNF-A-308A is associated with increased TNF- $\alpha$ expression and increased gastrin expression	TNF-A 308A is associated with higher levels of <i>H. pylori</i> infection and increased risk of gastric cancer [54,85]

Modified from J.G. Kusters et al. (2006) [72].

disorders. Elevated levels of homocysteine [26] and serum lipids [57,73], both independent risk factors for atherosclerosis, are associated with *Hp* infection [124]. An increased risk of CHD in patients with a systemic immune response to heat shock proteins (Hsps) has been demonstrated [79].

Hsp60 is mainly responsible for the development and progression of various immunopathological processes, which lead to chronic inflammatory diseases. Some studies [79,132] have shown an increased risk of coronary heart disease in patients with a systemic immune response to Heat shock proteins (Hsps). *Hp* infection may therefore concur to the development of coronary heart diseases.

#### 3.1.4.5. Adhesins and outer membrane proteins

Outer inflammatory protein A (OipA), as well as *cagPAI*, is involved in the activation of the RANTES promoter binding sites, including CRE, NF-IL-6 and NF-kB sites, leading to a high expression of this pro-inflammatory chemokine. This is supported by *in vitro* and *in vivo* experiments, which demonstrated that mucosal RANTES mRNA levels were higher when considering the infection with *cag PAI*+/*OipA*+ strains, followed by those with *cag PAI*-/*OipA*+ strains and then those with *cag PAI*+/*OipA*- strains, while RANTES mRNA levels were suppressed in *cag PAI*-/*OipA*-infections [71]. Moreover, the presence of functional OipA protein is associated with the expression of IL-8 and consequent gastric inflammatory infiltration, even in *cagA PAI*- strains.

Blood group antigen binding adhesion A (BabA), which is the best-characterized *Hp* adhesion protein, binds fucosylated Lewis b blood group antigen expressed on the cells surface. It has been shown that *Hp*

strains producing low levels of the BabA protein were associated with increased local inflammatory response and severe clinical outcome than BaA+ strains [134]. HomB is a member of the outer membrane protein family (OMPs) observed in highly virulent *Hp* strains and strongly associated with the presence of the *CagA* protein; it has been shown to promote *in vitro* and *in vivo* a pro-inflammatory response in gastric cells due to a high expression of cytokines as IL-8 [99].

The product of hom B, Hom B, is a virulence associated OMP candidate that is able to stimulate the immunological response of the patient and to contribute to inflammatory response and to bacterial adherence properties.

The mutant strains without hom B gene present reduced capacity to stimulate IL-8 by host epithelial cells and to bind to these cells [99]. Hom B, involved in the inflammatory response and in *Hp* adherence, can be considered a novel virulence factor.

#### 3.2. *Helicobacter pylori* infection and host susceptibility

Host response greatly affects the outcome of *Hp* disease, influencing and determining the pathology. The host response is not able to contrast or control the *Hp* infection but on the contrary it is involved in all the situations that worsen the clinical course of the illness. In fact, if *Hp* is not adequately treated, it remains lifelong in the infected subjects. In other words, host response contributes to determine the microorganism pathology.

Indeed, not only the characteristics of the pathogen but also host genetics play an important role in determining susceptibility to infections and severity of the disease. In recent years, the importance of host genet-

Table 3

Influence of IL-1B pro-inflammatory polymorphisms on the *Hp*-related infections

Host factors influencing <i>Hp</i> pathogenicity	
IL-1B pro-inflammatory gene polymorphisms	
<u>Present</u>	<u>Absent</u>
Corpus gastritis	Antrum gastritis
Hypochlorhydria	Normal or high levels of acid secretion
Gastric atrophy	Duodenal ulcer
Gastric adenocarcinoma	

ic polymorphisms in *Hp*-related pathologies has been demonstrated [45,59,106]. The most important host genetic polymorphisms are listed in Table 2.

Many of the pathogenic effects of *Hp* infection are associated with chronic active inflammation, which is controlled by a complex interaction of pro-inflammatory and anti-inflammatory mediators. Generally the pro-inflammatory genetic polymorphisms tend to increase the risk of development of gastric carcinoma. A good example of this is related to polymorphism of IL-1 gene such as IL-1 B (Table 3).

The level of acid secretion in the stomach determines the development of either duodenal ulcer disease or atrophic gastritis leading to cancer. IL-1 $\beta$  is a potent pro-inflammatory cytokine and the most patient's known inhibitor of acid secretion [21] High level of IL-1  $\beta$  leads to hypochlorhydria, gastric ulcer, formation of atrophic gastritis, intestinal metaplasia and increased risk of gastric carcinoma [34–36].

In contrast, if these polymorphisms are not present, the anti-inflammatory effects lead to high levels of acid secretion, antrum gastritis and duodenal ulcer [72]. Similar effects have been seen for polymorphisms in other inflammation-associated genes (Table 2), for example the genes encoding tumor necrosis factor alpha (TNF- $\alpha$ ) and IL-10. TNF- $\alpha$  is a pro-inflammatory cytokine and several polymorphisms of gene TNF-A are known. The TNF- $\alpha$  cytokine influences gastric production and lowers acid output by gastric parietal cells [119] being consequently associated with increased risk of gastric cancer [36].

Similarly, the level of IL-10 cytokine affects the gastric acid production. This is influenced by the haplotypes described for the IL-10 gene: the GCC (Guanine, Cytosine, Cytosine) haplotype stimulates the output of IL-10 cytokine producing an anti-inflammatory effect whereas ATA (Adenine, Timine, Adenine) haplotype lowers the IL-10 secretion producing a pro-inflammatory response with consequent increased risk of gastric cancer [83]. In any case, no single gene polymorphism can increase or decrease the risk of developing gastric cancer but, considering the marked hetero-

geneity of *Hp*, it is the combination of different pro- and anti-inflammatory polymorphisms, which strongly affects the disease outcome [105]. The natural course of *Hp* infection depending on the different levels of stomach acid production can be summarized in Fig. 4.

#### 4. Pre-endoscopy screening of *Helicobacter pylori* infection: Implication and advantages

Different invasive and non-invasive diagnostic tests are available for the diagnosis of *Hp* in the individual patient. The non-invasive tests obviate the need for endoscopy and can be surely more accepted by the subjects. Moreover the endoscopy has a high cost and provides a marked workload and medical expenses for the hospitals. So the strategy specifically followed in general practice is to avoid the endoscopy in patients at low risk of having major pathology. These patients could prevent prompt endoscopy and might safely undergo different managements.

It has been proposed [22,23,26] that younger patients with symptoms of dyspepsia with non-alarming symptoms could be screened non-invasively for the infection in order to reduce endoscopy procedure. In addition, non-invasive tests are suitable, other than for pre-endoscopy screening of younger dyspeptics, also for use in research and for epidemiological surveys as well as for confirming successful eradication after treatment and for screening asymptomatic population.

The pre-endoscopy screening is based on different methodologies (such as serological markers, molecular markers, etc.) that will be discussed in the present section.

##### 4.1. Serological markers

Serological testing has been recommended for initial pre-endoscopy or pre-treatment screening in dyspeptic patients. Serology is cheap and convenient and thus should be preferred in situations where the additional information yielded by an endoscopy is not needed. Patients are prone to undergo this analysis because it only requires a simple peripheral blood collection for the investigation of anti-*Hp* IgG, IgM and IgA antibodies. The serological tests are very commonly used for clinically diagnosis of *Hp*-related infections. In general, the serum levels of anti-*H. pylori* IgG antibodies increase in the presence of infection and can be used as a marker. On the other hand, even if anti-*Hp* IgA antibodies are less appropriate for this purpose [3,69],

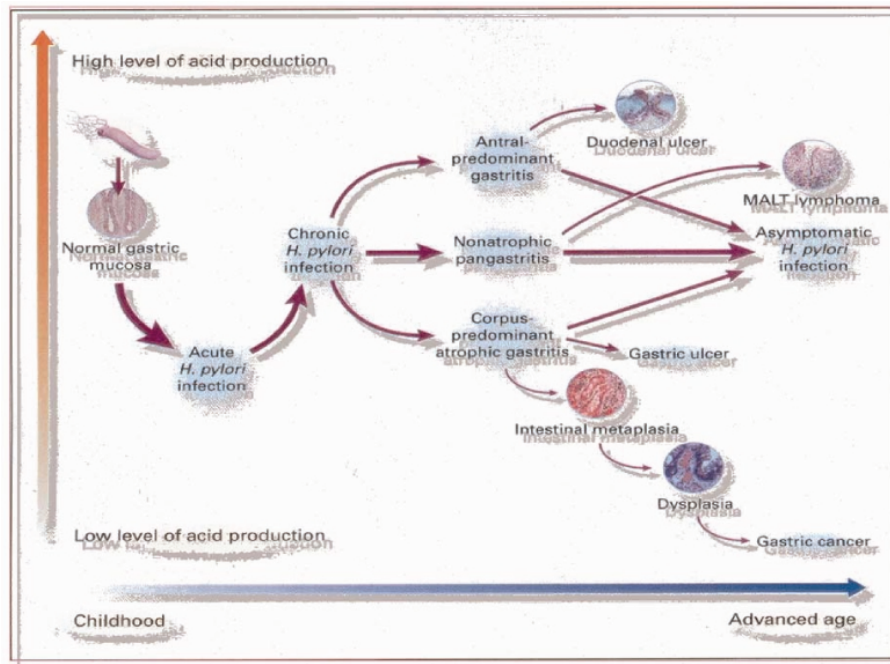


Fig. 4. Natural course of *Hp* infection from childhood to advanced age.

serological findings of anti-*Hp* IgA in symptomatic patients might have significant clinical value for the diagnosis of infection, especially if the patient is seronegative for IgG. The disadvantage for serology is that past or current infections are not distinguished owing to the fact that past infections may lead to false positive, so that this test cannot be used for determining therapy success after treatment even if successful eradication can follow a substantial drop in antibody title, using repeat serology after a delay post-treatment [3,69].

#### 4.1.1. Serology as diagnostic tool

The systemic response typically comprises a transient rise in IgM followed by a rise in specific IgA and IgG maintained throughout infection. The consideration that patients with IgG antibodies to *Hp* have a greater risk of peptic ulcer disease as a cause of their dyspepsia, has led to screen dyspeptic patients under the age of 45 years using *Hp* serology. Three strategies are proposed after serology screening:

- 1- Endoscopy of *Hp* seropositive patients and treatment of seronegative patients symptomatically.
- 2- Treatment of seropositive patients for *Hp* and endoscopy of seronegative patients.
- 3- Eradication of infection from *Hp* seropositive patients, treatment of seronegative patients symptomatically and endoscopy for those with recurrent dyspepsia.

The attitude in both gastroenterologists and general practitioners with interest in gastroenterology towards the current pattern of using pre-endoscopy *Hp* serology screening has been evaluated [78]. The most popular strategy among general practitioners is that of eradicating infection from seropositives and treating seronegatives symptomatically. In contrast, the most popular strategy among gastroenterologists is that of endoscopic seropositives and treating seronegatives symptomatically.

There is then wide variation in attitudes and practice between these two groups: general practitioners like more serological tests and strongly prefer eradicating infection in seropositives before addressing to endoscopy (mainly for cost consideration). On the contrary, the majority of gastroenterologists would endoscope seropositives before treating the infection.

In any case, it is recommended that non-invasive *Hp* testing should be used in place of endoscopy with all those testing positive being given anti-*Hp* therapy and those testing negative being treated symptomatically. The above strategy of "test and treat" used in clinical practice may include some inconveniences: expense morbidity from drug side effects and introduction of antibiotic resistance both in *Hp* and in other pathogens [10].

An important serological tool for pre-endoscopy screening in patients at risk of carcinoma includes the

Table 4  
Sensitivity, Specificity, Positive Predictive Value (PPV), Negative Predictive Value (NPV), Accuracy of IgG and IgA detection in serum

	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Accuracy (%)
IgG alone	64	83.7	82	66	73.1
IgA alone	72	65.9	72	67.4	69.8
IgG + IgA (both positive or negative)	86.6	74.2	74.2	86.6	80

Modified from A. Locatelli et al. (2004) [80].

### Serum *Helicobacter pylori*-specific IgG1 and IgG2 antibody in subjects with Gastric Cancer (GC), Duodenal Ulcer (DU), Chronic Gastritis (CG) and Reflux Esophagitis (RE)

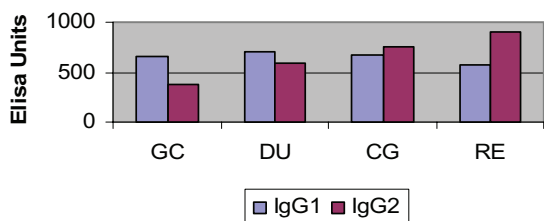


Fig. 5. Modified from Z. Ren et al. [109].

quantitative determination of the different subclasses of IgG.

In fact, a selective reduction of anti-*Hp* IgG subclass antibody is proven to occur in gastric carcinoma [109]. Cell-mediated immunity influences the outcome of infection including the development of gastric carcinoma (CG). The T-cell response comprises a secreted cytokine profile which influences the B-cell response including the production of the different IgG subclass antibody. In the adenocarcinoma, a fall in IgG level is demonstrated resulting to be particularly predictive of cancer [125]. This is thought to reflect premalignant gastric atrophy with loss of colonization and antigens stimulus [27]. A diminished IgG antibodies response due to low immunogenicity of *Hp* LPS or to the loss of *Hp* in some subjects evolving to GC, could reflect the premalignant phase of gastric atrophy. Significantly lower IgG2 levels are found in subjects with gastric carcinoma compared with those with reflux oesophagitis, chronic gastritis, gastric ulcer and peptic ulcer whereas IgG1 antibody remains at similar levels (Fig. 5). The levels of IgG 3 and IgG 4 are not affected and in most subjects are undetectable. The decreasing of IgG 2 subclass level noticed in patients with adenocarcinoma and not in other *Hp*-related pathologies depends on both the

switching of mucosal cytokine secretion and the different kinetics of IgG response to gastric colonization by B-lymphocyte that can be influenced by cytokine profiles in secreting different antibody patterns [61].

Consequently the patients showing low levels of IgG especially of subclass IgG 2 (below an established cut-off value) can be considered subjects at high risk of developing pre-malignant disease, gastric atrophy and adenocarcinoma [109]. These data show that above certain levels of antibody, irrespective of age, the risk of cancer is low and that primary endoscopy could be restricted to those with antibody values below this level. In this way, the endoscopy could be avoided, as initial investigation, in 42% of dyspeptic subjects [109]. The value of this test, as a predictive diagnostic tool in pre-endoscopy screening strategy is crucial.

In conclusion, the screening strategy based on *Hp* serological status, determined with the enzyme-linked immunoadsorbent assay (ELISA) and Western blotting (WB), in patients with uncomplicated, simple dyspepsia up to 55 years of age, is able to identify 95%-100% of patients with significant gastroduodenal lesions while potentially saving 47% of endoscopies [16].

#### 4.1.2. Sensitivity and specificity of serological tests

The concentration of serum IgG is reported to have sensitivity of 64%, specificity of 83.7%, PPV (Positive Predictive Value) of 82%, NPV (Negative Predictive Value) of 66% and accuracy of 73.1% for the diagnosis of *Hp* infection [80]. For the same purpose, serum IgA has the following values: 72.0%, 65.9%, 72.0%, 64.4% and 69.8% respectively [80]. If the serological tests are considered together (i.e. when both test are positive or negative), some of these values could increase: the accuracy is reported to be 80%, sensitivity 86.6%, specificity 74.2%, PPV 74.2% and NPV 86.6%. In synthesis, the serological tests are efficient in the diagnosis of the presence or absence of *Hp* infection and when used simultaneously, they are more efficient in accuracy, sensitivity and negative predictive value than when used alone. (Table 4). The detection of *Hp* IgA

and IgG antibodies in serum is useful in distinguishing between infected and uninfected patients whereas the concentration of antibodies in duodenal fluid is not suitable at this purpose [80,89].

#### 4.1.3. Advantages and disadvantages

Screening strategies, based on the serology used as marker of virulence, surely result to be very useful as reported above. The main advantage of serology is that it is a non-invasive and simple method for diagnosing *Hp* infections and for screening individuals at high risk to develop malignant disease. Furthermore, it reduces endoscopies taking also into account the patient's compliance. A drawback of using serology as predictive diagnostic marker of disease is that it could miss a proportion (even if irrelevant) of severe pathologies and underlying malignancy. However, in western countries, this is rare in patients less than 55 years of age presenting with dyspepsia in the absence of sinister symptoms [88].

#### 4.2. Molecular markers

Knowing in advance if a *Hp* strain in a specific patient is virulent or not is vital for the approach that the clinician should have towards the infected individuals. In other words, the presence of virulence determinants (such as CagA, VacA, Hsp60 proteins) can address the gastroenterologists to a correct and suitable therapy. For this aim, strain typing could be generally useful in pre-endoscopy screening; for example endoscopy might be unnecessary in young dyspeptic patients without severe symptoms who are infected with non-virulent strains. It would be better not only to treat young dyspeptic patients infected with virulent strains without performing an endoscopy but also to treat patients likely to develop ulcers or gastric malignancy before those conditions arise. For this purpose, the serology towards the virulence determinants can be used instead of invasive endoscopy.

##### 4.2.1. Vac-A and Cag-A

VacA serology is uncommon because there are some uncertainties about its interpretation owing to the mosaicism of antigens and to the variety of existing subtypes which are correlated to the different diseases (for example vacA s1a strains are more commonly associated with ulcer than vacA s1b strains or vacA s2). CagA serology is more reliable than VacA serology due to the strong immunogenicity and the less variability of CagA protein respect to VacA. CagA seropositivity

reflects the presence of cagA gene together with the cag PAI. Some problems linked to CagA serology could occur. First of all, the infection with CagA+ strains is common so that treating CagA seropositive subjects might result in unnecessary treatment even if it has been demonstrated [15,101] that people with CagA seropositive infection are at higher risk of ulcers or more severe pathologies than CagA- subjects.

A second problem concerns the fact that avoiding treatment for CagA- patients would lead to miss some infected individual patients who later develop malignancy.

Third, the presence of CagA- strains may be rare in some populations depending on geographical area. Further, it would be advisable to know, in CagA- subjects, if their risk of developing more severe disease such as carcinoma is higher than in uninfected people. If any significant risk is confirmed between CagA- infected and uninfected individuals, the treatment of CagA- patients would be strongly recommended.

In synthesis, if there is evidence that treatment of CagA+ reduces the possibility of subsequent *Hp*-related malignancy, CagA serology can be considered a viable test for selecting strains to treat [110,123].

The *Hp* infectious status is determined serologically using a commercially available enzyme-linked immunosorbent assay ELISA with a sensitivity and specificity of 96% and confirmed by Western blotting (WB) [16].

##### 4.2.2. HSP60

Antibodies to Hsp60 have been suggested as markers of chronic inflammation so the detection of anti-Hsp60 covers a crucial role as serological marker of strain-virulence and may therefore be good predictors for the risk of vascular diseases as well as it has been reported for *Chlamydia* species [86]. High levels of anti-Hsp60 antibodies may constitute a marker and/or a concomitant pathogenic factor of these pathologies [74].

The accurate definition of this new risk factor may lead to novel strategies for the prevention of ischemic heart disease since simple procedures such as the detection of anti-Hsp60 may be a good predictor of ischemic illness.

Wick et al. [130] demonstrated that the association between high levels of anti-Hsps60 antibodies and atherosclerotic vascular disease is due to an autoimmune reaction to endothelial cells that express high levels of Hsps in response to different stimuli such as free radicals, local infections, cytokines etc. Antibodies to Hsp60 are determined by ELISA test using a commercially available human hsp60 (Sigma Che. Co. Milan, Italy) [74].

Table 5  
Distribution of major virulence genes of *Helicobacter pylori* in various diseases

	Gastric carcinoma %	Duodenal ulcer %	Pre-pyloric ulcer %	Peptic ulcer %	GERD %	NUD %
vacA s1	85	64	100	100	50	50
vacA s2	14	35	/	/	50	50
cagA	100	78	100	50	100	66
cagE	100	85	100	100	100	83
cagT	100	92	100	100	100	83
hrgA	100	100	100	100	100	100

Modified from S.K. Tiwari et al. (2007) [122].

Table 6  
Detection of *H. pylori* in biopsies and in salivary secretions by multiplex PCR

	Symptomatic subjects (80)		Asymptomatic subjects (20)	
	N°	%	N°	%
Stomach biopsy	72	(90)	10	(50)
Saliva	70	(87.5)	12	(60)

Modified from S.K. Tiwari et al. (2005) [121].

#### 4.3. Multiplex PCR assay (Molecular Screening)

The molecular markers of virulence can also be easily detected by multiplex assays based on PCR. Multiplex PCR assay is an advancement, compared to uniplex or single locus PCR, because it is suited to diagnose and specifically identify virulence *Hp* strains and their main virulence genes cagA, cagE, cagT, vacA and hrgA. This method is able to genotype *Hp* isolates based on the main virulence genes. The analysis of cagA alleles as well as vacA is performed by polymerase chain reaction (PCR). The methodology for performing Multiplex PCR is reported by Tiwari et al. 2007 [123]. Reference strain *H. pylori* ATCC 49503 is used as a positive control whereas water for cell culture grade is used as a negative control [23,82]. This method results very useful in distinguishing five potential virulence genes also including the two subtypes of vacA signal region (s1 and s2). This new strategy, which not only predicts mere presence or absence of *Hp* infection but also gives information about its genetic heterogeneity, is highly recommended especially because it is a fast and reliable alternative to others methods and also can be employed even in highly contaminated samples.

Different genotypes were reported to be correlated to various infection kinds by Tiwari et al. 2007 [123]. In this study, they report the distribution of the genes in the different pathologies (Table 5). An important finding of this study is that hrgA gene results to have 100% prevalence among all disease groups irrespective of clinical category. This result differs from that obtained by Ando et al. 2002 [4] who reported a more

marked presence of hrgA in patients with cancer than in those with other pathologies. These discordant data can depend on different geographical areas considered in the two researches and on the need of examining a more large number of subjects. Higher prevalence of the genotype cagT +, hrgA +, cagA +, cagE + and vacAs1 + is found among patients with pre-pyloric ulcer (100%) and gastric carcinoma (85.7%) followed by duodenal ulcer subjects (60.7%). Overall, this genotype is present in 67% of the total subjects analysed with higher occurrence among those with ulceration and gastric carcinoma than among those with GERD (gastric oesophageal reflux disease) and NUD (non-ulcer disease). The genotype cagT +, hrgA +, cagA-, cagE + and vacAs2 subtype is least prevalent. The vacAs1 subtype is more correlated with the presence of cagA than the vacAs2 subtype and only 2.44% cagA- strains possess the vacAs1 allele. Then with reference to the clinical status, vacAs1 is prominent in patients with pre-pyloric ulcer (100%), gastric carcinoma (85%) and duodenal ulcer (64%). However, this study has been performed using gastric tissues (biopsies), which is an invasive method and cannot be used as a pre-endoscopy screening. The same authors in a previous attempt had reported saliva as one of the effective non-invasive specimen not only for the detection of *Hp* infection but also for genotyping the strain infecting [122]. The 16S rRNA gene of *Hp* is a highly specific target for amplification, able to confirm *Hp* infection. Positive amplification of *Hp* specific DNA may be considered as a direct evidence of the presence of the pathogen. Non-invasive methods for the rapid

Table 7  
Principal clinical applications of MBAA  
(Multiplex Bead Array Assays)

AUTOIMMUNITY
CANCER MARKERS
CYTOKINE QUANTITATION
GENE EXPRESSION
GENOTYPING

Modified from F.M. Elshai et al. (2006) [37].

diagnosis of *Hp* in salivary secretion of patients with various gastric diseases using 16S rRNA PCR analysis, result to be very useful in pre-endoscopy screening thus showing comparable results with those obtained when biopsies are used (Table 6). Consequently saliva of infected persons serves as a reliable non-invasive alternative to detect the presence of *Hp* infection compared to currently diagnostic invasive tests. Tiwari et al. 2004 [121] in another research also reported salivary secretion as a sample suitable for detecting cag PAI (pathogenicity island) of infecting *Hp* correlating this result with the disease status of the patients.

#### 4.4. Multiplex bead array assays and pre-endoscopy screening

A number of new methodologies and assays have been defined during the last years in order to have reliable, rapid, precise and cost-effective results for the management of many diseases. Furthermore, these methods include the use of non-invasive specimens such as serum and plasma being then a useful tool for pre-endoscopy screening. Multiplex bead array assays (MBAA) and Luminex X-map constitute advancement in detecting contemporaneously biomarkers in plasma and serum. They result comparable to ELISA method and in addition have the advantage of revealing, independently and quantitatively, a large number of analytes using an automated 96-well plate format. These methods also permit the molecular study of genetic variables involved in virulence mechanisms of important bacterial strains. The clinical applications of MBAA are reported in Table 7.

The most important application of this test is the quantitative detection of cytokines. The measurement of soluble cytokines and other analytes plays a pivotal role in *Hp*-related infections. In fact, in *Hp* diseases, a number of pro-inflammatory cytokines such as tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin-1 $\beta$  (IL-1 $\beta$ ), IL-6, IL-8, IL-2, IL-24 etc., is on the basis of the host immune response and of the immunopathology of this microorganism. Practically multiplex assays rely upon

the determination of soluble analytes in serum or plasma through the utilization of specific beads for each ligand with subsequent detection of the captured ligand by a second “reporter” antibody. Positive reaction is detected by the fluorescence where ELISA method uses enzyme amplification of a colorimetric substrate.

Problems for MBAA technique can arise for the multiplex nature of the test that can lead to cross-reactions and to anomalies in quantifying some analytes. Interferences can also occur in anti-cytokine antibodies, which may cross-react with other cytokines and interfering substances. Kits have been optimized to eliminate or minimize any artefact from multiplexing. Nevertheless the problem of interferences can exist.

Test ELISA has been considered as a “gold-standard” for the determination of the analytes in plasma and serum but MBAA test is comparable to it [37]. Even if these two tests have been correlated in many studies [24, 25, 37, 64, 100, 104], it can be difficult to evaluate the results because various investigators use different methods of comparison between MBAA and ELISA. Most of published studies [31, 56, 108] have shown good correlation and reproducibility between these two methodologies for the majority of cytokines tested even if the degree of correlation has varied widely. MBAA test has proven to be easy to perform, reliable, time saving and cost-effective so that its use in the clinical practice and in the research area is suggested [37].

Among various MBAA tests that generally incorporate automatic software able to evaluate the cytokine levels in the samples (plasma and serum), significantly reducing the complexity of the assay and requiring less user interaction, Luminex X-MAP technology covers an important role. It uses digital signal processing capable of classifying polystyrene beads (microspheres) dyed with distinct proportion of red and near-infrared fluorophores. A spectral address for each bead population can be defined by these proportions [33, 65]. Different detection reaction can be carried out simultaneously on various bead populations. Some recent applications with Luminex-based fluorescent microspheres include proteins quantitation [113] and polymorphism genotyping [19, 66].

In conclusion we can say that it is possible to measure, with these new methodologies, the level of important cytokines involved in *Hp* immunopathology. These results can make us know, through non-invasive methods, the pattern of cytokines involved in the infection, which accounts for the disease status and the strain virulence.

## 5. Conclusion

The presence of virulence factors listed above is seen to strongly affect the outcome of *Hp* infection. The combination of different virulence genotypes is the most important factor that strongly affects the bacterial virulence making some strains more pathogenic than others.

*Hp* has been reported to be extremely variable and this heterogeneity is involved in the ability of *Hp* to cause different diseases [9,31,81]. The *Hp* immunopathology is a very complex phenomenon in which both host immune response and microorganism factors strongly affect the disease outcome [72,111].

The host immune response does not eliminate the pathogen but in contrast is able to worsen the clinical course of the infection. Host gene polymorphisms with their anti- or pro-inflammatory effects determine the level of gastric acid production leading, on the one hand, to hypochlorhydria, gastric ulcer, atrophy, intestinal metaplasia and carcinoma, on the other hand, to high levels of acid secretion, antrus gastritis and duodenal ulcer [105]. *Hp* infection stimulates the production of many cytokines such as IL-1, IL-2, IL-8, IL-24, TNF- $\alpha$  etc. and the type IV secretion apparatus (cag PAI), which accounts for chronic and intense inflammation and for the promotion of cellular proliferation [18, 127].

The non-invasive tests as diagnostic tool in *Hp* infections of patients with various gastrointestinal disorders are strongly important because they make the endoscopy unnecessary in different situations. The pre-endoscopy screening may be performed principally through serological markers (detection of different kinds of immunoglobulines) or through molecular markers (presence of CagA or Hsp60).

For CagA detection, serology has proved to be useful, being CagA protein a factor with good antigenic properties, easy and reliable to perform and prone to reveal the presence of cag pathogenicity island [16]. Hsp60 is also a good antigen so that its detection can be performed through the appearance of specific antibodies against it [72]. Strain typing could also be useful in pre-endoscopy screening: in fact the invasive gastroscopy could be avoided in young populations with non-ulcer dyspepsia and with non-alarming symptoms. We would suggest the rapid and easy detection of virulent strains as a mean to avoid both the invasive techniques and the consequences of a long-lasting untreated infection. The best approach for this is to use the new and advanced multiplex PCR methods, which

could contribute to gain insights at the genotypic variability exhibited by this pathogen. Multiplex PCR assay by which the presence of various markers can be detected in a single reaction constitutes an important tool [121–123].

Other new methods such as new multiplex assays (Multiplex Bead Array Assays-MBAA) and Luminex-X map technology constitute a considerable advancement for genotyping *Hp* thus using non-invasive samples as serum, plasma and salivary secretions [25,37].

Further problems that should be more deeply examined concern the possible link that may exist between strains with more combinations of virulence determinants and antibiotic resistance that is known to be a crucial drawback in the disease treatment.

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