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Evaluation of a new vaccine based on pDNA and recombinant protein against *Helicobacter pylori*

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Abstract

*Helicobacter pylor*i is a bacterium capable of surviving and infecting a healthy human stomach and it is estimated that infect more than a half of world population. Despite of being almost always asymptomatic, in some cases, the infection can evolve to several gastric disease as chronic gastritis, peptic ulcers, gastric cancer and MALT lymphoma. Vaccination against *H. pylori* is a promising option due to emerging problems of antibiotics treatment. It is thought that oral immunization could be a good approach for a more effective protection against infections by *H. pylori*, creating a first line of defense in mucosal surfaces. Chitosan nanoparticles are a suitable vehicle for oral vaccines delivery due to its immunogenic and mucoadhesive properties, protecting the DNA and allowing high levels of transfected cells. Thus, this work aims to evaluate a new pDNA- and recombinant protein-based vaccine, with multi epitopes of different *H. pylori* antigens. Following production and purification of plasmid DNA and recombinant proteins, vaccines were formulated for oral and intramuscular administration with the antigens encapsulated with chitosan nanoparticles.

The type of immune response induced and the effectiveness of protective immunity elicited were assessed by ELISA, through analysis of specific IgGs, mucosal SIgA and cytokines levels produced by immunized BALB/C mice. When give by the intramuscular route, the formulated pDNA and recombinant protein-based vaccines efficiently stimulated the production of specific IgG2a and IgG1, which is supported by cytokines levels, revealing a better and balanced systemic immune response than oral immunizations. Nevertheless as expected, oral immunizations with either pDNA vaccines or recombinant protein revealed high levels of SIgA, showing to be effective in gastric mucosal immunization for a more protective immune response, contrasting with intramuscular immunizations which did not induce SIgA.

The immunization results showed that both pDNA and recombinant proteins vaccines encapsulated with chitosan nanoparticles are good candidates for the development of a future vaccine to prophylactic and therapeutic use to improve the eradication of *H. pylori* infections.

Keywords: *Helicobacter pylori*; vaccine; DNA vaccine; immunization; recombinant antigens.

Resumo

Helicobacter pylori é uma bactéria capaz de sobreviver e infectar o estômago humano saudável e estima-se que mais de metade da população mundial esteja infectada. Apesar de, na sua maioria, estas infecções serem assintomáticas, em alguns casos poderão evoluir para várias doenças gástricas como gastrite crónica, úlceras pépticas, carcinoma gástrico e linfoma MALT. A vacinação contra infecções por *H. pylori* poderá ser uma alternativa promissora, devido aos problemas emergentes dos tratamentos à base de antibióticos. Pensa-se que a vacinação oral poderá ser uma boa abordagem para uma imunidade protetora mais eficaz contra infecções por *H. pylori*, criando uma primeira linha de defesa ao nível das mucosas. Nanoparticulas de quitosano têm mostrado ser um vínculo adequado para vacinação por via oral, devido às suas propriedades imunogénicas e mucoadesivas, conferindo proteção do DNA, e permitindo níveis elevados de células transfectadas. Este trabalho visa avaliar uma vacina baseada em DNA plasmídeo e proteína recombinante, constituída por múltiplos epítopos de diferentes antigénios de *H. pylori*.

Após produção e purificação do pDNA e da proteína recombinante, foram formuladas vacinas para administração oral e intramuscular com os antigénios encapsulados em nanoparticulas de quitosano. O tipo de resposta imunitária gerada e a eficácia da imunidade protetora foi avaliada por ELISA, através da análise dos níveis de IgGs especificos, de SIgA e de citoquinas produzidas, presentes nos soros de ratos BALB/c imunizados. Quando administradas pela via intramuscular, as vacinas formulada com pDNA e proteína recombinante estimularam eficientemente a produção tanto de IgG1 como de IgG2a, resposta suportada pelos níveis de citocinas produzidas, revelando uma resposta imunitária sistémica mais consistente e equilibrada do que imunizações por via oral. No entanto, como esperado, imunizações por via oral revelaram níveis elevados de SIgA, mostrando-se eficaz na imunização da mucosa gástrica conferindo uma maior imunidade protectora, em contraste com imunizações por via intramusculares que não induziram secreção de IgAs.

Os resultados obtidos das imunizações mostraram que ambas as vacinas formuladas com pDNA e proteína recombinante são boas candidatas para o desenvolvimento de uma vacina futura para uso profilático e terapêutico de modo a erradicar infecções por *H. pylori*.

Palavras-chave: *Helicobacter pylori*; vacina; vacina de DNA; imunização; antigénios recombinantes.

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Abbreviations and symbols

Abs	Absorbance
AGS	Human gastric adenocarcinoma epithelial cell line
APCs	Antigen presentation cells
BB	Brucella broth
BCA	Bicinchoninic acid protein assay
BSA	Bovine serum albumin
CagA	Cytotoxin-associated gene A
Cag-PAI	cag-pathogenicity island
CS/DS	Chitosan/deoxycholate
CS/TPP/Alg	Chitosan/Tripolyphosphate/alginate
CMV	Cytomegalovirus
CT	Cholera toxin
DC	Dendritic cells
DNA	Desoxirribonucleotic acid
dsDNA	double strain Desoxirribonucleotic acid
DU	Duodenal ulcer
E. coli LT	*E. coli* heat-labile toxin
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal bovine serum
GC	Gastric Cancer
GroEL	Chaperonin
GU	Gastric ulcers
Homb	outer membrane protein
HpaA	Neuraminil-lactose hemagglutinin connection
IFN-γ	Interferon-gamma
i	*intermediate*-region
i1	*intermediate*-region polymorphism 1
i2	*intermediate*-region polymorphism 2
IgA	Immunoglobuline A
IgG	Immunoglobuline G
IgG1	Immunoglobuline G1
IgG2a	Immunoglobuline G2a
IL	Interleucine
IMAC	Imobilized metal ion affinity chromatography
IPTG	Isopropyl b-D-1-thiogalactopyranoside
ISCOMs	Immunostimulatory complexes
LPS	Lipopolysaccharides
LT	heat-labil toxin
m	*mid*-region
m1	*mid*-region polymorphism 1
m2	*mid*-region polymorphism 2
MALT	Mucosa-associated lymphoid tissue
MHC	Major Histocompatibility Complex
MHC I	Major Histocompatibility Complex type I

MHC II	Major Histocompatibility Complex type II
mRNA	mesenger Ribonucleotic acid
MW	Molecular weight
NAP	Neutrophil-activating protein
NBT	Nitro-Blue Tetrazolium
PAMPs	pathogen-associated molecular patterns
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
pDNA	plasmid Desoxirribonucleic acid
poly[A]	polyadenylation sequence
PPI	proton pump inhibitor
PRR	pattern recognition receptors
PUD	peptide ulcer disease
RNA	Ribonucleotic acid
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
s	*s*-region
s1	*s*-region polymorphism 1
s2	*s*-region polymorphism 2
T4SS	Type IV secretion system
TcL	T citotoxic lymphocites
Th	T helper cells
TLRs	Toll-Like Receptors
TNF-α	Tumor necrosis factor-alpha
TPP/Alg	Tripolyphosphate/alginate
Ty21a	Typhoid vaccine
UBT	Urea breath test
UreA	Urease alpha subunit
UreB	Urease beta subunit
VacA	Vacuolization cytotoxin A

1. Introduction

1.1. Historical perspective

Since the early of 20th century several investigators have reported the presence of spiral microorganisms in the stomach of several animals (Kusters *et al.*, 2006; Dubois, 2007). In 1906, Walter Krienitz was the first to report the observation of spiral bacteria in the human stomach, suggesting that spiral-shaped bacteria were causing gastric diseases, but it was not possible to make a consistent association between the presence of this bacteria and any specific disease. In 1983, *Helicobacter pylori* were successful isolated and cultured for the first time by Warren and Marshall. Self-ingestion experiments by Marshall demonstrated that these spiral bacteria can colonize the healthy human stomach and inducing inflammatory response in the gastric mucosa, developing a transient gastritis, as proved by gastric biopsy samples taken from B.J. Marshall (Marshall *et al*., 1985). This discovery was worth the 2005 Nobel Prize in physiology or medicine to Robin Warren and Barry Marshall for their "Discovery of the bacterium *Helicobacter pylori* and its role in gastritis and peptic ulcer disease." (Kusters *et al*., 2006). This knowledge showed that gastric colonization with *H. pylori* can lead a variety of gastrointestinal disorders, such as chronic gastritis, duodenal ulcers (DU) or gastric ulcers (GU), gastric mucosa-associated lymphoid tissue (MALT) lymphoma, and gastric cancer (GC). This discovery prompted efforts by other scientists in the study of the physiology, genetics, transmission and epidemiology of this bacterium (Kusters *et al*., 2006). The result was an increasing number of scientific manuscripts up to about 30 thousand manuscripts published in the past 20 years.

1.2. Helicobacter pylori

H. pylori is a gram-negative bacterium, spiral-shaped, microaerophilic, with 3 to 7 unipolar flagels, that allow the bacteria to move along the mucus layer to gastric epithelium and colonize the antral region (the most distal region) of the human stomach after oral ingestion (Amieva and El-Omar, 2008).

Most *H. pylori* organisms are free living in the mucus layer, but some organisms attach to the apical surface of gastric epithelial cells and after attachment always elicit an acute inflammatory response (gastritis) in the stomach mucosa (Velin and Michetti, 2010).

The stomach has a too acid and hostile environment unfavorable to the presence of most microorganisms, due to large amounts of gastric juice composed by digestive enzymes and hydrochloric acid (Amieva and El-Omar, 2008; Zhou *et al.*, 2009). *H. pylori* has a unique mechanism that allows it to survive a transient exposure to this acid environment of the stomach for a few minutes, by secreting a large amount of the enzyme urease that hydrolyses urea into ammonia and carbon dioxide, partially neutralizing the acidic pH of the stomach lumen (Kabir, 2007). Indeed, all

known gastric *Helicobacter* species are urease positive but also highly mobile through flagella. This motility permits rapid movement toward the more neutral pH of the gastric mucosa. Both factors are prerequisites for colonization of the human stomach and will allow trigger an inflammatory response in epithelial cells (Figure 1.2.1) (Kusters *et al*., 2006). The enzyme urease along with lipases, proteases and others virulence factors produced by the bacterium will disrupt the defenses of the mucus layer and will lead to the success of infection.

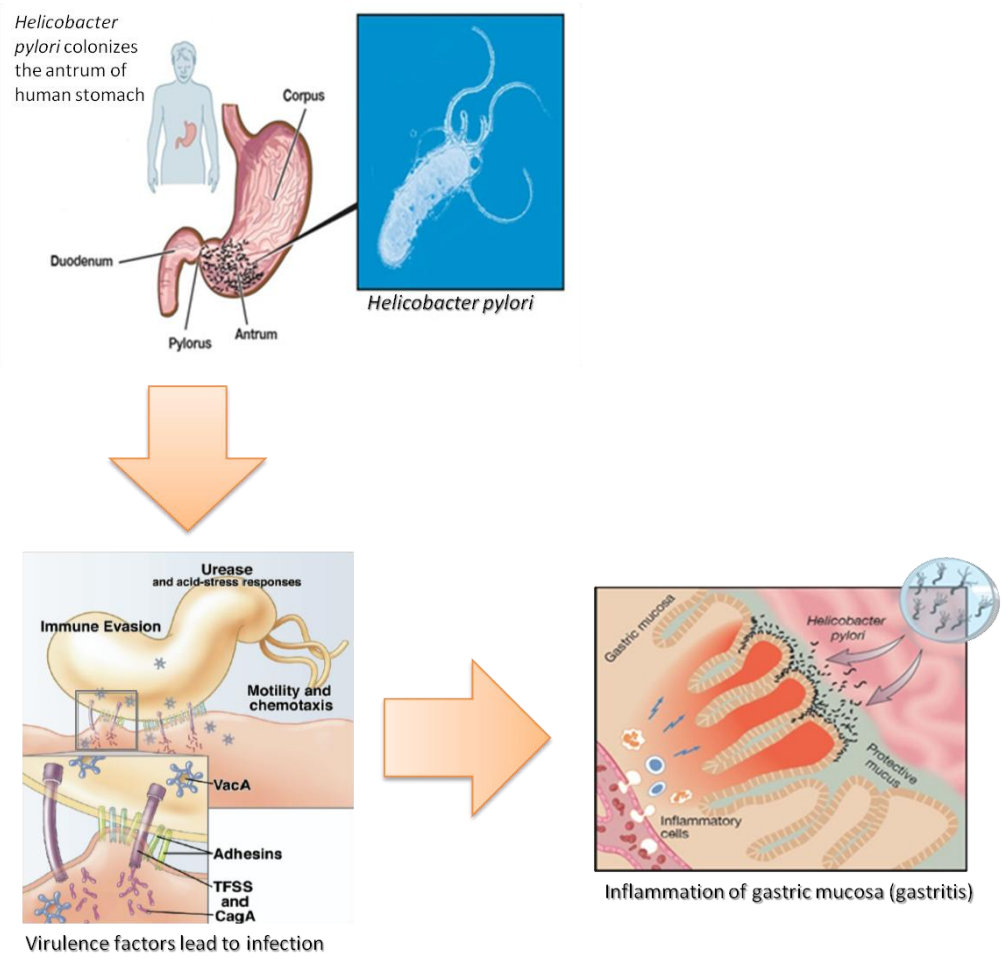

Figure 1.2.1 – *H. pylori* infection: Colonization and infection of the most distal part of the stomach, the antrum. Adhesion, urease and other virulence factors are important to *H. pylori* surviving in a transient exposure to low pH environments: to avoid the acid lumen of the stomach for long exposures, *H. pylori* swims toward the mucosal cell surface using their polar flagella and chemotaxis mechanisms. As near the epithelium, *H. pylori* actively adheres to the cell surface using a variety of specific adhesions that recognize glycoproteins on the host cell. Attachment to the host cells allows *H. pylori* to deliver toxins like VacA and CagA that deregulate some of host cell functions. This mechanisms lead to a consequent inflammatory response causing gastritis. Adapted from "Press Release: The 2005 Nobel Prize in Physiology or Medicine" and Amieva and El-Omar (2008).

1.2.1. Virulence factors

H. pylori has a high genetic variability among strains, due to a high rate of mutation and recombination, horizontal transfer of DNA and the lack of mismatch repair system of DNA. This leads to a high diversity of genes and a high number of alleles, essential for adaptation and colonization of the hostile environment of the stomach (Suerbaum and Michetti, 2002). Bacterial virulence factors play an important role, since the virulent strains are more aggressive and increase the risk of developing severe clinical manifestations. These virulence factors also play structural and critical roles and functions such as adhesion, invasion, colonization and virulence. Some antigens have been extensively studied and tested, known to be involved in the pathogenesis of the infection and shown to be safe and promising, due to its pathogenic importance, immunogenicity and representativeness in a large number of strains. These antigens include urease, the Neuraminil-lactose hemagglutinin connection (HpaA), the vacuolating cytotoxin (VacA), the cytotoxin-associated antigen (CagA), the neutrophil-activating protein (NAP) and others (Del Giudice *et al.*, 2009; Velin and Michetti, 2010).

Urease is an enzyme that catalyzes urea into ammonia (NH_3) and carbonic acid (H_2CO_3), which in turn is converted into CO_2 and H_2O, and is crucial for the survival of the *H. pylori* in the human stomach. This enzyme is present in all strains and because of that its activity is used for detected *H. pylori* infections as in urea breath test (UBT) (discussed in section 1.7.) and gastric biopsy samples (urease test). It is composed by 12 urease alpha (UreA) and 12 urease beta (UreB) subunits with molecular mass of approximately 27 kDa and 60 kDa each, respectively, being the UreB subunit most commonly used for immunization (Begue and Sadowska-Krowicka, 2010; Calvet *et al.*, 2010).

The virulence factor HpaA or adhesion A is a protein that works as an adhesion present in flagellar filament of flagella and on the bacterial surface, essential to colonization and infection. Studies with *hpaA* mutants showed this protein is necessary for motility and for establishment of a stable colonization. HpaA is highly immunogenic and leads to induction, maturation and antigen presentation by dendritic cells (Lundstrom *et al.*, 2001; Voland *et al.*, 2003; Carlsohn *et al.*, 2006).

HomB is a *Helicobacter* outer membrane protein (OMP) that permits the attachment of the bacteria to the gastric epithelium to avoid the bacteria elimination by peristaltic movements. Bacterial adherence to ephitelial cells leads to an induce of inflammatory response through activation of IL-8, leading to PUD. This makes this protein a co-marker for strains associated with peptic ulcers, as well as with the presence of other *H. pylori* disease-related genes like *cagA*, *babA* and *vacA.* (Oleastro *et al.*, 2008).

Vacuolization cytotoxin VacA, is an exotoxin that is involved in various mechanisms of virulence such as induction of cytoplasm vacuolization in epithelial cells that can cause disruption of the epithelial barrier due to formation of pores, membrane channels and cellular damage, targeted to the mitochondrial membrane where it causes release of cytochrome c and induces apoptosis (showed at

Figure 1.2.1.1) and phagocytosis inhibition by macrophages (Suerbaum and Michetti, 2002; Wilson and Crabtree, 2007).

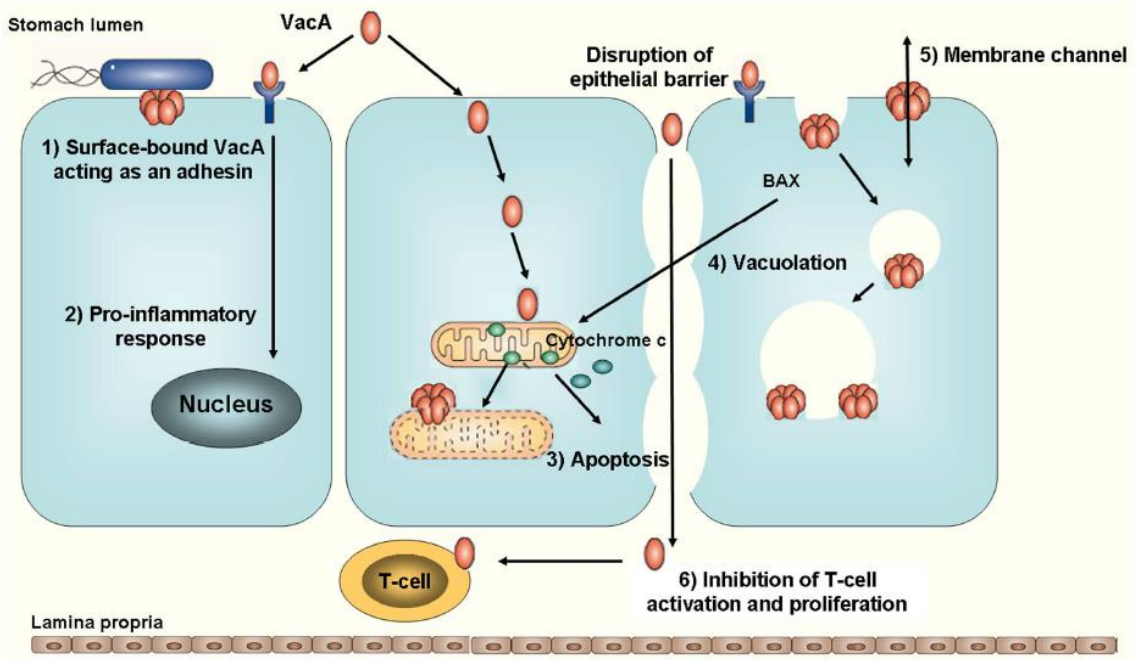

Figure 1.2.1.1 - Mechanisms of action of VacA on host gastric epithelial cells and its effect on immune cells. Surface bound VacA may function as an adhesion (1), while secreted VacA may bind to a host receptor and induce pro-inflammatory response (2) or induce apoptosis mediated by cytochrome c (in green) release (3), or induce vacuolation by alterations in endocytic vesicles (4), or form a membrane channel leading in exit of nutrients and ions to the extracellular space (5), or achieve the lamina propria where it binds to immune cells causing immune system modulation (6). Adapted from Cover and Blanke (2005) and Jones *et al.* (2010).

The *VacA* gene is present in all known *H. pylori* strains but the activity of the protein depends on this polymorphism of two regions of the gene, *s-region (s)* and *mid-region (m).* Each *s* and *m* regions present two polymorphisms, *s1* and *s2*, and *m1* and *m2*. Strains having *s1/m1 vacA* gene have the highest vacuolization activity, when *s1/m2* strains present VacA activity but has a restricted number of affected cells, and activity of the protein is absent in presence of *s2/m2* strains. The *S2/m1* genotype is very rare (Letley and Atherton, 2000; McClain *et al.*, 2001). VacA vacuolization activity is codified by *s-region* while the toxin binding to epithelial cells is related to *m-region*. A third variable region, located between the *s-* and *m-region*, was identified as intermediate (*i*) *region*, and *i1* is associated to the vacuolization form and *i2* is associated to the non-vacuolization form. Was observed that while all *s1/m1* strains were of type *i1* and *s2/m2* were type *i2*, *s1/m2* could be both *i1* and *i2*, varying in their toxicity (Rhead *et al*., 2007).

VacA gene was identified as mostly common and encodes a highly antigenic protein due to induce the production of pro-inflammatory cytokines, such as TNF-α, IL-6 and IL-8 in immune cells, and showed to have an immunosuppressive effect on them. It will inhibit T cell activation interfering with calcium-signaling events inside the cell and prevented activation of the calcium-dependent phosphatase calcineurin, inhibit the proliferation of T and B lymphocytes and will interfere with phagocytosis and antigen presentation (Backert *et al*., 2010).

The *cag*-pathogenicity island (*cag*-PAI), a 40kb DNA segment of the *H. pylori* genome, encodes both CagA protein and type IV secretion system (T4SS). This system translocates the CagA protein into the epithelial cells through a pilus responsible for the delivery of bacterial proteins such as CagA, inside the host cells, during infection (Covacci *et al.*, 1993; Censini *et al.*, 1996; Vitoriano *et al.*, 2011). The entry of CagA, will lead to increased inflammation and to activation of adaptative immunity, disruption of tight junctions, cytoskeleton rearrangements, oxidative stress that leads to apoptosis and DNA damage, and induce secretion of IL-8 cytokine activating Dendritic Cells and IL-12 cytokine, that will stimulate Th1 cells too (Vitoriano *et al*., 2011). CagA protein is one of the most important and well-characterized virulence factors of *H. pylori*. For that reason it is essential for stimulating the immunity to protect the host against *H. pylori* infections.

When injected in the host cell, CagA is phosphorylated and interacts with several epithelial cells effectors proteins of the host. Additional bacterial factors are injected through the T4SS, such as peptidoglycan, that also interact with host proteins. These interactions with host protein effectors by *cag*-PAI genes products, results in the deregulation of signalling pathways associated with host cell motility and elongation, proliferation, inflammatory response, disruption of cell junctions and phagocytosis inhibition. It was demonstrated that *cag*-PAI positive strains induces the development of gastritis and GU, when compared to mutants for *cag*-PAI-encoding proteins (Tegtmeyer *et al*., 2011).

1.3. Epidemiology of *Helicobacter pylori* infection

It is estimated that more than a half of global population is infected with *H. pylori* and its prevalence differs according to geographic location. Within the same country, the prevalence of *H. pylori* is influenced by patients' socioeconomic status, age, gender and genetic predisposition. Among countries, the prevalence is higher in developing countries were achieves 80-90%, when compared to developed countries with 10-60% of prevalence (Figure 1.3.1) (Del Giudice *et al.*, 2009; Khalifa *et al.*, 2010). The prevalence of infection is especially higher in the rural developing areas in contrast to urban developed ones because in rural environments people are probably exposed to an increased number of infectious sources such as contaminated food and water, or intensive contact between infants and non-parental caretakers and poor sanitation and hygiene cares (Vale and Vitor, 2010).

The route of transmission of *H. pylori* is not completely understood but is thought that new infections occur as a consequence of direct human-to-human transmission or by environmental contamination. There are evidences that infection by *H. pylori* can occur by gastro-oral, oral-oral and fecal-oral routes, but these data are inconclusive (Vale and Vitor, 2010).

Infection occurs typically in early childhood, frequently transmitted within families through mother-to-child transmission, but the role of these vehicles in *H. pylori* transmission remains to be clarified (Kabir, 2007). Unless treated, colonization usually persists during lifelong (Kusters *et al*., 2006).

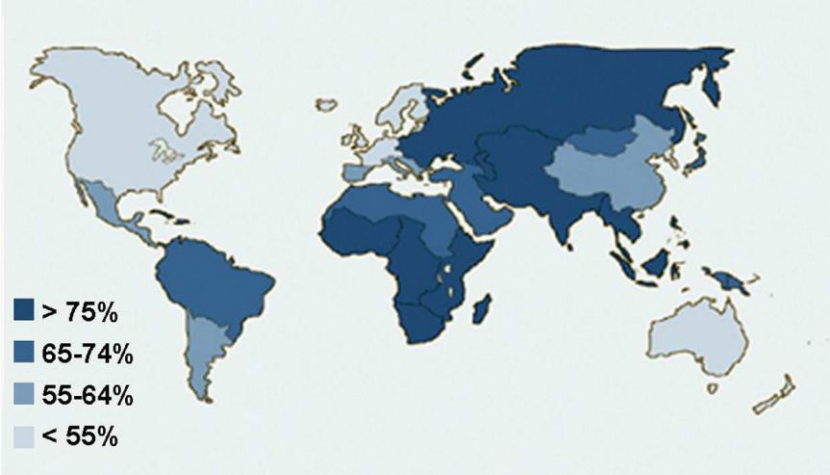

Figure 1.3.1 - Prevalence of *H. pylori* infection around the world in asymptomatic adults (Crew and Neugut, 2006).

Most infections remain asymptomatic, however, in some cases and if not treated the infected may develop for a chronic gastritis, gastro-duodenal ulcer, gastric adenocarcinoma or mucosa-associated lymphoid tissue lymphoma, depend on the inherent properties of the bacterial strain, virulence factors, genetic predisposition and the immunological response of the host. Gastric cancer is the fourth most common cancer and the second most common cause of cancer death in the world (Jemal *et al*., 2011). Since 1994 the World Health Organization classifies the bacterium as a type I carcinogen (Kabir, 2007; Lima and Rabenhorst, 2009).

1.4. *Helicobacter pylori* Infection and Immune system

The innate immune system represents the first line of defence against pathogens and provides an unspecific response. After colonization by *H. pylori*, the pattern recognition receptors (PRR), like Toll-Like Receptors (TLRs), are the first involved in the recognition of conserved microbial constituents called pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharides

(LPS), peptidoglycan (PG), flagellins, RNA and DNA molecules. TLRs are expressed by epithelial cells, including gastric epithelial cells and by specialized antigen presenting cells (APCs) such as macrophages and dendritic cells (DC). Activation of TLRs triggers several signaling pathways, including activation of cytokines production, stimulate the migration of neutrophils, and induce the activation of macrophages and maturation of DCs in the gastric mucosa, leading to an inflammatory response (Kindt *et al*., 2006).

APCs will trigger and stimulate the adaptive immune response through the ability to capture, process and present antigens to other cells, presenting the peptides fragments of antigens connected to surface molecules of Major Histocompatibility Complex (MHC) class I and II. Intracellular antigens (like viral proteins) are presenting by molecules of MHC I which activate T CD8+ lymphocytes (CTLs) and extracellular antigens are endocyted and presenting to molecules of MHC II which activate T helper (Th) CD4+ lymphocytes (Kindt *et al*., 2006).

Macrophages are one of those APCs and are also involved in the amplification of the inflammatory immune response producing more cytokines, such as IL-1, TNF-α and IL-6, and along with DCs are activators of adaptive immunity producing cytokines such as IL-12, that stimulate differentiation of Th lymphocytes (Kawai and Akira, 2011). Other APCs involved in activation of adaptive immunity are DCs. These cells, after capture and process antigens, which are transported linked to molecules of MHC class I and II, migrate to secondary lymphoid organs, leading to activation of T lymphocytes and differentiation of B lymphocytes thereby initiating a specific immune response against the antigens of the pathogen (Wilson and Crabtree, 2007).

The adaptive immune system is the second line of defence and is able to provide highly specific responses against pathogens. After presentation of antigens to the specific surface receptors linked to MHC class II, both humoral and cellular immune responses in the infected host are triggered.

T lymphocytes are divided into two lineages: T helper (Th) lymphocytes that express CD4 +, stimulated by MHC II, and cytotoxic T lymphocytes (CTL) expressing CD8+ to the surface, stimulated by MHC I (Kindt *et al*., 2006).

The cytokines produced by APCs such as IL-12, will stimulate activation and recruitment of lymphocytes and the development of T helper (Th) response. Two types of T CD4+ lymphocytes will be stimulated: Th1 lymphocytes and Th2 lymphocytes. Th1 cells are induced in the presence of intracellular pathogens and mediate the cellular immune response through the production of a set of cytokines that include interferon (IFN)-γ, tumor necrosis factor (TNF)-α and interleukin (IL)-2. These cytokines will activate the CTLs and enhance phagocytosis. Thus CTLs will eliminate the infected cells by a mechanism mediated by antibodies. In the other hand, Th2 are stimulated in the presence of extracellular pathogens, mediating the humoral immune response, characterized by production of cytokines such as IL-4, IL-5, IL-6, IL-10 and IL-13 (Wilson and Crabtree, 2007).

After activation, Th cells will stimulate B lymphocytes to produce immunoglobulins. Associated to Th1 response are induced IgG2a (and IgA) and to Th2 response are expressed IgG1.

The CD_4^+ T cells activated by antigens linked to MHC II molecules are crucial to protection against *H. pylori* infections. It is known that *H. pylori* induces a polarized Th1 response and being a non invasive microorganism was expected that induce a mediate Th2 response, but this is not observed (Suerbaum and Michetti, 2002). Studies have shown that *H. pylori*-infected patients have an increased of IFN-γ producing T-cells, consistent with Th1 cytokine response and mucosal T-cells produce high levels of Th1 cytokines such as IFN-γ and IL-2, unlike Th2 cytokines such as IL-4 and IL-5 that has not been detected in the gastric mucosa of infected individuals (Peek *et al.*, 2010; Velin and Michetti, 2010). The persistent *H. pylori* infections typically result in Th1-polarized responses, whereas successful *H. pylori* immunization could result in Th1/Th2 balanced response (Algood and Cover, 2006). It could then be concluded that the immune system is unable to clear off *H. pylori* infection. Studies have shown that *H.pylori* is capable of compromise the host immune response through virulence factors that interfere with T-cell proliferation (Velin and Michetti, 2010).

Thus, the immune response production in presence of *H. pylori* is inefficient to eliminate the infection and will contribute to the tissue damage like apoptosis and DNA damage, duo to the inefficient immune response by Th2 cells which leads to continuous neutrophil and macrophag activation, consequent inflammation persistence and constant production of cytokines such as IFN-γ (Wilson and Crabtree, 2007).

1.4.1. Mucosal immunity

M cells are the major effector cells in the mucosal lymphoid tissues, taking up the antigens or microorganisms by phago-, endo-, or pinocytosis and delivery to the APCs. The mucosal lymphoid tissues can be divided into effectors sites and inductor sites. Antigens are presented the inductor sites leading to an activation of immune system cells. The effector sites are the locals where the antibodies and the immune cells act. The main sites of induction of mucosal immune system are the MALT components, which include the gut associated lymphoid tissue (GALT), the bronchus-associated lymphoid tissue (BALT), the nasopharynx associated lymphoid tissue (NALT) and the genital mucosa-associated lymphoid tissue (GENALT). At the MALT, some PPRs interact with PAMPs of the microorganisms for the translocation the bacteria across the epithelial barrer (Azizi *et al.*, 2010).

The main effector cells at mucosal surfaces are constituted by $CD8^+$ and $CD4^+$ T. The APCs like macrophages, DCs and B lymphocytes process and present antigens to activate $CD4^+$ T cells and $CD8^+$ T cells. Therefore, the proximity of APCs to the T and B lymphocytes at the induction sites of mucosa are essential for the success of an efficient immune response (Wilson-Welder *et al*., 2009).

1.5. *Helicobacter pylori* associated diseases

H. pylori is therefore considered the main etiologic factor in chronic gastritis, PUD (peptide ulcer disease) and GC. The infection, despite being asymptomatic in most individuals, can evolve and cause different gastric disorders, such as those shown in Figure 1.5.1. In most of symptomatic infected individuals the colonization by *H. pylori* can cause acute gastritis which if not treated will progress to a chronic gastritis that will affect both antrum and corpus. In 10-15% of infected patients, chronic gastritis occurs only in gastric antrum and these cases will result in DU outcomes, characterized by excessive acid and gastrin secretion in antral zone of the stomach. Another gastric phenotype is characterized by gastritis in corpus zone of the stomach, which occurs in 2-5% of the infected individuals that leads to GU with hypochlorhydria and acid reduction and may evolve into GC (Amieva and El-Omar, 2008; Schubert and Peura, 2008; Konturek *et al.*, 2009).

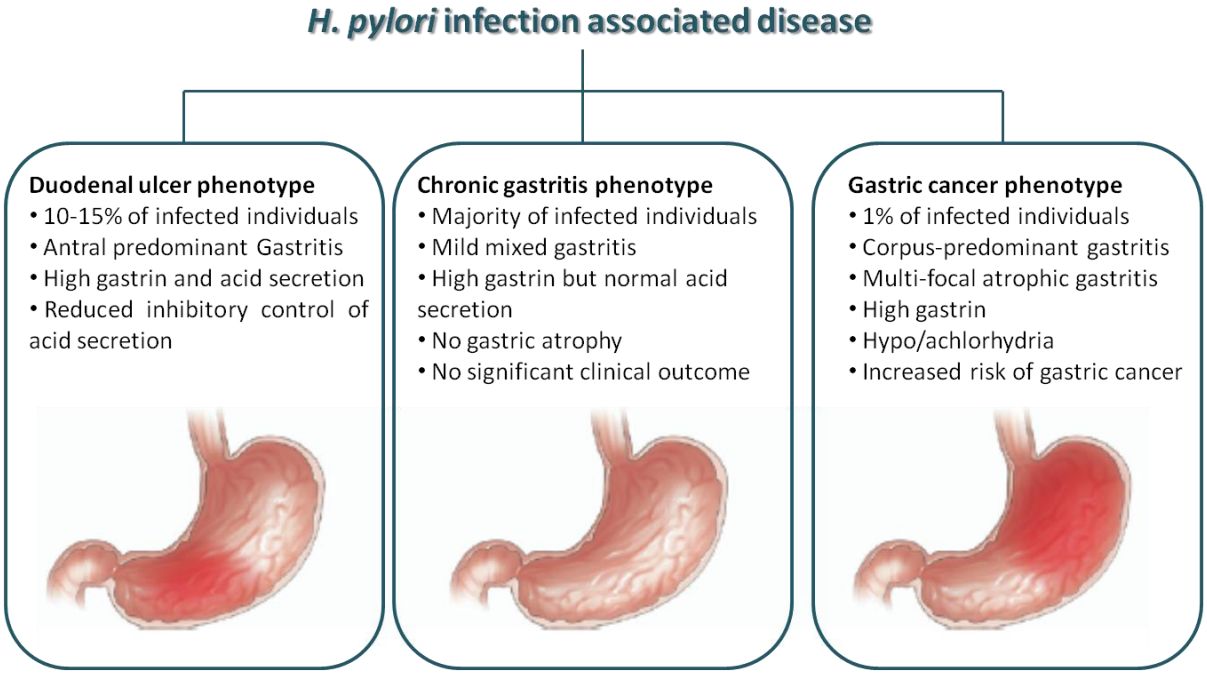

Figure 1.5.1 – Chronic *H. pylori* infection: Chronic *H. pylori* infection can lead to different gastric diseases such as chronic gastritis, duodenal ulcer and gastric cancer. Adapted from Amieva and El-Omar (2008).

1.6. Diagnosis and Treatment

Infection with *H. pylori* can be diagnosed by both non-invasive and invasive methods. Invasive tests are based on endoscopic biopsy of the gastric mucosa, collecting samples for diagnosis of gastritis, ulcerogenic lesions, gastric atrophy, intestinal metaplasia and GC lesions. To confirm the

presence of *H. pylori* tests are performed such as urease test, bacterial culture and histological analysis of the biopsy samples. In addition, *H. pylori* can also be detected using molecular methods, such as PCR and real-time PCR applied to specific *H. pylori* genes (Suerbaum and Michetti, 2002).

Non-invasive tests include the urea breath test (UBT), serologic tests, search of stool antigens and urine tests. The commonly used is the urea breath test (UBT) and is based on the abundant *H. pylori*-derived urease activity in the stomach (detection of $^{13}CO_2$ and $^{14}CO_2$). For routine diagnostic the most used test is UBT with an accuracy of >90%, while for large number of samples, such as epidemiological studies, faecal antigen test is most appropriate, although less accurate (Suerbaum and Michetti, 2002).

Current triple therapies for *H. pylori* eradication include two antibiotics in combination with a proton pump inhibitor (PPI). Commonly used antibiotics for *H. pylori* eradication therapy include macrolides (usually clarithromycin), tetracycline, amoxicillin and imidazoles (predominantly metronidazole) as the first-line therapies. Antimicrobial resistance is the primary cause of treatment failure, but antibiotic treatment has others emerging problems such as antibiotic side effects, re-infections, and recurrence and has high-costs (Zhou *et al*., 2009).

For these reasons is essential to develop new therapies and other therapeutics approaches such as an effective preventive and therapeutic vaccine against *H. pylori,* that stimulates an effective humoral response as well as cellular response, capable of prevent and effectively eliminate the infection.

1.7. Vaccines

Thought to be therapeutic vaccination the solution for clearance and eradicate infection by *H. pylori,* efforts are being made in toward to develop a vaccine that has a prophylactic use to prevent infection and a therapeutic use to eradicate an ongoing infection (Kabir, 2007). Vaccination presents a major benefit/cost ration and has major advantages such as financial, while avoiding re-infections and decrease antimicrobial resistances.

Some prophylactic and therapeutic vaccine strategies using a wide variety of *H. pylori* antigens have been reported (Del Giudice *et al.*, 2009) and will be referred to in the following sections.

1.7.1. Types of vaccines

Several clinical studies have been made in order to develop an efficient vaccine capable of induce a protective immune response for eradicate ongoing infections and in order to test the immunogenicity, safety and efficacy of potential *H. pylori* vaccines.

Vaccines with attenuate *H. pylori* are extendedly study, due to have the advantage to having all the *H. pylori* antigens, but these vaccines can lead to safety problems due to mutations that lead a reversion on virulence or to an insufficient attenuation, which may ultimately induce the infection (1.7.1.1.2). Alternatively there are inactivated whole killed vaccines that such as attenuated vaccines have the advantage of containing all bacteria antigens, but without the danger of virulence reverting. Studies with *H. pylori* inactivated whole-cells combined with LT adjuvant showed a significant rise in specific IgA antibodies, but did not show success in eradicating the pre-existing infection (Wilson and Crabtree, 2007). Despite inactivated whole-cell vaccines have the advantage of elicit immune response against all antigens known and unknown of the bacteria, this can be also a disadvantage since it contains other dangerous components of the bacterium that could induce a cross-reacted immunity responses (Kabir, 2007; Wilson and Crabtree, 2007; Ingolotti *et al.*, 2010).

The protection induced by several *H. pylori* antigens was described, including recombinant antigens and strain-specific virulence factors of the bacterium. In addition to urease, protection with CagA, VacA, NAP and GroEL has been demonstrated (Wilson and Crabtree, 2007), but it has been concluded that given the genomic variability and variation of *H. pylori* antigens, an optimal vaccine must be contain multiple antigens. As an example, recombinant *H. pylori* urease with *E. coli* LT (*E. coli* heat-labile toxin) orally administrated to *H. pylori* infected volunteers, demonstrated the immunogenicity of the vaccine through the observation of a reduction in bacterial load in vaccinated individuals compared with controls, but the eradication of the infection was not accomplished and the degree of gastritis remained unchanged (Kabir, 2007).

Other approach for immunization against *H. pylori* has been delivering antigens in live vectors such as attenuated *Salmonella typhimurium* and modified poliovirus. Salmonella-based recombinant vaccine expressing urease was orally administrated but most of the volunteers presented specific IgG to the Salmonella vector and none of them showed any detectable mucosal or humoral immune response against urease. Vaccines vectors using poliovirus genomes (replicons) also have been described as a protective way to immunized mice: capsid genes were replaced with urease B subunit and administered by systemic route and showed both prophylactic and therapeutic efficacy against *H. pylori*, clearing an established infection in mice (Wilson and Crabtree, 2007).

Although these types of vaccines have proven their efficacy in inducing some type of immune response, they do not retain the security requisites needed and the strong humoral immune response induced alone do not provide protective immunity against subsequent contacts with the pathogen (Shedlock and Weiner, 2000).

1.7.1.1. DNA based vaccines

On other hand, there are the DNA vaccines, whereby naked plasmid DNA, introduced into the host, is taken up by eukaryotic host cells and the encoded antigen is expressed (Figure 1.7.1.1.1). The gene sequence of the antigens of interest is inserted in a bacterial plasmid backbone. The transcriptional unit containing the nucleotide sequence should be under the control of an eukaryotic housekeeping gene promoter and should be followed by a polyadenylation sequence (poly[A]) to ensure the stability of the mRNA molecule and its translation. One of the most commonly used is the cytomegalovirus (CMV) promoter. The plasmid backbone should also contain an origin of replication for amplification the plasmid in bacteria and an antibiotic resistance gene to enable the selective growth of transformed bacteria. Thereby, there is a commercially available plasmid approved by the Food and Drug Administration, "pVAX1" (Invitrogen) specially designed for DNA vaccines (Ingolotti *et al.*, 2010).

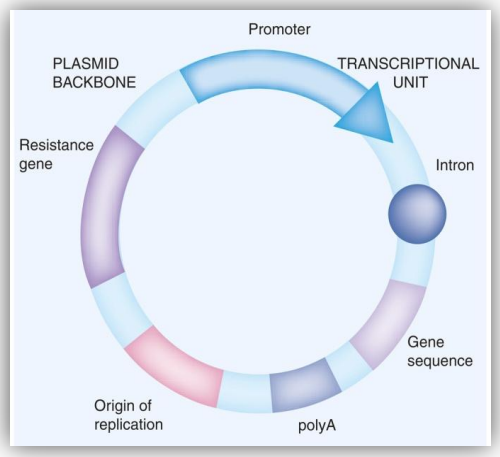

Figure 1.7.1.1.1 – Plasmid DNA backbone scheme for DNA vaccines with gene sequence of interest, polyA sequence, promoter and origin of replication, and antibiotic resistance gene for selection of competent bacteria (Ingolotti *et al.*, 2010).

The advantage of DNA vaccines, compared to protein-based vaccines (figure 1.7.1.1.2), is the possibility to triggering both humoral and cellular immune response, so that the infected host can quickly and effectively respond to the infection and efficiently eliminate the pathogen. DNA vaccines stimulate the antigen presentation both by MHC I to the $CD8^+$ T cells (TCL) and by MHC II to the $CD4^+$ T (Th) (Wilson and Crabtree, 2007).

In a simplistic view of the DNA vaccine mechanism, the DNA plasmid upon transfection is translocated to the cell nucleus and uses the host cell machinery to transcribe and translate the genes. The resulting proteins are recognized by the host cells as non-self and undergo cleavage resulting in fragments that will be presented on the cell surface by MHC I. Moreover, the antigenic protein secreted by the transfected host cells can be taken by APCs, which present it on the cell surface MHC II. Also, apoptotic transfected cells can be engulfed by APCs which will present the antigen through MHC I and MHC II. This will stimulate the both naïve $CD8^+$ T cells and $CD4^+$ Th cells. Activated

$CD4^+$ Th cells and a cascade of cytokines will activate B cells which induce antibody production. The $CD8^+$ T cells will lyse transfected cells which presenting antigens through MHC I causing the release of more antigens and $CD4^+$ Th cells activated by DCs, which will repeat this cycle of activation. So both T and B cells migrate to the site of immunization and are re-stimulated (Kutzler and Weiner, 2008; Ingolotti *et al.*, 2010).

To improve the efficacy of DNA vaccines could be encoded only some epitopes, instead the whole antigen in order to avoid potential dangerous components of the bacterium such as those sharing homologies with self-antigens and thus avoid cross-reactive immune responses against host epitopes. These vaccines have the advantage of including only the potential epitopes selected, considering only the PAMP highly conserved among strains. (Del Giudice *et al.*, 2009; Zhou *et al.*, 2009; Moss *et al.*, 2011).

Studies suggest that a DNA-prime and peptide-boost vaccine designed with multiple *H. pylori* epitopes has a therapeutic efficacy in mice previously infected with *H. pylori*. Has showed that the multi-epitope vaccine induced a broad immune response that lead to a significant reduction of *H. pylori* colonization. So these studies suggest that further development of an epitope-based mucosal vaccine against H. pylori can potentially lead to a novel approach to prevent *H. pylori* associated diseases (Moss *et al.*, 2011).

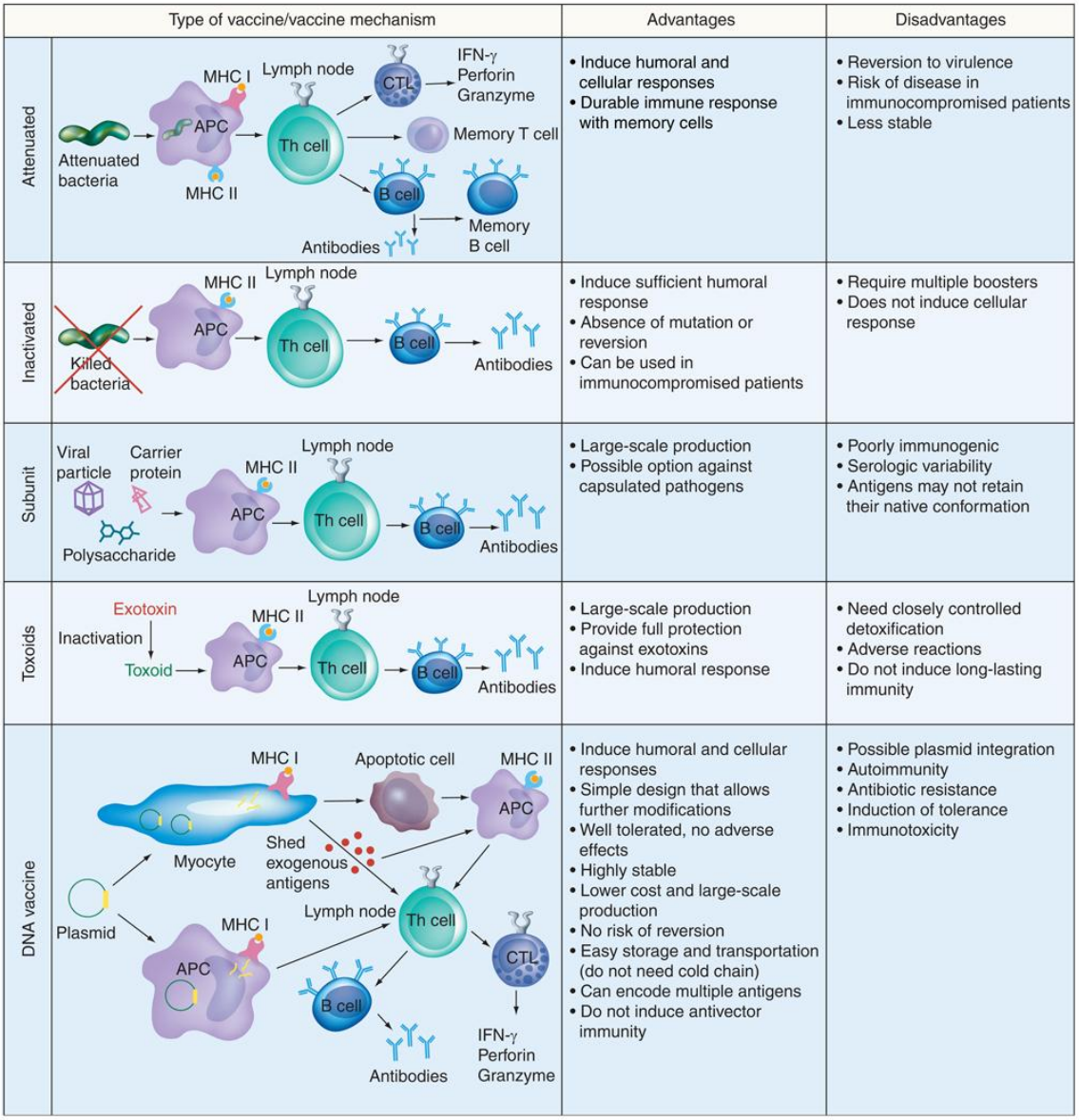

Figure 1.7.1.1.2 – Resume of the Mechanisms of action of different types of vaccination: attenuated vaccines, inactivated vaccines, subunits vaccines, toxoids vaccines and DNA vaccines; Advantages and disadvantages (Ingolotti *et al*., 2010).

A variety of factors could affect the frequency of integration of plasmid DNA vaccines into host cells, including DNA sequences within the plasmid, the expressed gene product (antigen), the formulation, delivery system and route of administration. A proper route of administration and a suitable delivery system could provide a better and highly immune response against infections *H. pylori*. So, in this and others studies in our research group, effords were made for chose a proper route of administration and develop an appropriated delivery system for enhance the potential of immune protection induced by our vaccines.

1.7.2. Routes of vaccination

Most vaccines are administered through parenteral administration, such as intramuscular or subcutaneous. However, mucosal vaccination presents several advantages since most pathogens infect their host through mucosal membranes, as the respiratory tract, gastrointestinal, vaginal and urinary tract, suggesting mucosal vaccination is essential to create a first line of immunization at mucosal level. Thus, mucosal vaccines have the benefit of not only stimulate the mucosal immunity as well as trigger systemic immune response, resulting in a stronger protective immunity. Other advantages include non-invasiveness, which improve patient compliance, avoiding the use of needles, ease self-administration, low costs and ease production and application, and low risks of needle infections (Vajdy *et al.*, 2004; Sijun and Yong, 2009; Azizi *et al.*, 2010; Chadwick *et al.*, 2010; Cadete *et al.*, 2012).

Recent studies indicate that oral route is the ideal mucosal route for induction predominantly secretory IgA antibody in gastric mucosa to confer effective for protection against *H. pylori* infections, preventing direct contact of the bacteria with epithelial cells (Strugnell and Wijburg, 2010; Velin and Michetti, 2010).

Some studies have been made but with reduced success (Moss *et al.*, 2011), since mucosal inoculation require a successful delivery system for the vaccines efficiency, which constitutes a challenge because mucosal membranes are constituted mainly of epithelial tissue highly vulnerable with viscous mucus secretion that acts as a barrier against pathogens and against vaccine antigens. These disadvantages may decrease the lifetime of antigens and difficult the determination the amount that effectively penetrates the mucosa (Neutra and Kozlowski, 2006).

Thus, these vaccines have to be capable of effectively penetrate the mucus epithelial cells without any damage, penetrate through the epithelium and interact with APCs or directly with MALT. For the success of mucosal DNA-based vaccines it is essential to use suitable vaccine formulations working both as adjuvants and delivery systems (Del Giudice *et al*., 2009).

It is known that vaccination through mucosal membranes requires potent adjuvants or delivery systems to enhance the immunogenicity of the antigens as well as to protect the antigens form degradation and to prolong the contact with the epithelium.

1.7.3. Adjuvants and Delivery systems

Adjuvants are molecules, compounds or macromulecules that do not have any specific effect of antigen but can be co-administered with pathogen-derived antigens improving the efficacy of vaccines with less reactive antigens and enhance a longer and stronger specific immune response, without causing immune reaction against themselves. They are essential to confer enhanced antigen

immunogenicity, through a proper transport and delivery system for presenting antigens to specific cells of the immune system and to confer ability to prolong drug release. On the other hand, have to be cost-effective, easily produced and present high stability (Vajdy *et al*., 2004).

Adjuvants can be divided into antigen-binding systems such as emulsions, micro and nanoparticles, immunostimulatory complexes (ISCOMs) and liposomes, and immunostimulatory adjuvants such as lipopolysaccharides (LPS), monophosphoryl lipid A, etc (Shahiwala *et al*., 2007).

The chemical composition of each vaccine is critical to defining the type of immune response that will develop, stimulating a specific type of antibodies of interest secreted by B cells, and stimulating specific cytokines secreted by T cells. This cascade of events can be controlled through the combination of antigens associated with adjuvants (Vajdy *et al.*, 2004; Shahiwala *et al.*, 2007).

It is well known that some antigens in their soluble form are not recognised by APCs and therefore do not induce a protective immune response. The therapeutic use of particulate carriers for the development of protective immune responses is currently one of the most promising strategies to fight infectious diseases (Shedlock and Weiner, 2000; Florindo *et al.*, 2009).

There are a few adjuvants approved for human use. These usually raise issues of potential toxicity, intolerable reactogenicity and side effects. A number of experiments with *H. pylori* vaccines with different adjuvants, such as *E.coli* heat labile enterotoxin (LT), mutant LT, cholera toxin (CT), typhoid vaccine Ty21a and aluminum hydroxide have been described, but none of these adjuvants showed protective immunity and some were unable to eliminate ongoing infection (Kabir, 2007). Aluminum hydroxide is the widely used adjuvant, with reduced side effects but has the disadvantage of triggering an unbalanced response essentially the prevalence of Th2 type with humoral component, having reduced stimulation of the cellular component. Other approach to delivering *H. pylori* vaccines is the use of attenuated *Salmonella typhimurium* and modified poliovirus, but none of them succeeded in eradication of *H. pylori* without triggering a successfully immune response in the host (Kabir, 2007; Velin and Michetti, 2010; Moss *et al.*, 2011).

Accordingly, therapeutic use of particulate carries for the development of protective immune responses is currently one of the most promising strategies against infectious diseases. It is believed that particulate systems for delivery the antigens could be the most advantageous because they confer antigen protection against mucosal enzymes, promote the sustained release of antigens increasing the time of contact between antigens and APCs, and possibility of increased time of retention in the administration site through adhesion. Since particulate systems have dimensions similar to pathogens, they are easily recognized and phagocytosed by APCs (Florindo *et al*., 2010).

Chitosan nanoparticles are thought to be a suitable vehicle for oral DNA vaccine delivery because it is one of the most promising polymers for drug delivery through mucosal routes. Chitosan is biocompatible and biodegradable, as well as mucoadhesive and permeation-enhancing. Its mucoadhesive characteristics allow prolonged exposures of the antigens with the mucosal epithelium. It has been extensively used in drug and vaccine formulations to be administered by mucosal routes.

Studies reported no effects on cell viability, high levels of gene expression in the epithelial cells of both stomach and intestine. Nanoparticles made of chitosan, showed stability under physiological conditions of the intestine and also for short periods of time in the stomach. For these reasons chitosan particles are good candidates for the development of novel gastrointestinal drug and DNA delivery systems. Nanoencapsulation of DNA into chitosan nanoparticles showed efficient nanoparticles binding to cellular membranes leading to transfection and effective immune system stimulation triggering both humoral and cellular responses (Boyoglu *et al*., 2009). Other studies show that intranasal administration with tetanus toxoid-loaded chitosan nanoparicles increases humoral response as compared to the soluble antigens.

1.8. Aims

The ultimate aim of this research project, where the work of this thesis is included, is to develop a prophylactic and therapeutic efficient multigenic DNA-based vaccine against *H. pylori*. In this direction, this work aims to evaluate a pDNA- and recombinant protein-based vaccine encoding multi epitopes from different *H. pylori* antigens capable to induce an effective humoral as well as cellular immune response against *H. pylori* infections and capable of eradicating the pathogen. For that propose, a plasmid DNA was produced, encoding the specific epitopes of selected antigens, and expressed in an appropriated expression vector. To evaluate the immune response triggered after vaccination, the recombinant proteins and plasmid DNA were purified and encapsulated in a specific nanoparticulate delivery system and the immune response was assessed by ELISA. The results could contribute to the positive development of an efficient prophylactic vaccine against *H. pylori* infections. Positive results will lead to a new approach in the development of novel vaccines for other infectious diseases.

2. Materials and Methods

2.1. Antigen production

2.1.1. Cell transformations

Escherichia coli XL1-Blue cells (Stratagene,USA) (200 μL), made competent by $CaCl_2$ treatment, were transformed by heat shock method with two plasmids vectors pQE30 (6T) (Qiagen, Germany) and pVAX1 (6T-plamid) (Invitrogen, UK), containing the recombinant gene for the following fragment antigens of *H. pylori* CagA, UreB, HpaA, VacA, GroEL, Homb (Figure 2.1.1.1).

The positive clones were selected in agar with ampicillin for clone 6T and kanamycin for 6T-plasmid. The cells were expanded and a cell bank was produced and preserved with 20% glycerol at -80ºC. Each production either of protein or plasmid was obtained from the same cell bank.

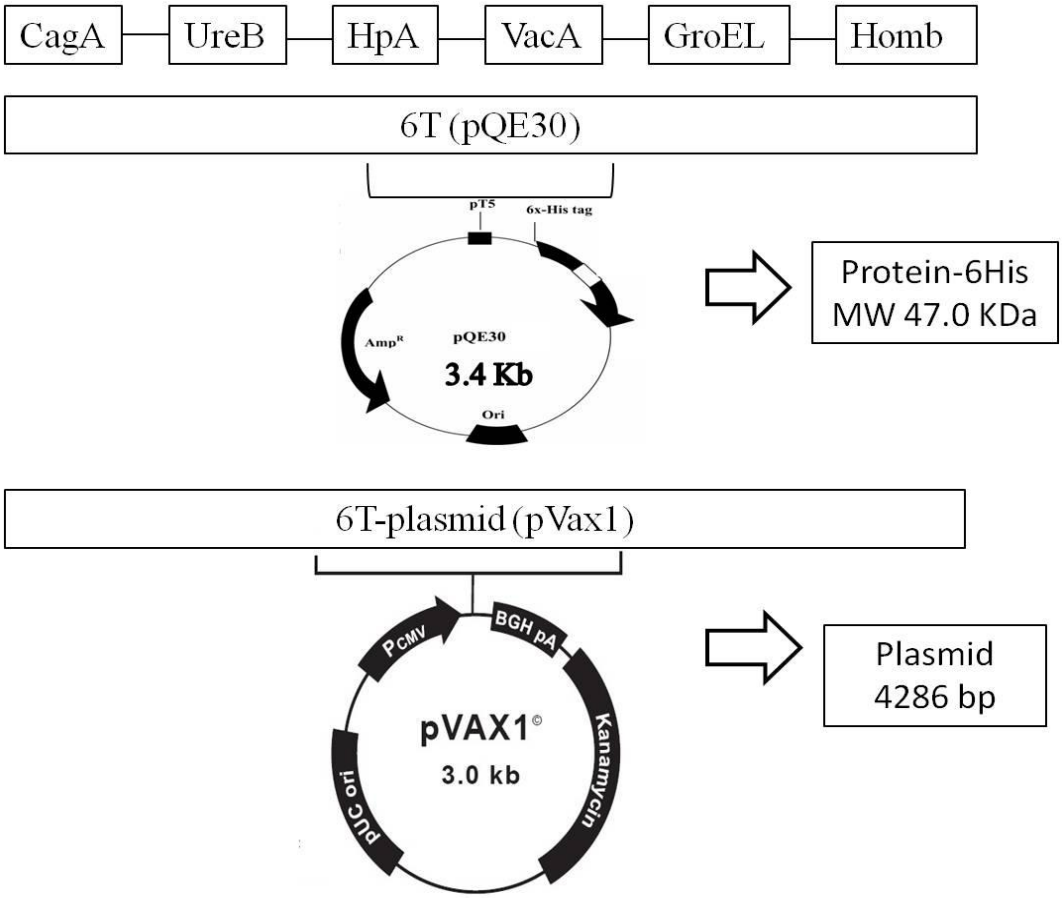

Figure 2.1.1.1 - *H. pylori* DNA and protein vaccines. Schematic representation (not to scale) of the *H. pylori* vaccine construction.

2.1.2. Cell culture growth

Cell culture growth of *E. coli* XL1-Blue cells with 6T recombinant vector, was followed by measuring the absorbance at 610nm for 30 hour after inoculation of 1L of tryptic soy broth medium (Table 2.1.2.1) (Biokar, France) containing 100mM ampicillin at 37°C, under agitation (250 rpm).

Table 2.1.2.1 - Tryptic soy broth composition (Sterilized at 121ºC, 20min)

For 1 L of medium:	
- Tryptone	17.0 g
- Papaic digest of soybean meal	3.0 g
- Glucose	2.5 g
- Dipotassium phosphate	2.5 g
- Sodium chloride	5.0 g

pH of the ready-to-use medium at 25°C: 7.3±0.2.

2.1.3. Optimization of production conditions of 6T recombinant protein

E. coli XL-1 Blue expressing 6T recombinant protein was cultivated in agitated flasks with tryptic soy broth culture medium containing 100 mM of ampicillin and cultivated at 37 ºC after induction with isopropyl b-D-1-thiogalactopyranoside (IPTG) collecting samples for 1h, 2h and 3h. The final concentration of IPTG added in culture medium was 1 mM.

The expression of 6T protein was evaluated by SDS-PAGE and western blot using anti-His antibody, after cell homogenization in denaturing buffer (8 M urea at pH 8.00).

2.1.4. 6T recombinant protein purification

The expression of recombinant protein was carried out as follows. *E. coli* XL-1 Blue transformed using pQE30-6T plasmid was grown at 37ºC, after 3 hours of induction with IPTG, in 2 L of culture media, overnight, until mid-log phase (Abs_{610nm}=0.600). Cells were harvested, washed with ice-cold 10 mM phosphate buffer, pH 7.4 and frozen for further use. For each assay, 1 g (wet weight) of cell pellet was resuspended in 10 mL lysing buffer (8M urea, 50 mM sodium phosphate, 500 mM NaCl, 1% (v/v) Triton X-100, 30 mM Imidazole, pH 8.0) and homogenized with an ultrasound probe for 3 cycles of sonication for 5 min. each, for cells lysis. Insoluble material was separated by centrifugation at 30,000g for 30 min at 4 ºC (Beckman 64R, USA). The supernatant was purified in a HisTrap FF 1 mL column (GE Healthcare) using a manual method as follows:

The protein was loaded onto the column twice times and washed with 10 bed column volumes of a solution containing 8M urea, 50 mM sodium phosphate, 500 mM NaCl, 1% (v/v) Triton X-100, 30 mM Imidazole, pH 8.0. Bound proteins were eluted with 8M urea, 20 mM sodium phosphate, 500 mM NaCl, 1% (v/ v) Triton X-100, 250 mM Imidazole, pH 8.0 solution. The eluted proteins were analyzed by 10% (w/v) SDS–PAGE under reduced conditions, by western blot using anti-His antibody following the NuPAGE Novex Bis-Tris Mini Gels Technical Guide (Invitrogen, UK) and further quantified by the BCA Protein Assay kit (Pierce, USA) using BSA as standard.

The purified protein from the 2.5 mL nickel column was desalted on Sephadex G-25 medium pre-filled PD-10 column (GE Healthcare) using 100 mM Heppes buffer pH 7.4.

Samples taken along the purification steps were analyzed in 10% SDS-PAGE gel followed by Western Blot to confirm the presence of recombinant protein in different solutions filtered.

After this process, the obtained proteins are frozen with 10% of trealose and then lyophilized. This was the protein used in *in vivo* assays and ELISA analysis.

2.1.5. SDS-PAGE

To confirm the presence and the structural integrity of proteins extracted and purified, the samples were analyzed by electrophoresis in pre-casted 10% (w/v) polyacrylamide gel (NuPAGE®, Invitrogen, UK) and run at a constant voltage of 200 V for 35 min using a Electrophoresis Power supply EPS 3501XL system (GE Healthcare Life Science, USA) under denaturing conditions, through comparison with BenchMarck™ pre-stained molecular markers in the range of 6-180 kDa (Invitrogen, UK) and purified proteins. After migration, proteins were visualized by SimplyBlue™ SafeStain solution (Invitrogen, UK).

2.1.6. Western Blotting

Samples were transferred from polyacrylamide gel onto the PVDF membrane using a semi-dry transfer system (Hoefer Semiphor Amersham, GE Healthcare Life Science, USA) for 1 h at 0.8 mA/cm^2 membrane. The membrane was then washed and blocked by its incubation with 10% (w/v) skimmed milk powder (Merck KGaA, Germany) dissolved in 10 mM PBS at pH 7.4 containing 0.05% (v/v) of Tween 20 (PBST; Sigma Aldrich, Co., Germany), for 1 h under constant agitation in an orbital shaker (100 rpm). The membrane was further incubated for 2 h at room temperature with mouse anti-6XHis tag protein diluted in the blocking buffer (1:1700), under constant agitation. After washing, the membrane was incubated with a goat anti-mouse IgG conjugated to phosphatase alkaline (Sigma Aldrich Co., Germany), diluted 1:2000 in blocking buffer for 1 h at room temperature. The capacity of

anti-mouse 6XHis tag protein to recognize the recombinant 6T protein was revealed calorimetrically using the substrate kit of alkaline phosphatase SIGMA FAST™ BCIP (5-bromo-4-chloro-3'-indolyphosphate p-toluidine salt) and NBT (nitro-blue tetrazolium chloride) (Sigma Aldrich, Germany), in accordance to the kit instructions.

2.1.7. Protein quantification

The protein quantification was made by BCA (bicinchoninic acid assay), using bovine serum albumin (BSA) 1mg/ml as standard protein. Successive dilutions were made in 96-well plates of 1:2 using bicinchoninic acid and the plates were incubated 1hour at 37ºC. The BCA protein assay is based on the reduction of Cu^{2+} to Cu^{1+} by protein in an alkaline medium. The Cu^{1+} ion reacts with bicinchoninic acid forming an intense purple-coloured reaction product. The absorbances are detected at 565nm with increasing protein concentration in spectrophotometer (Infinite M200, Tecan, Austria) and the absorbance values obtained compared with the standard curve of BSA protein standard in order to calculate the protein concentration in mg/ml of sample.

2.1.8. DNA Plasmid extraction and purification

Recombinant 6T-plasmid *E. coli* strain was inoculated in 1 L of tryptic soy broth medium (Biokar, France) with 100mM kanamycin, at 37ºC/250 rpm until 10h of growth, which typically corresponds to the transition from logarithmic into stationary growth phase. Cells were isolated by centrifugation and cell pellet washed with phosphate buffer saline at pH 7.4 (10 mM PBS, Invitrogen, UK). The pDNA extraction and purification was performed according to the procedure of Mega QIAfilter Plasmid purification kit (QIAGEN Inc., Germany) as shown in Figure 2.1.8.1. This protocol is based on an alkaline lysis followed by vacuum filtration to clear bacterial lysates to allow binding of plasmid DNA to QIAGEN anion-exchange resin. The RNA, proteins and low molecular weight particles are first removed by washing using gravity flow. Plasmid DNA is eluted in a high salt buffer. Desalting is performed using isopropanol precipitation.

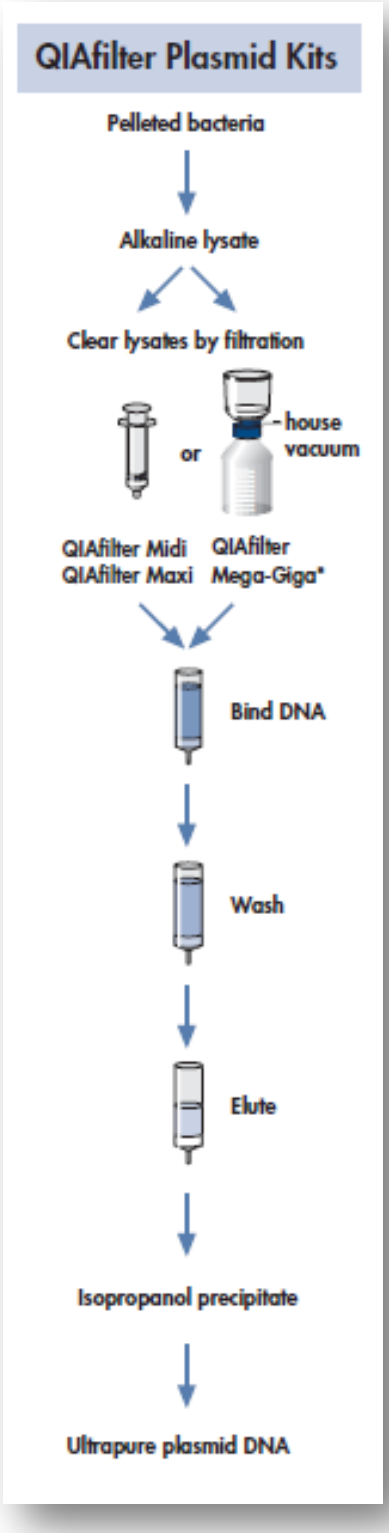

Figure 2.1.8.1 – QIAfilter Plasmid Kits procedure. Adapted from "QIAfilter Plasmid Purification Handbook" of QIAGEN Inc, Germany.

2.1.9. Electrophoresis

To confirm the presence of extracted plasmid DNA, samples collected throughout the purification steps were run in 1% agarose gel containing ethidium bromide. The gel is constituted by 1% of agarose dissolved by heating in TAE 1X buffer (1,14 mL glacial acetic acid; 2mL 0.5M EDTA and 4.8 g/L Tris-base, pH 8.0). To this solution was added ethidium bromide (0.3 µg/µl) and the samples were run at constant voltage of 60 V.

2.1.10. Quantification of plasmid DNA

The quantification of purified plasmid DNA was performed using the Quant-iT™ Picogreen® assay kit (Molecula Probes™, Invitrogen, UK) according the manual instructions (PicoGreen® dsDNA Quantitation Reagent and Kits).

A serial dilution of the stock DNA standard solution with TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5) was performed yielding eight concentrations ranging from 0.015 µg/mL to 2 µg/mL. The samples were also serial diluted. Aliquots (100 µL) of each preparation, standard and samples, was placed in duplicate into a white 96-well plate (Griner, Germany) for measurement. The working PicoGreen® reagent (100 µL) was added to each measurement well. The blank solution was prepared identically, but without DNA. The prepared 96-well plate was incubated for 5 min at room temperature, and fuorescence was measured in a fluorescence microplate reader (Infinite M200, Tecan, Austria) at 480 nm excitation wavelength and 520 nm emission wavelength. The average value of blank measurement was subtracted from the duplicate measurements made at each standard concentration. The results were plotted and a linear regression was performed on the standard curve to calculate the DNA concentration in each sample.

2.2. In vivo Assays

2.2.1. Nanoparticle preparation

For intramuscular (i.m.) immunisation, recombinant protein or plasmid were encapsulated in chitosan/sodium deoxycholate (CS/DS) (1:1 w/w) nanoparticles. These were prepared using an ionotropic gelation technique, previously described by Cadete *et al*. (2012). Briefly, chitosan and sodium deoxycholate were separately dissolved in ultra-pure water in order to obtain 1mg/mL solution. Nanoparticles were formed when 1 volume of DS solution was added drop wise to one volume of CS solution and magnetically stirred at 300 rpm for 15 min at room temperature. For the nanoencapsulation, pDNA and protein was added to DS solution before the addition to CS solution.

For oral vaccination the antigens, recombinant protein or plasmid, were encapsulated in chitosan/tripolyphosphate/alginate (CS/TPP/Alg) (20:0.25:1 w/w/w) nanoparticles. These were prepared using thr ionotropic gelation technique previously described in Gonçalves *et al*. (2011). Briefly, chitosan (low molecular weight), sodium tripolyphosphate and sodium alginate (low viscosity) were separately dissolved in ultrapurified water in order to obtain respectively 2.5mg/mL, 10mg/mL and 3 mg/mL solutions. The alginate solution was added to the TPP and this was added drop wise to the chitosan solution. For encapsulation the solution of recombinant protein or plasmid was added to TPP/Alg solution before the addition to CS solution.

2.2.2. Immunization studies / Vaccination

Ten groups of female BALB/c mice (n=5/group), were used in the *in vivo* studies, which were performed in strict accordance with the Directive of 24 November (mº86/609 EEC), the Portuguese laws D.R. nº 31/92, D.R. 153 I-A 67/92 and all following legislation.

Mice were immunized on day 1 and boosted on day 22, either by the oral or the i.m. routes, with 50 µg of encapsulated protein and/or pDNA . Controls were administrated with equivalent doses of non-encapsulated protein and DNA, with empty nanoparticles and PBS. All doses were freshly prepared in sterile PBS (pH 7.4) under aseptic conditions, just before animal dosing and boosting. Group 1 received encapsulated pDNA by the oral route; Group 2 received free pDNA (in solution) by the oral route; Group 3 received encapsulated recombinant protein by the oral route; Group 4 received free protein (in solution) by the oral route; Group 5 received encapsulated pDNA by the i.m. route; Group 6 received free pDNA (in solution) by i.m. route; Group 7 received encapsulated recombinant

protein by i.m. route; Group 8 received free recombinant protein (in solution) by i.m. route; Group 9 was vaccinated with empty nanoparticles by i.m. route ; Group 10 received PBS (table 2.2.2.1).

Table 2.2.2.1 - Immunization groups with recombinant antigens 6T and 6T-plasmid by oral and i.m. routes.

Groups	Antigens	Dose/presentation antigen	Via
1	6T-plasmid encapsulated in CS/TPP/alginate nanoparticles	2x50µg/encapsulated	Oral
2	6T-plasmid in solution	2x50µg /free	
3	6T recombinant protein encapsulated in CS/TPP/alginate nanoparticles	2x50µg /encapsulated	
4	6T recombinant protein in solution	2x50µg /free	
5	6T-plasmid encapsulated in CS/DS nanoparticles	2x50µg /encapsulated	i.m.
6	6T-plasmid in solution	2x50µg /free	
7	6T recombinant protein encapsulated in CS/DS nanoparticles	2x50µg /encapsulated	
8	6T recombinant protein in solution	2x50µg /free	
9	CS/DS empty nanoparticles	Empty nanoparticles	
10	Naive	Naive	———

2.2.3. Sample collection and analysis

Blood samples of each mouse were collected every two weeks from the tail vein after the first immunization, during 4 months. Blood samples were centrifuged at 18,000×g for 20 min at 4ºC and serum was collected and stored at -20ºC until analysis (Figure 2.2.3.1). The serum antibody levels were determined by an enzyme-linked immunosorbent assay (ELISA) using purified 6T recombinant proteins (described in section 2.1.).

To quantify the cytokines production and the mucosal immune response, spleens and intestines were collected (Figure 2.2.3.1), from ethically sacrificed animals according to a previously described method (Figueiredo *et al*., 2012a).

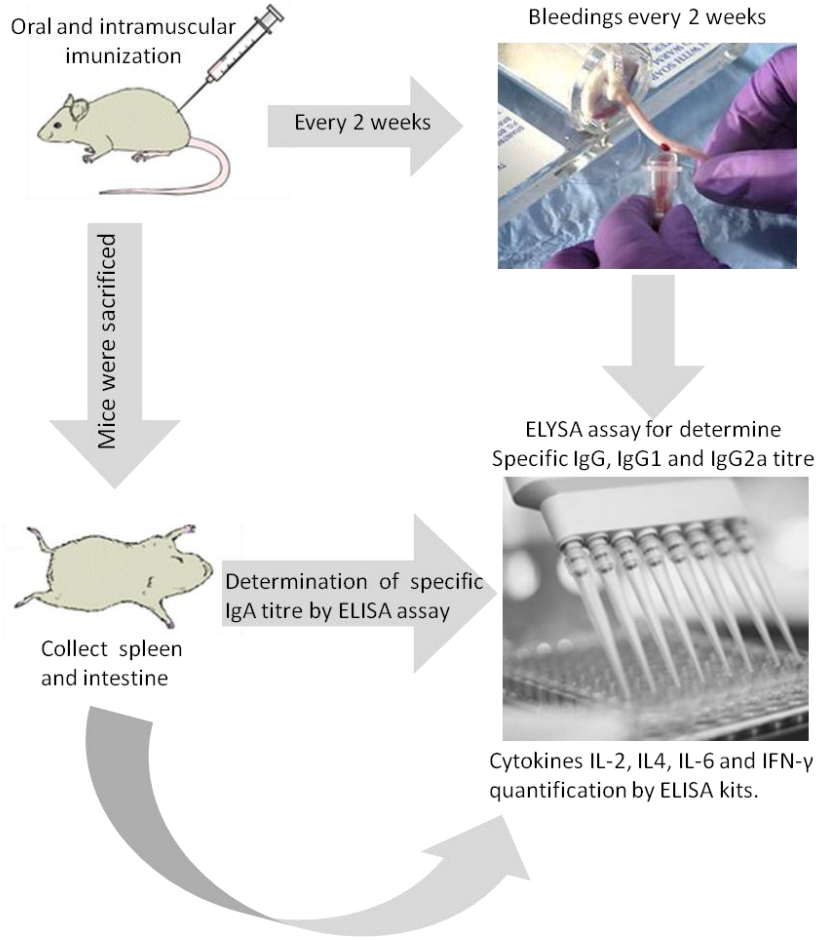

Figure 2.2.3.1 – Sample collection and immunoassays scheme. After oral and i.m. immunization, blood samples were collected every 2 weeks, and antibody titres measured by ELISA. Spleens and intestines were collected for determination of specific IgA and specific cytokine titres using specific ELISA techniques.

2.2.4. Quantification of immune response by ELISA

The antibody responses (IgG, IgG1 and IgG2a) to recombinant protein (6T) and plasmid (6T-plasmid) were determined according to a previously reported method (Figueiredo *et al*, 2012a). ELISA plates (Microlon®, High binding flat bottom plates, Greiner, Germany) were coated overnight with 5.0 µg/mL 6T recombinant protein enzymatic in 100 mM sodium carbonate buffer (pH 9.6), washed and afterwards blocked with a 5% (w/v) skimmed milk powder (Merck KGaA, Germany) dissolved in 10 mM PBS, pH 7.4 containing 0.05% (v/v) of Tween® 20 (PBST; Sigma Aldrich, Co., Germany). Plates

were washed again and sera were tested by serial two-fold dilutions. Sera obtained from naive mice were used as a control. Horseradish peroxide conjugate goat anti-mouse IgG, (Sigma, Pool Dorset, UK), IgG1 and IgG2a (Serotec, UK) (diluted 1:1000) were applied as secondary antibody.

Finally, the substrate OPD (SigmaFAST™ OPD Kit, Sigma Aldrich, Germany) was used to develop the plates, the colour reaction was stopped after 15 min by adding 2.5N H_2SO_4 to the wells, and absorbance was read at 490 nm.

For analysis of intestine IgA, the ELISA method was essentially the same, with some modifications. The intestines were homogenized in 0.9% sodium chloride with protease inhibitor PMSF 1 mM and 0.5% Tween® 20. The homogenization was performed using an ultrasound probe (3 × 5 min intermittent pulsed for 30 s each, in an ice bath). The homogenized tissues were centrifuged at 20,000×g for 30 minutes, at 4ºC (Allegra 64R, Beckman, USA), and the supernatant was frozen at −80ºC until freeze-drying. After freeze-drying, proteins were reconstituted with 500 µL of sterile water and added directly, in triplicates, to the plate wells. The horseradish peroxide conjugate goat anti-mouse IgA (1:2000; Serotec, UK) was used to detect this antibody and OPD substrate was then added as mentioned above. The mean OD was determined for each treatment group and used directly to compare mucosal response. The titres reported are the reciprocal of serum dilutions that gave an optical density 5% higher than the strongest negative control reading.

2.2.5. Cytokine production studies

Mice were sacrificed and spleens were aseptically collected into test tubes containing RPMI medium (Gibco, UK). After washing with RPMI medium, spleens were transferred to RPMI complete medium (RPMI culture medium supplemented with 10% fetal bovine serum (FBS), 20mM L-glutamine, 105U/L penicillin and 100mg/L streptomycin) and spleen cells were dispersed with the aid of two needles. Cell suspension was centrifuged at 200×g for 10 min and the pellet resuspended in RPMI complete medium to perform another centrifugation. Cells were counted in a haemocytometer diluted in trypan blue and the cell concentration was adjusted to $2x10^6$/100µL in RPMI complete medium. Aliquots of 100 µL of cell suspension were added to each well of a cell culture plate (Greiner, Germany) containing 100 µL of RPMI complete medium and other plate containing 100 µL of RPMI complete medium with 5 µg/mL of antigen (6T). Plates were incubated in 5% (v/v) CO_2 incubator for 72 h at 37ºC. Supernatant was collected and stored at -80ºC for subsequent analysis. Cytokines IL-2, IL4, IL-6 and IFN-γ were then quantified using appropriate kits: Mouse IL-2 ELISA Kit, Mouse IL-4 ELISA Kit, Mouse IL-6 ELISA Kit and Mouse IFN-γ ELISA Kit (RayBiotech, USA).

2.2.6. Statistical analysis

Results are expressed as mean±standard deviation (SD). The results of animal experiments are presented as mean ± standard error of the mean (SEM). Statistical analysis of the experimental data was performed using the nonparametric Mann-Whitney test and significance was defined for p value <0.05.

2.3. Agglutination assays

In this study, nine *H. pylori* strains from the collection of the National Institute of Health Dr. Ricardo Jorge (Lisbon, Portugal), primary isolates from Portuguese colonized patients, were inoculated in Brucella agar medium plates (Table 2.3.1) at 37ºC under microaerophilic environment (CampyGen; Oxoid, Hampshire, UK) for approximately 48 hours. Agglutination tests were then performed with these strains and sera from immunised mice to checking for the presence of neutralising antibodies.

Table 2.3.1 – *H. pylori* selective medium composition (per litre)

Agar	17g
Brucella broth (BB)	28g
Fetal Bovino Serum (FBS)	50 mL

Agglutination tests were performed according with the BSOP TP 3 as issued by Standards Unit, Department for Evaluations, Standards and Training, UK. Briefly, all bacteria were tested after total biomass was recovered from the growth plates with a 10^{-3} ml plastic loop and resuspended in 200µL of PBS (pH 7.2). Twenty µL of bacterial suspension were placed on glass slides, against black background, and 10 µL of serum were added followed by homogenization. Each serum was diluted 1:10 and 1:40 and each dilution was tested with each *H. pylori* strain. Negative controls were performed with 20 µL of bacterial suspension only. Agglutinations were graded as follows: (+) agglutination observed, (-) no agglutination observed and (?) inconclusive observation.

3. Results and Discussion

3.1. Antigen production

3.1.1. Cell transformation

E. coli XL1-Blue cells were transformed with two plasmids pQE30 (6T) and pVAX1 (6T-plasmid). The genetic sequence cloned into the plasmid vectors, coding for the recombinant proteins and pDNA, contains the immunogenic and conserved sequences of the following *H. pylori* antigens: CagA, UreB, HpaA, VacA, GroEL and Homb. It was demonstrated that *H. pylori* vaccines comprising a single recombinant antigen were only partially effective (Liu *et al*., 2011). Therefore, it is thought that effective immunity against *H. pylori* infections could be achieved by a combination of various antigens involved in the pathogenesis of infection. So fragments of these six different antigens of *H. pylori* were chosen as candidate antigens to vaccine design in order to confer protective immunogenicity against infections of a large range of *H. pylori* strains. These six fragments of antigens were selected based on an extensive research that is part of an ongoing research project. Due to intellectual property constraints the sequences and the methods used to obtain the genetic constructs cannot be fully described herein. Nevertheless, its description remains beyond the scope of this dissertation.

The plasmid vectors pQE30 (6T) is a prokaryotic vector that allows protein expression in prokaryotic cells, whereas pVAX1 (6T-plasmid) is a eukaryotic vector under the promoter of cytomegalovirus that permits the expression of the protein antigen in host eukaryotic cells, in order to facilitate the induction of cellular immunity (Zhang *et al*., 2003).

3.1.2. Cell culture growth

The successful expression of recombinant proteins in *E. coli* depends on the optimization of the methods used. Optimization of the protein expression is essential for the maximization of protein-yield, for successful expression and for protein integrity.

Previous studies have indicated that the time of induction is important for optimal expression of a cloned gene. It is also important to control growth phases of *E. coli* culture for inferring the beginning of exponential phase, because it is in the early exponential phase that cell growth starts, more nutrients are available and thus more protein will be expressed. An early induction of protein expression at lag phase can lead to up to 50% inhibition of biomass production. Existing no recipes or formulas for optimal expression of proteins, it is necessary to optimize the best protocol for each protein and for each culture (Olaofe *et al*., 2010).

In order to determine the stage of cell cultivation suitable for optimal expression of recombinant proteins, *E. coli* 6T culture in shack flask was incubated at 37ºC. Sampling was performed every hour

and the culture growth was followed by measuring the optical density at 610 nm over 30 h of incubation (Figure 3.1.2.1).

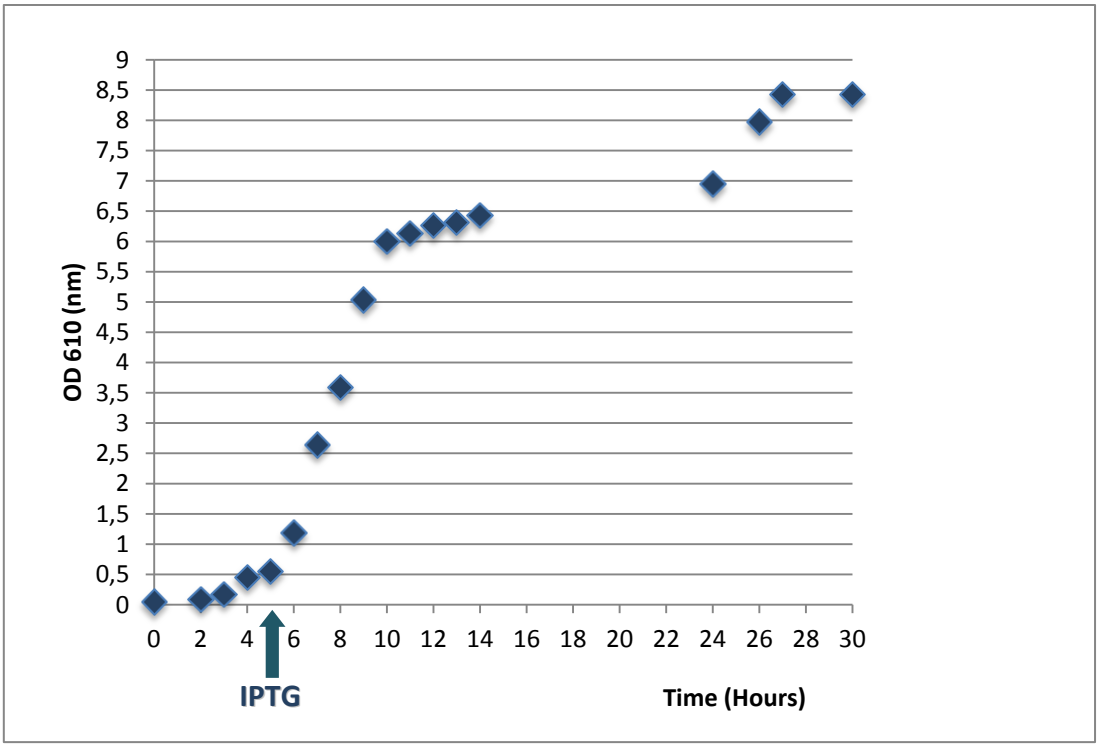

Figure 3.1.2.1 – Cell growth curve of *E. coli* XL1 Blue expressing 6T recombinant protein. Arrow indicates the time of IPTG addition.

Through the growth curve we are able to confirm that after five hours of incubation culture begins the exponential grow, where the OD_{610nm} is 0.600 as shown in Figure 3.1.2.1.At this stage, the cells initiate a period of exponential cell division and consequently initiate protein synthesis. According to Olaofe *et al*. (2010), the early exponential phase is the best time to induce protein expression. After 10 hours of incubation the bacterial culture reaches the stationary phase, stabilizing the cell growth.

3.1.3. Optimization of protein expression conditions

The expression of recombinant proteins in cells in which they do not naturally occur is termed heterologous protein production (Weng *et al.*, 2006). Protein concentration increased by a factor of approximately two with IPTG addition in early exponential phase compared to cultures without inducer (Olaofe *et al.*, 2010). For this reason recombinant protein expression was performed by addition of isopropyl-beta-D-thiogalactopyranoside (IPTG) to a final concentration of 1mM after five

hours of culture incubation when OD_{610}=0.600. The aim is to optimize a feeding induction protocol for protein expression by host strain.

The IPGT, an analogue of lactose, is an inductor of genetic transcription which when added to the cell culture increase synthesis of T5 RNA polymerase enzyme, which binds to the T5 promoter initiating the expression of the cDNA of interest (figure 3.1.3.1). The promoter system of T5 RNA polymerase is the most efficient prokaryotic system, heavily regulated, allowing only the expression of recombinant protein with T5 promoter when added IPTG and allowing high levels of expression (Weng *et al*., 2006).

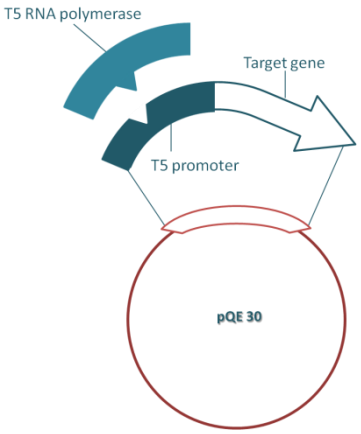

Figura 3.1.3.1 – Expression system of pQE30 recombinante proteins induced by IPTG.

Our aim was to empirically optimize expression protocol for our 6T host strain. So in order to evaluate which incubation time is required after IPTG addition, to maximize the recombinant proteins synthesis, samples were taken over 3 hours (time 0, 1, 2 and 3) post-induction. These samples were then analysed by SDS-PAGE and Western Blot (Figure 3.1.3.2).

From the results obtained (Figure 3.1.3.2) it can be observed a band of approximately 49 kDa similar in size to 6T recombinant protein, since this protein has a molecular mass of 47kDa. Western Blot analysis confirmed a signal sequence at ~49kDa that corresponding to 6T recombinant protein. The six histidines tagged to 6T recombinant proteins act as an protein epitope detected by anti-HistTag antibodies in Western Blot (Ramos *et al*., 2004). It indicates that there was induction and expression of the desired 6T-protein successfully. The 115kDa bands observed in SDS-PAGE and in Western Blot correspond to the insoluble fraction of the expressed protein. Both soluble and insoluble fractions of the protein were expressed and can be use as antigens for vaccine formulation, since they retain their immunogenicity.

After induction (T1, T2 and T3), the intensity of the 49 kDa and 115 kDa bands increased over time, suggesting the quantity of protein expressed increases over time, stabilizing after 3 hours of induction with IPTG, without significant differences in the amount of protein expressed, as compared to T2.

At time zero (T0) a basal expression of the 6T proteins is detected. These proteins are highly regulated by T5 promoter with the induction of IPTG, but might be activated by other pathways (Weng *et al*., 2006).

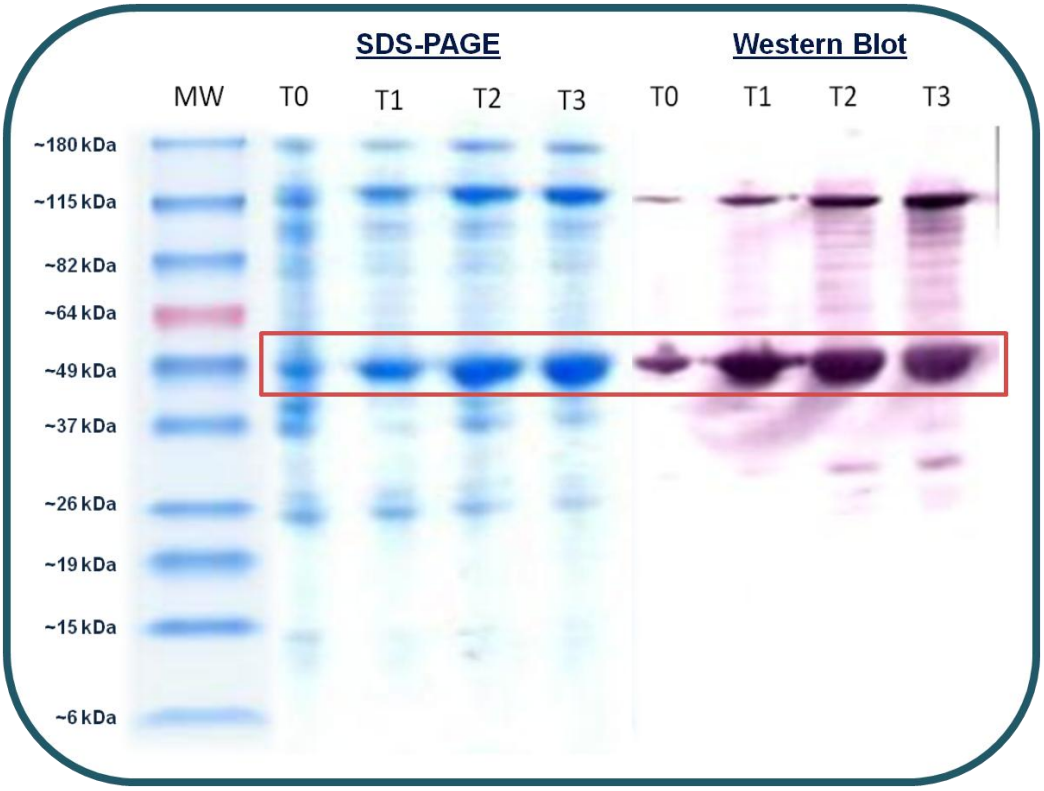

Figure 3.1.3.2 - SDS-PAGE and Western Blot of 6T-protein extract processed with anti-HistTag antibodies. MW – pre-stained Molecular Weight Markers; T0 – sample before induction with IPTG; T1 – sample after 1 hour of induction with IPTG; T2 - sample after 2 hours of induction with IPTG; T3 - sample after 3 hours of induction with IPTG.

The induction of recombinant protein expression usually results in impaired growth rates and low increase in cell mass (Sorensen and Mortensen, 2005), which correlates inversely with the rate of synthesis of recombinant proteins. Hence, expression for longer times would not be advantageous because the growth rate of cells expressing proteins decreases over time, leading to stagnation of rates of specific protein expression and reducing process productivity (Teich *et al.*, 1998). This is due to the fact that expression of proteins stops when cell growth reaches the stationary phase and nutrient concentration decreases, thus greatly reducing cell division and protein expression (Olaofe *et al.*, 2010).

Taking into account the results obtained for maximizing the expression of recombinant proteins, the ideal incubation time after induction with IPTG is 3 hours.

3.1.4. Protein purification

The pQE30 plasmid expressing recombinant 6T protein permits rapid protein purification through a 6XHis-tag fused to the expressed proteins, which has affinity to the nickel-affinity chromatography

column. Affinity chromatography allows to recover proteins with a purity level higher than 95% (Terpe, 2003). Histidine-Tag is the preferred purification system for protein purification, especially when knowledge of the biochemical properties of the protein is poor (Arnau *et al.*, 2011), just as in this case. It allows easy purification and specific detection of recombinant proteins by immunological methods such as Western Blot. Other advantage of Hist-Tag, described by Ramos *et al.* (2004) and Catanzariti *et al.* (2004), is that fusion proteins can be purified under denaturing conditions since the interaction between consecutive histidine residues and the affinity nickel column is stable in 8 M urea, which is a strong protein denaturant. In the case of recombinant 6T-protein, the protein refolding is not essential since it is not necessary to maintain protein function. Only epitopes immunogenicity has to be maintained and displayed upon antigenic presentation. Purification in denaturing conditions allows increasing protein purity because it permits a better exposure of HistTag at the protein surface, while decreasing protein aggregation (Triccas *et al.*, 1998; Terpe, 2003).

After 3 hours of induction of protein expression with IPTG 1mM, the culture was centrifuged and lysed. Expressed proteins were purified through immobilized metal ion affinity chromatography (IMAC) in Hist Trap FF 1mL nickel column (GE Healthcare, England). Six consecutive histidine residues tagged to the recombinant proteins allow specific binding of the histidine to metallic ligands, such as nickel, retaining the protein in the column. This procedure was performed in denaturing conditions, since the buffers used during purification contain 8M Urea.

The SDS-PAGE and Western Blot were used to analyse the samples from the recombinant protein purification and identification process, using specific anti-histidine antibodies (Figure 3.1.4.1). Western Blot confirmed a signal sequence at ~49 kDa that corresponds to the 6T recombinant protein (MW 47 kDa). Is also revealed a 115 kDa band, corresponding to the insoluble fraction of the recombinant protein expressed. Overproduction of heterologous proteins in *E. coli* could lead to misfolding and segregation into insoluble aggregates known as inclusion bodies. Another reason is the linking of histidine tails with each other forming agglomerates of proteins. Even under denaturing conditions (8 M Urea) these aggregates are difficult to eliminate, as observed in 115 kDa band. Accordingly Han *et al*. (2011), the high-level of expressed inclusion bodies may also be useful for purification because aggregates are easily isolated due to the histidine-tag located at the protein surface, and formation of inclusion bodies protects the protein from proteolytic attack. In this case, 6T protein does not need to be soluble and retains its function, as long as it can be used directly as an antigen since it retains its immunogenicity.

Flow-through sample, analysed in line F of Figure 3.1.4.1, shows a large amount of other proteins extracted from the bacteria, but there was no corresponding specific reaction at ~47 kDa with anti-histidine antibodies in western blot. Therefore, protein degradation or premature translation product at lower molecular weights may also occur since they cannot be detected by Western Blot. Degraded proteins were not retained in the column, since plenty is found in flow-through solution but are absent in wash solution (W). The column was washed with Binding buffer with low concentrations of

imidazole (30mM) for cleaning up non-specific binding proteins and minimizing binding host cell proteins. Low imidazole concentrations permits minimizing nonspecific binding host cell proteins and other impurities present in cell lysate (Terpe, 2003). In Figure 3.1.4.1, line W suggests that part of 6T-protein is released from the column during the washing step, because imidazole competes with proteins for binding to the nickel, so imidazole itself may have impaired the binding of some 6T-proteins leading to their release. Future optimization of the imidazole concentration will have to be performed for improving the amount of histidine-tagged purified protein. Thus, the optimized imidazole concentration ensures the best high purity (low binding of others proteins) / high yield (strong binding of histidine-tagged proteins) balance (Terpe, 2003).

The histidine-tagged proteins retained in the nickel column were eluted with Elution buffer containing high imidazole concentrations (250mM). As imidazole and histidine contain a similar radical, high concentrations of imidazole allow, by competition, the release of histidine-tagged proteins bound to the column (Terpe, 2003). Figure 3.1.4.1 shows strong bands at ~47 kDa and ~115kDa molecular weights, indicating 6T proteins were successfully purified and the purity level increases along the 3 elutions, since no other contaminants are detected in Western Blot (E2 and E3 samples).

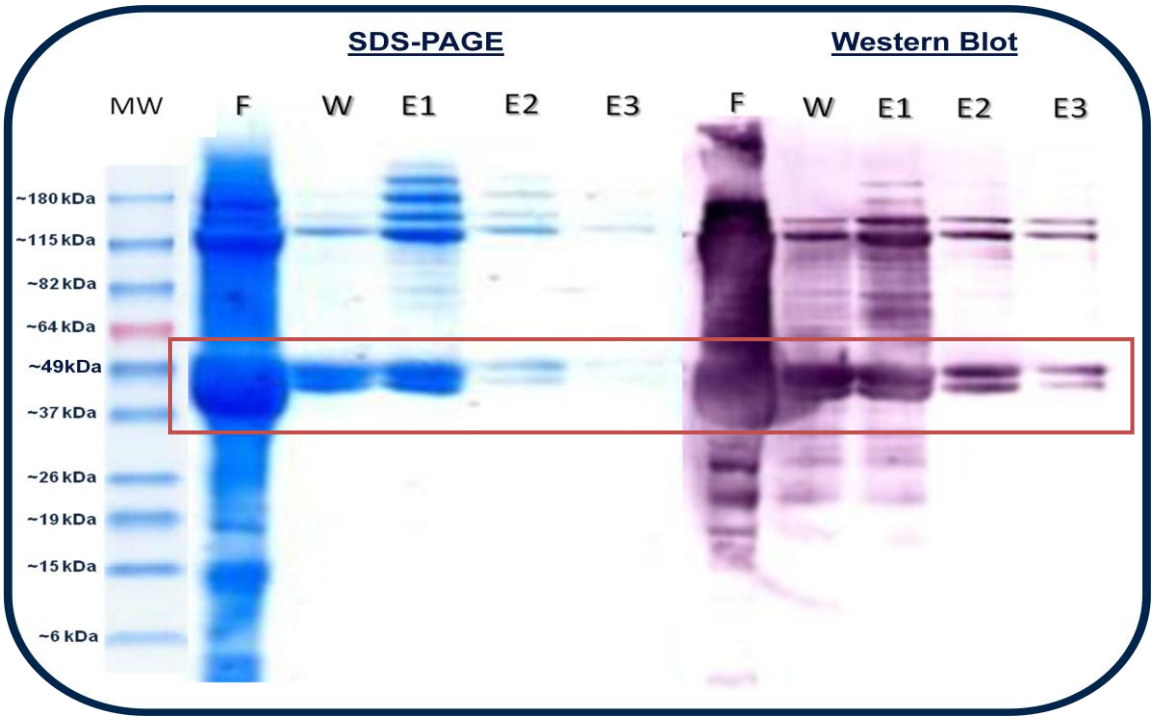

Figure 3.1.4.1 –SDS-PAGE (10% polyacrylamide gel) and Western Blot of samples from purification process of recombinant 6T protein. **(**MW- molecular weight standards; F – Flow-through sample; W – wash sample; E1 – first elution sample; E2 – second elution sample; E3 – third elution sample).

Some protein was lost during the purification process. However it was possible to purify the desired 6T-proteins with high degree of purity by affinity chromatography. Therefore, nickel-affinity chromatography enables a strong adsorption onto the stationary phase, allowing an efficient removal of other components before the protein is desorbed from the column, as well as a high yield of purification of the desired protein (Han *et al.*, 2007; Arnau *et al.*, 2011).

After affinity chromatography the protein is in a denaturing buffer containing 8M urea and 250mM of imidazole, thus a new step was needed for buffer exchange. The buffer exchange was performed through PD-10 desalting column containing *Sephadex G-25 Medium* (GE Healthcare, EUA), which allows separation of substances with high molecular weight (Mr=5000 Da) from substances with low molecular mass (Mr=1000 Da). The PD-10 desalting column was used to separate small molecules such as salt and other impurities, from the proteins of interest. Molecules smaller than the largest pores in the Sephadex matrix will be retained within the pores, therefore are eluted after the large molecules. The purified proteins are eventually diluted whenever it is desalted. Protein recovery was made using 100mM HEPES buffer. This buffer is more effective in maintaining protein structure at low temperatures and in maintaining pH levels during freeze drying, to prevent protein denaturation.

The protein was quantified before and after desalting using the BCA method, taking into account that 2.5 mL of protein applied to the column PD-10 is eluted with 3.5 mL of the new buffer solution (dilution factor of 1.75 fold). So, BCA method (section 2.1.7.) is based on the conversion of Cu^{2+} to Cu^{+} under alkaline conditions. The Cu^{+} is then detected by reaction with BCA. Since BCA is stable under alkaline conditions, this assay has the advantage of being carried out as a one-step process. This reaction results in the development of an intense purple colour, detected at 565nm of absorbance. Since the production of Cu^{+} is a function of protein concentration, this may be determined spectrophotometrically by comparison with known amounts of standard proteins (Krieg *et al.*, 2005; Walker, 2009).

In this study, the BCA assay has the advantage of being unaffected by denaturing agents such as urea (Walker, 2009), so it is suitable for quantifying 6T purified protein in 8 M Urea.

Table 3.1.5.1 – Amounts of purified protein obtained before and after buffer exchange and desalting.

Buffer	Protein Concentration (µg/mL)	Total Protein (µg/g cells wet weight)
8M Urea	310	4.655
100mM Hepes	129	2.714
Yield	58%	

There was protein lost during the desalting with PD-10 column (Table 3.1.5.1) and the yield of this step of purification was 58% (w/w).

3.1.5. Plasmid DNA purification

Large-scale produced pDNA must be available for vaccine formulation as a highly purified molecule, without any traces of RNA, endotoxins and proteins. Accordingly, anion-exchange chromatography provides a good selectivity for pDNA purification in the presence of RNA, endotoxins and residual protein (Stadler *et al.*, 2004).

In order to determine the plasmid yield after purification, samples were collected during the plasmid DNA purification procedure and analysed by electrophoresis in 1% agarose gel stained with ethidium bromide (Figure 3.1.6.1). The size of plasmid DNA determines the gel migration pattern, but there are other factors that may influence this pattern. Plasmid DNA, under some conditions, can assume others isoforms such as super coiled form that tends to migrate faster than circular plasmid DNA.

Figure 3.1.6.1 shows a filtered lysate (A1) containing supercoiled and open circular plasmid DNA, as expected, and others isoforms of supercoiled plasmid DNA; a flow-through fraction (A2) containing slight bands corresponding to different isoforms of pDNA that not bind to the QIAGEN Resin; a fraction (A3) after washing the QIAGEN resin with a specific buffer to remove all contaminants. It is evident that there was no DNA loss during this process since no band is observed and that pDNA is pure without any contaminants. The A4 sample corresponds to the elution fraction of DNA before being precipitated with isopropanol. Only after being precipitated, DNA is observed in electrophoresis gel and therefore is not seen in A4 sample in agarose gel. Lastly, Figure 3.1.6.1 shows the samples taken after purified DNA precipitation with isopropanol (A5 and A6), presenting strong bands at 4.0 kb and at 2.0 kb, which correspond to recombinant 6T-plasmid at circular form and super coiled, respectively. The intensity of emitted fluorescence, that was proportional to the amount of DNA, we can confirm that after purification we obtained a large amount of plasmid DNA purified with success in both circular and supercoiled form. In this study, supercoiled pDNA could be more advantageous for a more efficient uptake and higher transfection of host cells.

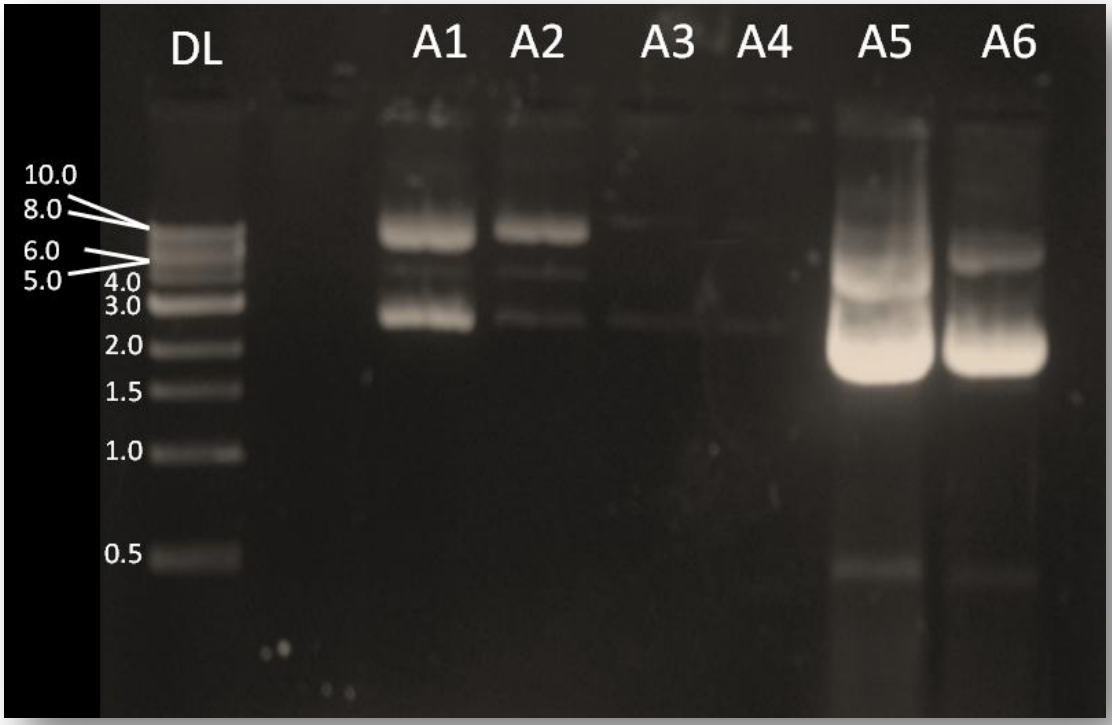

Figure 3.1.6.1 – Confirmation of the purification of plasmid DNA by agarose gel electrophoresis. DL - DNA ladder containing the molecular weight standards; A1 – sample of cleared lysate; A2 – sample from the flow-through faction; A3 – sample from washing; A4 – sample from the eluate; A5 – sample obtained after precipitation with isopropanol; A6 – sample obtained after washing with 70% ethanol.

Quant-iT^{TM} Picogreen$^{®}$ dsDNA, a fluorochrome that selectively binds dsDNA, which becomes intensely flourescent upon binding double-stranded DNA in solution, was used for plasmid DNA quantification. The brightness of this reagent is due to its high quantum yield and large molar extinction coefficient (Singer *et al.*, 1997).

In order to calculate the amount of plasmid DNA extracted and purified, to subsequent encapsulation and vaccine production, a Picogreen® assay kit was used according to the manual instructions. The amount of purified 6T-plasmid precipitated after centrifugation and afterwards redissolved from tube walls (plasmid residual) was quantified.

Table 3.1.7.1 – Total amount of purified 6T-plasmid.

[plasmid]	1769µg/mL
[plasmid]$_{residual}$	648 µg/mL
Total [plasmid]	285.5 µg/ g cell wet weight

3.2. In vivo assays

Several prophylactic vaccines against *H. pylori* infection have been developed but have failed to confer protective immunity or eliminate an ongoing infection by *H. pylori* (Algood and Cover, 2006; Zhou *et al.*, 2009; Velin and Michetti, 2010; Liu *et al.*, 2011). Accordingly, it was thought that a multi-epitope vaccine based in both DNA and recombinant protein as antigens could bring advantages and confer effective protection against *H. pylori* infection.

Here we present our initial findings on the efficacy of this construct as a prophylactic vaccine in a mouse model.

3.2.1. Nanoparticle characterization

The delivery of nanoparticles by oral route involves overcoming several physiological barriers such as enzymatic degradation, low gastric pH, crossing the mucus layer and cellular uptake.

To overcome these barriers, chitosan nanoparticles appear as promising oral drug carrier system, due to its mucoadhesive properties. Such bioadhesiveness will enable a higher interaction between the carrier nanoparticle and the membrane epithelium, allowing a more effective uptake and transfection of the antigens (Aebischer *et al.*, 2010; Gonçalves *et al.*, 2011; Cadete *et al.*, 2012).

Chitosan binds to cell membranes, promoting transcellular and paracellular permeability showing high transfection efficiency, and for these reasons it has been used as a biomaterial for non-viral gene delivery system (Gonçalves *et al.*, 2011; Cadete *et al.*, 2012). The pDNA encapsulated in chitosan nanoparticles are taken up by cells endocytosis, macropinocytosis or phagocytosis in intracellular vesicles, where a small fraction of the DNA is released into cytoplasm and migrates into the nucleus, and is expressed (Cadete *et al*., 2012).

Particle size, DNA and antigen loading, and route of administration are the main characteristics that could influence the interaction with mucosal tissues, particle uptake, translocation and transport to the lymphatic nodes (Florindo *et al.*, 2009). Four types of nanoparticles were formulated, incorporating pDNA or recombinant protein antigens. CS/DS nanoparticles were used for intramuscular vaccination and CS/Alg/TPP nanoparticles were used in oral immunization. The physicochemical characteristic of nanoparticles were evaluated previously by (Gonçalves *et al.*, 2011) and are summarized in Table 3.2.1.1.

Table 3.2.1.1 – Charecteristics of nanoparticle used in immunization studies. Values are expressed as mean±S.D. (n=3).

Nanoparticles		**Mean size (nm)**	**pDNA/CS (w/w)**	**Encapsulation efficiency (%)**
CS/DS	pDNA	242 ± 8	32	100
	Protein	894 ± 6	-	70 ± 10
CS/Alg/TPP	pDNA	195 ± 1	15	100
	Protein	328 ±7	-	70 ± 8

In a previous work (Gonçalves *et al.*, 2011) it has been demonstrated, that chitosan/alginate nanoparticles can protect the encapsulated plasmid DNA from nuclease degradation, whereas pDNA is degraded when in direct *in vitro* exposure to the same environment. Moreover, nanoparticles containing DNA were able to transfect different cell lines, where high expression of genes was achieved, while showing low toxicity levels (Cadete *et al*., 2012).

3.2.2. Immunization studies

In order to measure and evaluate the immune response elicited by recombinant proteins and pDNA, BALB/c mice were immunized twice by oral and i.m. routes with recombinant proteins or pDNA, either nanoencapsulated or in solution. Animals were treated at day 1 (priming) and boosted at day 22 (see Table 2.2.2.1). Throughout the experiment, all immunized mice remained healthy. There were no adverse reactions after immunization including lethargy, weight loss, etc. (data not shown). Results were restrictively compared using nonparametric Mann-Whitney test.

As mentioned above (section 1.5.), despite it remains unclear which type of protective immune response does confer protection against *H. pylori* infections, there is consensus that CD4+ T cells are essential for this protection (Algood and Cover, 2006; Moss *et al.*, 2011). Interestingly *H. pylori* infections elicit only a Th1 polarized immune response, so the immune system is incapable of generating an efficiently balanced immune response. Therefore, it is thought that an effective therapeutic vaccine should induce a balanced Th1 and Th2 immune response for successfully eradicate *H. pylori* infections (Suerbaum and Michetti, 2002; Vajdy *et al.*, 2003; Zhou *et al.*, 2009).

To assess whether administered vaccines are leading or not to a balanced immune response, specific IgG isotypes were evaluated in immunised mice. The sera from negative controls (groups 9 and 10) were used as baseline to obtain the titre values for the treated groups. Two weeks after prime vaccination, immunized mice started to produce specific *H. pylori* IgG types (Figure 3.2.2.1).

It is important to point out that boosters at day 22 elicited a significant increase in antibody levels, which were maintained at the end of the study for almost all vaccinated groups (Figure 3.2.2.1).

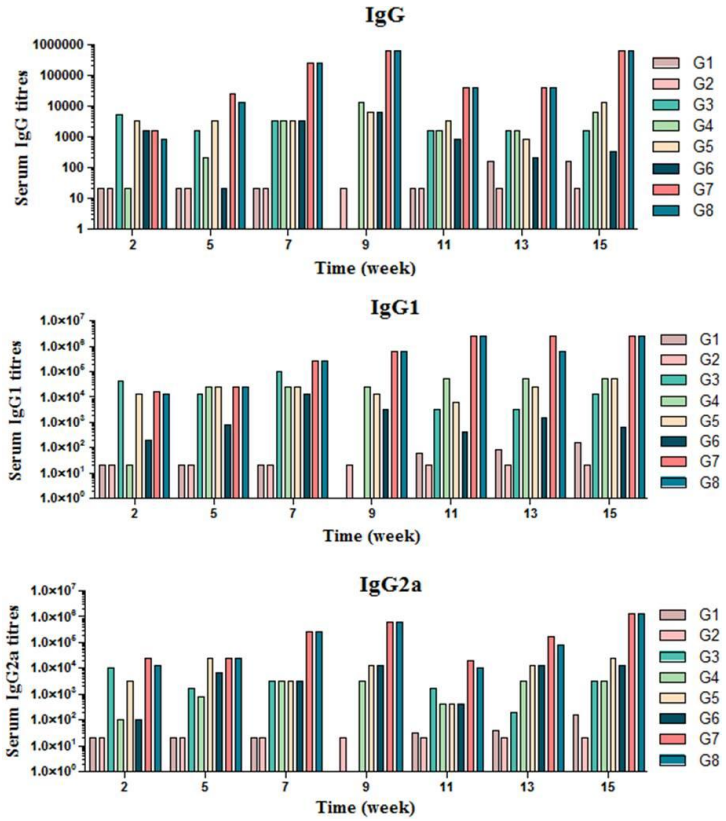

Figure 3.2.2.1 - Serum anti-*H. pylori* specific IgG, IgG1 and IgG2a titres induced after mice immunization with: G1 – nanoencapsulated pDNA by oral route; G2 – free pDNA in solution by oral route; G3 – nanoencapsulated recombinant protein by oral route; G4 – free recombinant protein in solution by oral route; G5 – nanoencapsulated pDNA by i.m. route; G6 – free pDNA in solution by i.m. route; G7 – nanoencapsulated recombinant protein by i.m. route; G8 – free recombinant protein in solution by i.m. route. Data represent a preliminary assessment concerning serum pool of each group (n=5).

Through specific IgG titres the type of response that is being generated can determined, because IgG1 is stimulated by a Th2 type immune response, whereas IgG2a is produced in a Th1 type response (Peek *et al.*, 2010; Velin and Michetti, 2010). So, high levels of both IgG1 and IgG2a indicate the stimulation of a balanced immune response.

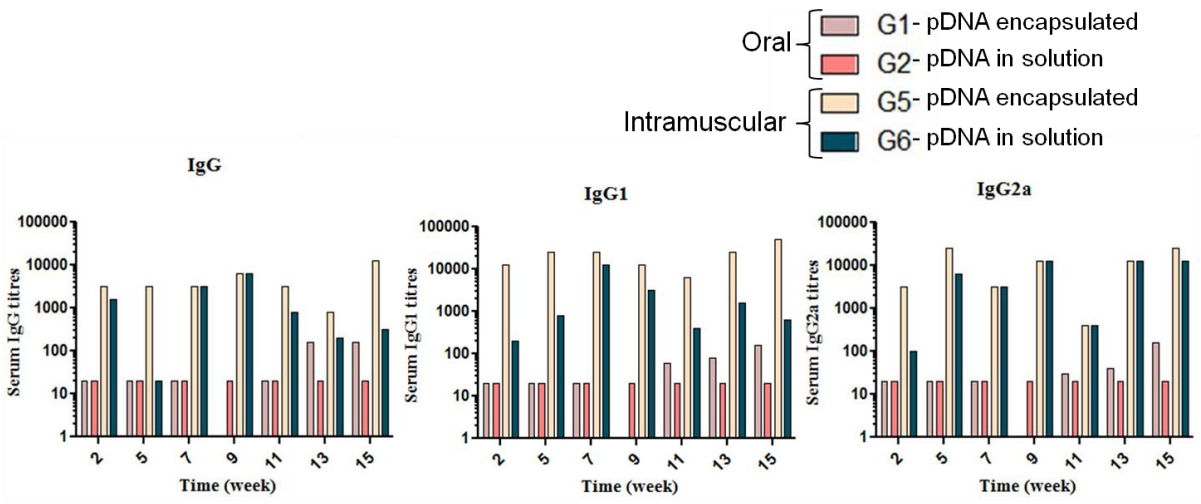

Figure 3.2.2.2 – Serum anti-*H. pylori* specific IgG, IgG1 and IgG2a titres induced by immunization with pDNA by both oral and i.m. route. Data represent a preliminary assessment concerning serum pool of each group (n=5).

It can be observed in Figure 3.2.2.2 that an effective immune response was triggered only by i.m. immunization with pDNA-based vaccines. Encapsulated and free pDNA administrated orally (G1 and G2) resulted in lower levels of all IgG isotypes when compared with groups immunized i.m., without significant differences among both oral groups (p=0.2500) and among Ig2a/IgG1 antibodies rate (p=0.1736).

Significant differences in the response to pDNA administered i.m. (G5 and G6) were found in IgG2a/IgG1 rate (p=0.0452). While pDNA in solution induced a strong Th1 response expressed by high levels of IgG2a, i.e. similar to immune response induced by natural *H. pylori* infections with inefficient infection elimination, nanoencapsulated pDNA induced a balanced Th1/Th2 immune response. This suggests a high immunogenicity of the multi-epitope antigen. In addition, it confirms the properties of CS nanoparticules, such as mucoadhesiveness and permeation-enhancement, allowing a more effective response through a prolonged interaction with the host cells surface, and consequently a high level of DNA transfection to host cells, as showed in Cadete *et al*. (2009).

Comparing both administration routes (Figure 3.2.2.2), pDNA encapsulated in chitosan nanoparticles administered i.m. showed to induce a higher, balanced and prolonged immune response by high levels of specific IgG antibodies.

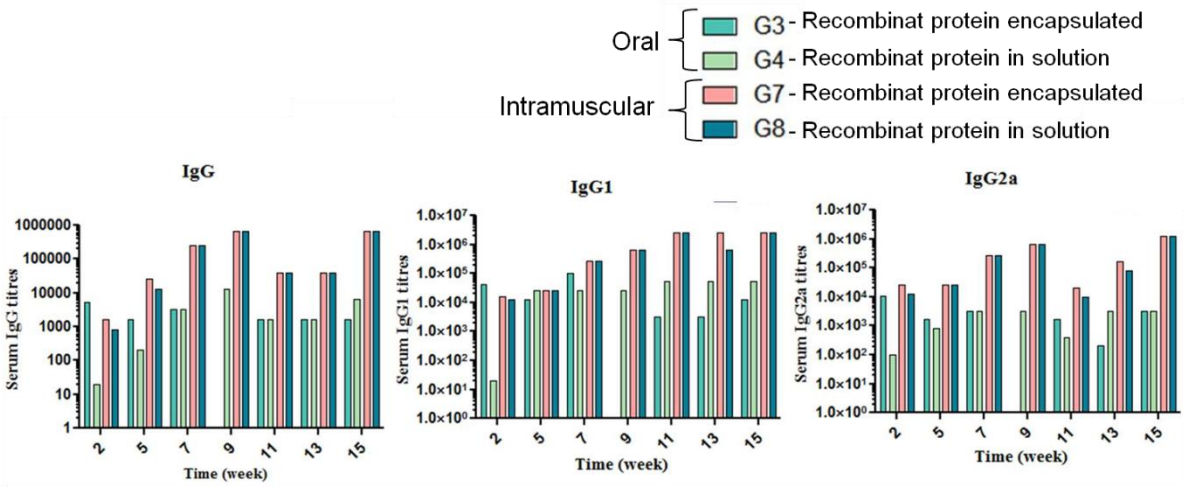

Figure 3.2.2.3 – Serum specific anti-*H-pylori* IgG, IgG1 and IgG2a titres stimulated by immunizations with 6T recombinant protein by oral (G3 and G4) and i.m. route (G7 and G8). Data represent a preliminary assessment concerning serum pool of each group (n=5).

Data in Figure 3.2.2.3 shows that there were significant differences between oral and i.m. immunizations. Recombinant proteins administered by i.m. route elicited a high and balanced immune response, confirming the strong potential of these vaccines for protection against *H. pylori,* as reported in previous studies by (Figueiredo *et al.*, 2012b). Other studies have showed the potential of intramuscular immunizations as protective (Zhou *et al.*, 2009; Azizi *et al.*, 2010; Liu *et al.*, 2011).

Oral immunization with these antigens could also induce an immune response with IgG1 predominance, showing the ability to induce a Th2 polarized response different from those induced by natural *H. pylori* infections. Further studies must be performed to confirm if this Th2 polarized response could confer protection against *H. pylori* infection, because it is controversial whether humoral response contributes to protective immunity (Zhou *et al*., 2009).

No significant differences were observed between both delivery systems, indicating that 6T recombinant protein is highly immunogenic and capable of inducing a strong immune response without any delivery system or adjuvant.

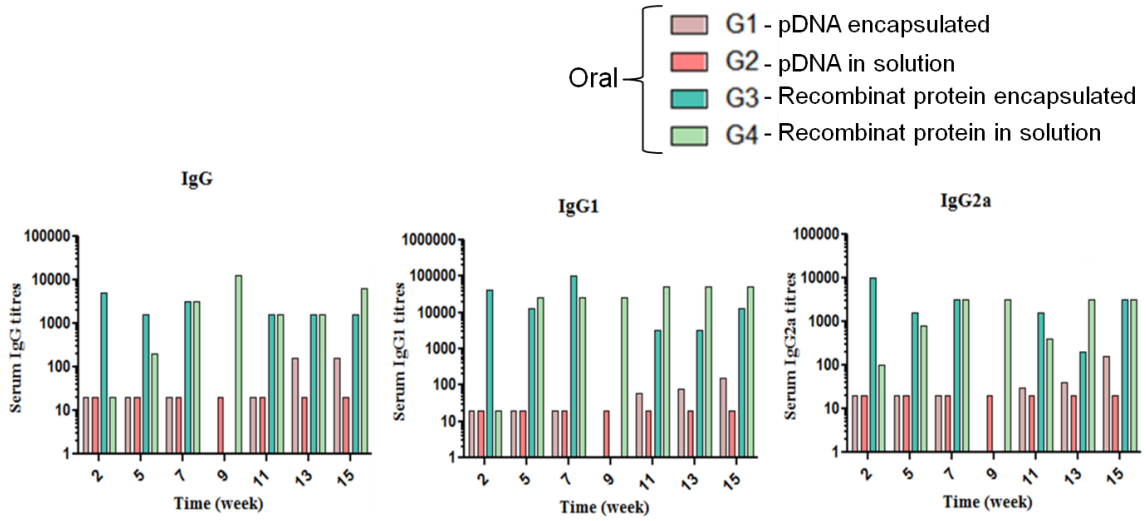

Figure 3.2.2.4 – Serum specific anti-*H. pylori* IgG, IgG1 and IgG2a titres from immunized mice with pDNA and 6T protein by the oral route. Data represent a preliminary assessment concerning serum pool of each group (n=5).

After oral immunization only recombinant proteins showed an efficient stimulation of specific IgG, IgG1 and IgG2a antibodies, whereas pDNA showed lower IgG isotypes titres. Significant differences (p=0.0056) were found between pDNA vaccines and those prepared with *H. pylori* recombinant antigens. These results lead to the conclusion that the nanoparticulate carriers used may not be suitable for the hostile environment of the stomach and may undergo degradation by the low pH environment and enzymes present in the stomach.

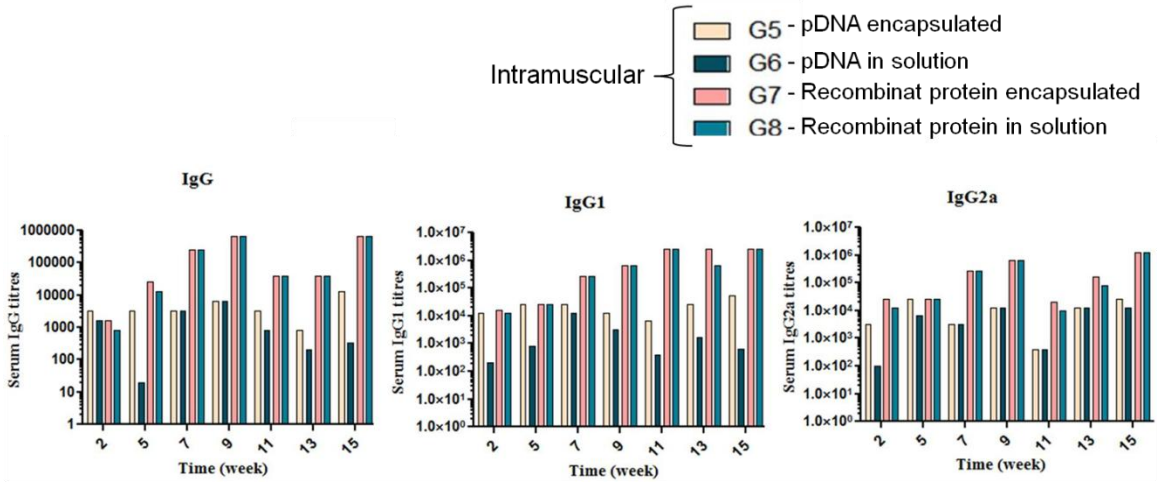

Figure 3.2.2.5 – Serum anti-*H. pylori* specific IgG, IgG1 and IgG2a titres of mice immunized by i.m. route, with pDNA and recombinant proteins, both encapsulated and in solution. Data represent a preliminary assessment concerning serum pool of each group (n=5).

Both i.m. administered nanoencapsulated pDNA and recombinant proteins induced strong Th1 and Th2 immune responses (Figure 3.2.2.5). Nevertheless, it was observed that recombinant proteins elicit a higher and more balanced immune response than that obtained with pDNA. This indicates that i.m. route could be a good approach for immunization, inducing of a strong systemic immune response by pDNA or recombinant proteins. No significant differences ($p=0.8376$) were found between IgG2a/IgG1 ratio in immune response triggered by recombinant protein vaccines administered intramuscularly, indicate a mixed Th1/Th2 immune response stimulation.

In the present study the two types of vaccines induced an effective immune response, where both Th1 and Th2 types were stimulated, except for pDNA vaccine administered orally. It was expected that a major IgG2a/IgG1 rate would result from pDNA vaccination, but it was the protein-based vaccines that induced higher IgG levels. Nevertheless, both vaccines were capable of inducing both Th1 and Th2 immune responses, being the highest responses triggered by the proteins given by i.m. route.

After i.m. administration chitosan nanoparticles slowly release the encapsulated pDNA, promoting a prolonged presentation of the antigens to APC cells. Moreover, the results demonstrate that these nanoparticles are efficient in stimulating a lasting immune response against *H. pylori* antigens, and act as a reservoir of the antigens since there was a prolonged immune response over 15 weeks. This is also due to their immunological adjuvant properties and immunogenic characteristics, such as mucoadhesiveness and penetration enhancing activity into host cells.

However, oral vaccination with nanoencapsulated pDNA did not induce a strong immune response, probably due to incapacity of penetrate the stomach mucosa or resist to the acid pH and enzymes present in the stomach (as aforementioned).

In general, it seems that CS particulate systems allowed the development of continuous and sustained immune response when compared with antigen solutions. Besides, both recombinant proteins and pDNA strategies for immunization showed promising results with a consistent Th1/Th2 immune response emerging as new approaches from vaccination against *H. pylori*. In that way, more studies will have to be done for increasing the efficacy of the delivery systems and selecting the best administration route. A heterologous immunization seems to be a potential approach since recombinant proteins showed higher capacity to stimulate both Th1 and Th2 responses by both routes of administration and pDNA showed the ability to induce mixed responses after i.m. administration. It also shows that the choice of routes to deliver plasmid DNA for obtaining higher and efficacious immunogenicity of the expressed antigens is rather restricted (Bohm *et al*., 1998).

3.2.2.1. Local IgA immune responses

As *H. pylori* infects their hosts by oral route and establishes an infection in the stomach, it was thought that oral immunization could provide a fist line of defence. Oral immunization was thought for mucosal immunity stimulation in order to induce a stronger systemic immune response against *H. pylori*, to confer a more protective immunity.

Other studies showed that local secretory IgA (sIgA) production at mucosal level, could be very important for establishing a first line of defence at the mucosal surface of the stomach in order to stimulate lymphocytes proliferation that will promoting the production of more specific IgA blocking *H. pylori* adhesion (Vajdy *et al.*, 2003; Moss *et al.*, 2011). The inhibition of bacterial adhesion may contribute to eliminate the infection. On the other hand, mucosal immunization will also promote systemic humoral and cellular immunity (Kabir, 2007; Zhou *et al.*, 2009; Moss *et al.*, 2011; Figueiredo *et al.*, 2012a).

To assess the success of the mucosal immunization, the sIgA levels present in intestines of vaccinated BALB/c mice, were evaluated (Figure 3.2.2.1.1).

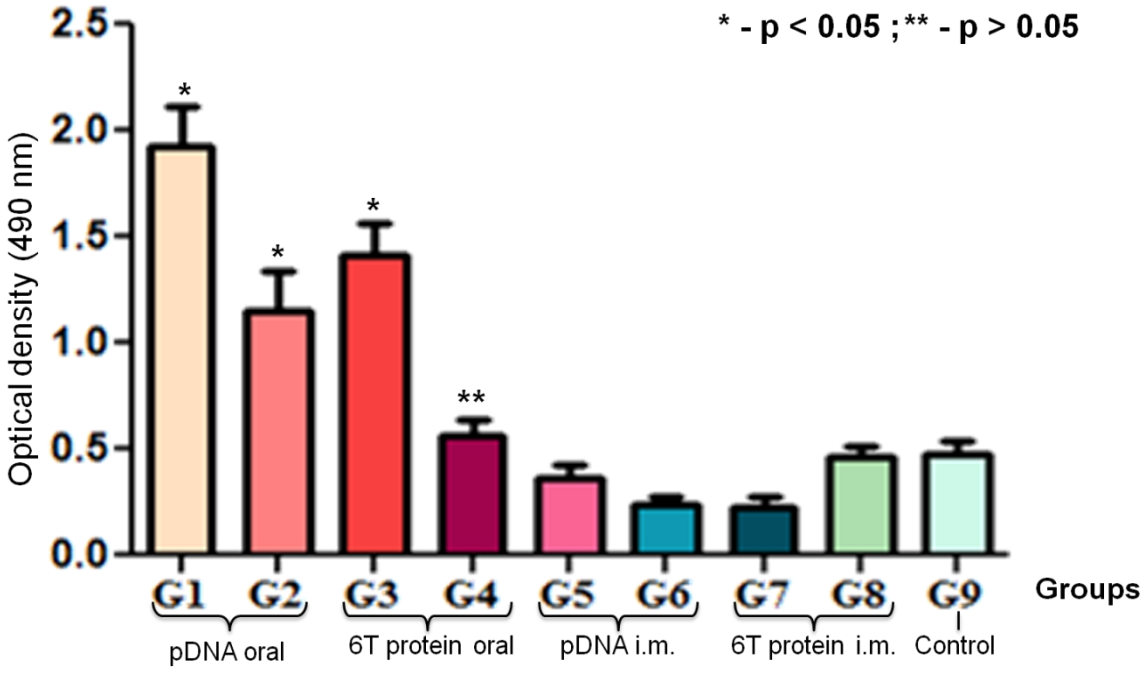

Figure 3.2.2.1.1 - sIgA levels in intestine homogenates of mice immunised by i.m. and oral route with: G1 – nanoencapsulated pDNA by oral route; G2 – free pDNA in solution by oral route; G3 – nanoencapsulated recombinant protein by oral route; G4 – free recombinant protein in solution by oral route; G5 – nanoencapsulated pDNA by i.m. route; G6 – free pDNA in solution by i.m. route; G7 – nanoencapsulated recombinant protein by i.m. route; G8 – free recombinant protein in solution by i.m. route (n=5; mean ± SD).

As expected, results showed a high level of local immunization by oral route, when compared with negative control (G9). Nanoencapsulated pDNA administered orally showed the greatest level of SIgA stimulation, showing a significant difference (p=0.0079) from the negative control. On the other hand, pDNA in solution also stimulated SIgA, but at levels much lower than those elicited by nanoencapsulated pDNA, despite showing no significant difference (p=0.0556).

Encapsulated recombinant proteins administered orally also induced high level of sIgA, when compared with negative control (p=0.0079), and present significant differences (p=0.0079) when compared with immunizations with recombinant proteins in solution (G4) by oral route. This indicates these antigens are not capable of inducing an effective immunity without the presence of an adjuvant or a delivery system capable to protect them from degradation and to increase immunogenicity and uptake by the host cells. Hence, chitosan nanoparticles are essential for efficient oral antigen presentation at epithelial cells, due to their capacity of adhesion to epithelium leading prolonged exposures to APC. Free recombinant proteins delivery orally (G4), present the same mucosal response that the group immunized with empty chitosan nanoparticles (G9). All other groups showed very low levels of sIgA, since they were not administered via mucosal and then induced no mucosal response, as expected.

Therefore, locally produced sIgA is thought to be important to prevent and control this endemic disease, mainly in their route of entry, being complemented by systemic immune response (Kabir, 2007). Some studies have shown the protective effect of mucosal immunization against H. Pylori, eradicating previous Helicobacter infections in mice (Vajdy *et al.*, 2003), providing eradication of ongoing infections and evidence that re-infection following oral vaccination could be prevented. As in this study, no cellular immunity was detected after oral immunizations, indicates that this route of immunization is not inducing the type of immunity found in mice prophylactically immunized. So, a positively approach in this study, could be heterologous immunization with pDNA administered orally and recombinant proteins administered intramusculary. The responses generated by heterologous vaccination may show whether there are indeed differences between the responses generated by oral vaccination and heterologous oral / i.m. vaccine.

The mainly difference that distinguishes our study, which confers advantages, is the fact that we use a vaccine comprising different antigens from different strains of *H. pylori* which will provide protection against a greater number of strains.

3.2.2.2. Cytokine production

The T lymphocytes expressing CD4+ at cell surface, stimulated by MHC II, release different types of cytokines. Through analysis of which type of cytokines is produced, we can infer what type of response is being stimulated. So T helper cells were divided according to the type of cytokines

secreted: Th1 cells mainly secret IL-2 and IFN-γ, stimulating a cellular immune response promoting synthesis of IgG2a, while Th2 cells express IL-4, IL-5, IL-6, IL-10 and IL-13, stimulating a humoral immune response promoting secretion of IgG1 (Florindo *et al*., 2009; Figueiredo *et al.*, 2012a; Wilson and Crabtree, 2007)

As seen before, CD4+ T cells are essential for protection against *H. pylori*. During *H. pylori* infection, only Th1 immune response is stimulated a cellular response being ineffective in eradicating this infection. Studies suggest that an induction of a well-equilibrate and balance Th1/Th2 immune response together with mucosal immunity, could eradicate the infection and confer protection (Vajdy *et al.*, 2003; Strugnell and Wijburg, 2010; Moss *et al.*, 2011).

In order to understand what type of immune response is actually induced by each vaccine formulation, the supernatants of cells extracted from the spleens of immunized mice were collected to quantify the cytokines produced in response to vaccines administered (Figure 3.2.2.2.1). Group 2 immunized with pDNA in solution by oral route and Group 4 immunized with recombinant proteins in solution by oral route were not analysed.

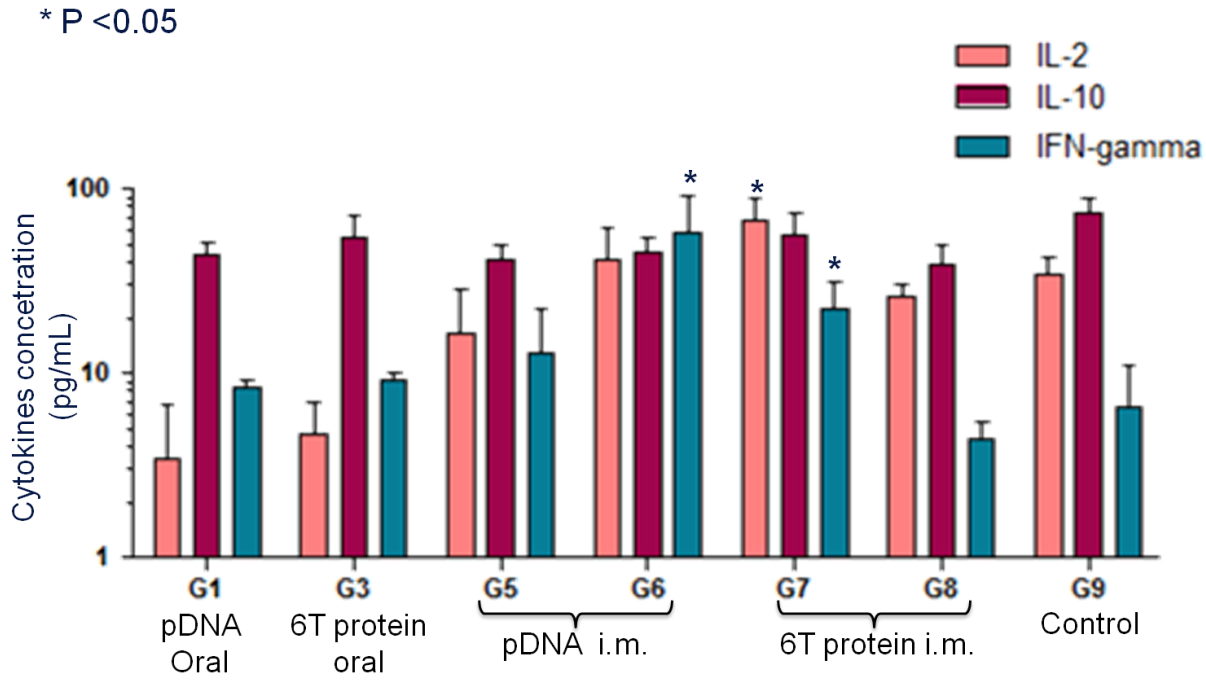

Figure 3.2.2.2.1 - Cytokine levels after splenocyte stimulation with *H. pylori* antigens (Note: G2 and G4 were not analysed). Columns with a (*) show significant differences ($p<0.05$) from the negative control (G9).

Figure 3.2.2.2.1 shows that all the immunized mice groups developed a humoral immune response, due the high levels of IL-10, including group 9 which is the negative control immunized with empty nanoparticles. This indicates that the nanoparticulate system used is highly immunogenic

and could induce both humoral and cellular responses, as shown by the high levels of IL-2 and IL-10, thus confirming the previous reports from our research group (Cadete *et al.*, 2012; Figueiredo *et al.*, 2012b).

On the other hand, immune response resulting from oral vaccination did not show significant differences between Group 1 (nanoencapsulated pDNA) and Group 3 (nanoencapsulated recombinant protein), stimulating essentially a Th2 polarized response, due to the chitosan nanoparticules.

The pDNA administered by i.m. route, either encapsulated or nonencapsulated, showed significant differences ($p<0.05$) among them, with free pDNA (G6) inducing a higher balanced immune response than encapsulated pDNA. Group 6 showed significant differences in IFN-γ when compared to negative control (G9) ($p=0.0079$), proved to be more efficient in induce a stronger and balanced immune response. Group 7, immunized with encapsulated proteins by i.m. route, induced a balanced Th1/Th2 immune response by IgGs isotypes levels, in agreement with high and equilibrate levels of cytokines. Significant differences were observed in IFN-γ ($p=0.0079$) and IL-2 ($p=0.0079$) when compared to negative control.

These results indicate that both antigens were able to induce an immune response by i.m. route, stimulate both Th1 and Th2 responses, which is in line with the levels of specific IgGs previously analysed (Section 3.2.2.).

These results substantiate that the epitope-based vaccine described here, should support the immune system fighting against *H. pylori* infections and prevent *H. pylori* colonization and infection.

3.3. *Helicobacter pylori* agglutinations assays

Sera from the last sampling of vaccination groups were tested against a total of nine *H. pylori* strains. The presumptive results of the agglutination tests are shown in Table 3.3.1.

The aim of this approach is to verify if antibodies present in sera of immunized mice (section 2.2.2.) specifically reacted with different, clinically relevant, *H. pylori* strains.

Maeland *et al.* (1997) demonstrated that almost all *H. pylori* strains used show strong agglutination with at least one of the two sera from infected mice. But a small percentage of *H. pylori* strains showed autoagglutination. These tests are based on the variability of the surface-localized antigens of each *H. pylori* strain, which function as agglutinogens in presence of specific antibodies against specific *H. pylori* strain antigens. These results allow identifying the majority of cultured *H. pylori* strains by agglutination testing (Maeland *et al*., 1997).

It should be noticed that this is a presumptive test and that as in all agglutination tests, it is very difficult to conclude if there was bacterial agglutination with sera tested, due to the negative control (*H. pylori* strain only) that almost always stays with a cloudy appearance and small agglomerates with bleached centre. Even with sera from negative control mice (immunized with empty nanoparticles –

G9) it was difficult to understand whether or not there was agglutination of bacteria. This may be due to the fact that *H. pylori* cultures may form a biofilm when in suspension (Vale and Vitor, 2010).

Table 3.3.1 – Results from agglutination tests with *H. pylori* bacterial suspension against sera of immunized mice. (+) indicates positive results; (-) indicates negative results where no agglutination is observed; (?) indicates inconclusive results.

Sera groups	**G1**		**G2**		**G3**		**G4**		**G5**		**G6**	
Strains / **Dilutions**	1:10	1:40	1:10	1:40	1:10	1:40	1:10	1:40	1:10	1:40	1:10	1:40
B1A	-	-	-	-	-	-	-	-	-	-	-	-
B6A	+	+	?	?	+	+	+	+	+	+	+	+
B8A	+	+	+	+	-	-	?	?	-	-	+	+
BAPOUI	+	+	+	+	+	+	+	?	?	?	+	+
P1	-	-	-	-	-	-	-	-	-	-	-	-
26695	+	+	+	+	?	?	+	+	+	+	+	+
HS11	+	-	+	+	+	-	-	+	+	+	-	?
B45	+	+	+	+	+	+	+	+	+	+	+	+
B3C	-	-	-	-	-	-	-	-	-	-	-	-

	G7		**G8**		**G9**	
Strains/Dilutions	1:10	1:40	1:10	1:40	1:10	1:40
B1A	-	-	-	-	-	-
B6A	+	+	+	+	+	+
B8A	+	+	+	+	+	+
BAPOUI	+	+	+	+	+	+
P1	+	-	-	-	-	-
26695	+	+	+	+	+	+
HS11	+	+	+	+	+	-
B45	+	?	?	+	+	+
B3C	-	-	-	-	-	-

In B1A and B3C (Table 3.3.1) no agglutination was observed with any sera used, indicating the sera from immunized mice do not contain antibodies against the antigens present in these strains. In all of the others strains agglutinations were observed with all the sera of immunized mice, even with the sera from negative control groups (G9).

These agglutination tests based on visual observation are rather subjective and presumptive, depending largely on operator interpretation. So, in order to confirm if the antibodies present in sera of

immunized mice effectively react with antigens of different *H. pylori* strains, Western Blot must be performed in future studies.

4. Conclusions

Despite decades of research, the immune response to *H. pylori* and the correlation of successful immunity necessary for protection and eradication of *H. pylori* remain poorly understood. The immunity generated after *H. pylori* infections always show inadequate ability in eradicating the infections and due to the continued process of inflammation the infection may progress to chronic gastritis, peptic ulcers or gastric cancer (Algood and Cover, 2006; Wilson and Crabtree, 2007; Zhou *et al*., 2009; Moss *et al*., 2011).

Current therapies used in treatment of *H. pylori* infections comprise combination of antibiotics in conjunction with proton pump inhibitor, leading to several problems such as re-infections, side effects and antimicrobial resistance. Accordingly, it is unanimous that therapy-based vaccines anti-*H. pylori* are the best approach to eradicate the infection and prevent new infections.

Several vaccine approaches are being made, using a wide range of strategies such as the use of whole cell lysates, or specific recombinant antigens, but previous studies in animal models provided poor or no immunity against *H. pylori* (Kabir, 2007; Zhou *et al.*, 2009; Liu *et al*., 2011). So a multi-epitope based vaccine was constructed based on most common virulence factors among *H. pylori* strains and on most virulence antigens essential for successful infection by *H. pylori*. The antigens epitopes chosen were cloned into two different plasmid vectors so that recombinant protein is expressed in both prokaryotic and eukaryotic cells. In order to formulate pDNA- and recombinant proteins-based vaccine, the recombinant proteins were produced in *E. coli* transformed cells with recombinant pQE30 plasmid and then purified through a nickel-affinity chromatography column. The purification process was achieved successfully confirmed by SDS-PAGE and Western Blot, protein has been purified either as soluble or insoluble. However 6T protein does not need to be soluble to retain its function, it can be used directly as antigen since it retains its immunogenicity. The pDNA was also extracted from *E. coli* transformed cells with recombinant pVAX plasmid and then purified through Mega QIAfilter Plasmid purification kit (QIAGEN Inc., German). The pDNA was successfully purified, without any other components as shown by electrophoretic analysis. The two antigens, pDNA and the recombinant proteins, were assessed as a therapeutic vaccine in a mouse model.

The assessment of systemic titres of specific IgG, IgG1 and IgG2a antibodies reveals a better immune response after i.m. immunization. Moreover, recombinant protein encapsulated in nanoparticles present a high and more balanced immune response than that obtained with encapsulated pDNA. Cytokine levels support the evidence for a more relevant immune response after i.m. immunization, which is in agreement with IgG titres. The chitosan nanoparticles also showed high levels of IL-2 and IL-10 that confirm the immunological adjuvant properties of these nanoparticles. Intramuscular immunization with encapsulated proteins results in a well-balanced immune response confirmed by the presence of the characteristic Th1/Th2 cytokines and specific IgG titres.

The oral vaccination shows a higher stimulation of sIgA, which can be a good result for controlling the infection and preventing the colonization of the gastric mucosa. In order to confirm this

findings a challenge assay with *H. pylori* will be needed to study if there is colonization and infection or not, after oral vaccination.

The finding that *H. pylori* infections stimulate a Th1-polarized immune response that showed ineffective in fighting against these infections, suggest that a balanced Th1/Th2 immune response are important to mediate an effective immunity against *H. pylori* in order to protect and eradicate infections. On the other hand, there is consensus of our research group that a mucosal immunity could confer a fist line of defence at mucosal level leading to prevent new infections. So, , we developed a multi-epitope pDNA- and recombinant protein-based vaccine that showed an efficient induction of both Th1 and Th2 response, with high IgG1 and IgG2a titres together with high cytokine levels, mainly by i.m. route. On the other hand, oral vaccination induced high levels of SIgA indicating an effective stimulation of mucosal immunity, conferring protection at this level. Duo to the high stimulation of systemic immunity by i.m. immunization and high stimulation of mucosal immunity by oral immunization, a DNA-prime/peptide boost could be a good approach for future immunization studies.

In conclusion, this study shows that a multi-epitope vaccine is a promising candidate for the development of an adequate immunity against *H. pylori* infections. Ongoing studies will evaluate the best routes of immunization and the best approach to improve protective immunity. Ultimately, we hope that these studies will lead to a therapeutic and prophylactic effective vaccine for human use.

Following this work, it would be interesting, in future studies, to compare the responses generated by an oral vaccination and a heterologous oral/ i.m. vaccination, in order to infer what is the best approach, since our objective was the development of a vaccine for oral administration, due to its advantages in administration, security, storage and stability. It would be also interesting to evaluate the protective immunity conferred by this vaccine, by performing an experimental infection in immunized animals. After proven successful immunization of mice, the next step would be to evaluate the potential of the vaccine in prime *H. pylori* hosts, the humans.

Agglutination slide tests made showed agglutination of some *H. pylori* strains with most sera from immunized mice, but also agglutination of certain strains of *H. pylori* with sera from immunized mice with empty nanoparticles without the presence of antigens. Agglutination slide tests will have to be optimized in order to obtain more concrete and reliable results and must be confirmed by Western Blot to confirm the presence of anti-*H. pylori* antibodies in sera of immunized mice.

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