Universidade do Minho
Escola de Engenharia

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On the routes of Helicobacter pylori transmission among the humans

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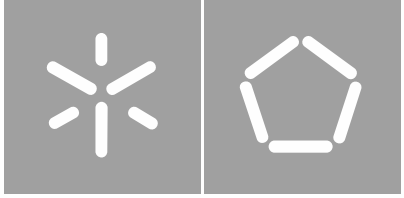

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On the routes of Helicobacter pylori transmission among the humans

Tese de Doutoramento
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Professora Doutora Maria João Vieira e da
Professora Doutora Céu Figueiredo

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Abstract

Helicobacter pylori is a spiral, microaerophilic, Gram-negative bacterium that colonizes the human stomach and has been associated with the pathogenesis of chronic gastritis, peptic ulcer disease and gastric carcinoma. Since the isolation of *H. pylori*, numerous studies have been published addressing the prevalence and epidemiology of the infection, the relationship with disease, the identification and characterization of virulence factors and their role in pathogenesis. Nevertheless, the routes of transmission of this bacterium are still a matter of controversy. Both epidemiologic and microbiologic data support direct person to person contact as responsible for the most successful *H. pylori* colonizations. The most relevant routes of person to person transmission are the gastro-oral, oral-oral, and fecal-oral routes. There is also a growing amount of data reporting the identification of *H. pylori* in external environmental reservoirs, most significantly in water. The majority of studies that have investigated drinking water, or drinking water-related conditions, as a risk factor for *H. pylori* infection support a relationship between these parameters. Therefore, exposure of humans to *H. pylori* from water may not be neglectable. As such, this Thesis explores aspects of *H. pylori* routes of transmission, considering both the person to person transmission and the human exposure to *H. pylori* from water environments.

In Chapter 1, the literature is reviewed focusing on several aspects of *H. pylori*, with major emphasis on methods for diagnosis, prevalence and routes of transmission of the infection. The selection of the methods used for *H. pylori* detection is of utmost importance for achieving the best results. In Chapter 2, a fluorescence *in situ* hybridization (FISH) method for the rapid detection of *H. pylori* using a novel peptide nucleic acid (PNA) probe was developed. Laboratory testing with different bacterial species, including other *Helicobacter* spp., showed that this probe is highly specific for *H. pylori* strains. In addition, the PNA-FISH method has been successfully adapted for detection of the pathogen in bacterial smears and in paraffin-embedded gastric biopsies.

The routes of *H. pylori* transmission consider the oral cavity as the means of entry of the bacteria in the human host. In Chapter 3, the PNA-FISH assay was used, together with other *H. pylori* detection methods, to evaluate whether the oral cavity, specifically adenoids and tonsils, may constitute an extra-gastric reservoir for *H. pylori*. Sixty-two children from the North of Portugal were included in the study, and the presence of *H. pylori* in adenoids and in tonsils was evaluated in a total of 101 surgical specimens.

Results showed that detection methods such as the rapid urease test and immunohistochemistry that have a high specificity for gastric samples, originate false positive results in samples from polimicrobial environments as the adenotonsillar tissue. In all cases of adenoid and tonsillar specimens analysed *H. pylori* detection was negative, even in children that had a gastric infection assessed by serology. Therefore, is it likely that the adenotonsillar tissue does not constitute an extra-gastric reservoir for *H. pylori*, or at least a permanent one.

Also considering the importance of the oral cavity in *H. pylori* transmission, in Chapter 4, the influence of the exposure of *H. pylori* to saliva and its consequences in the survival and infection capacity of the bacteria were evaluated. The culturability of saliva exposed *H. pylori* was assessed, and it was observed that only at exposure times higher than 24 hours the bacteria loses culturability. Furthermore, contact with saliva did not alter the ability of *H. pylori* to adhere to and to induce IL-8 secretion by the host cells within the period that bacteria remain viable. This led to the conclusion that only long times of exposure to saliva affects the properties of *H. pylori*. One can speculate that, since saliva is constantly being swallowed, after *H. pylori* enters the oral cavity it can rapidly reach the gastric environment and, since adhesion properties are not altered by the contact with saliva in this time period, bacteria are viable and able to colonize the gastric mucosa.

In Chapter 5, and considering exposure of humans to *H. pylori* from environmental sources like water, water exposed *H. pylori* was evaluated regarding its culturability and the capacity to produce structural components of pathogenicity like the *cag* type IV secretion system (T4SS). Further, water exposed *H. pylori* were assessed for their capacity to adhere to host cells and to induce inflammation and apoptosis in those cells. When exposed to water, *H. pylori* loses the culturability, the ability to induce host cell inflammation and apoptosis, which can be attributed to the non-functionality of the T4SS. Nevertheless, water-exposed *H. pylori*, although to a lesser extent, are still able to adhere to the host cells, an important property that might allow the bacterium to colonize the gastric epithelium. Overall, water-exposed *H. pylori* showed a decreased interaction with the host and from the standpoint of the microorganism, attenuation of inflammation and of cell apoptosis may be beneficial in the sense that it may improve the likelihood for the establishment and persistence of the infection. It is therefore possible that *H. pylori* from water environments recover their capacity to colonize and to infect when reaching the gastric environment.

Resumo

Helicobacter pylori é uma bactéria espiralada, microaerofílica, Gram-negativa que coloniza o estômago humano e está associada à etiopatogénese da gastrite crónica, úlcers péptica e carcinoma gástrico. Desde o isolamento de *H. pylori*, vários estudos foram publicados e tanto os dados epidemiológicos como os microbiológicos suportam o contacto directo pessoa a pessoa como o responsável pelas colonizações de *H. pylori* mais bem sucedidas. As vias mais relevantes na transmissão pessoa a pessoa são as vias gastro-oral, oral-oral e fecal-oral. Há também cada vez mais evidências da presença de *H. pylori* em reservatórios externos ambientais, principalmente na água. A maior parte dos estudos que investigaram a água potável, ou condições associadas à água potável, identificaram estes parâmetros como factores de risco para a infecção por *H. pylori*. Assim sendo, a exposição de humanos a *H. pylori* presente na água, não deve ser negligenciada. Em suma, esta Tese explora aspectos das vias de transmissão de *H. pylori*, considerando a transmissão pessoa a pessoa bem como a exposição humana a *H. pylori* presente na água.

No Capítulo 1, foi feita uma revisão da literatura englobando diversos aspectos da infecção por *H. pylori*, com maior ênfase nos métodos de diagnóstico, prevalência e vias de transmissão da infecção. A selecção do(s) método(s) utilizados para detectar *H. pylori* é de extrema importância para a obtenção de resultados inequívocos. No Capítulo 2, foi desenvovido um método de hibridação *in situ* usando fluorescência (FISH) para a detecção rápida de *H. pylori* usando uma nova sonda de ácido nucleíco peptídico (PNA). Testes laboratoriais com espécies bacterianas, incluindo espécies que não *Helicobacter* spp., mostraram que esta sonda é altamente específica para a as estirpes de *H. pylori*. Além disso, o método de PNA-FISH foi adaptado com sucesso para detecção de *H. pylori* em esfregaços bacterianos e em biopsias gástricas incluídas em parafina.

As vias de transmissão de *H. pylori* consideram a cavidade oral como o meio de entrada da bactéria no hospedeiro humano. No Capítulo 3, o método de PNA-FISH foi usado, juntamente com outos métodos de detecção da bactéria, para avaliar se a cavidade oral, especificamente as adenóides e as amígdalas, podem constituir um reservatório extra-gástrico para *H. pylori*. No estudo foram incluídas 62 crianças do Norte de Portugal, tendo sido determinada a presença de *H. pylori* nas adenóides e amígdalas num total de 101 amostras cirúrgicas. Os resultados mostraram que métodos de detecção como o teste rápido da urease e imunohistoquímica, que são altamente específicos em amostras

gástricas, originaram falsos positivos em amostras de ambientes polimicrobiais, como é o caso do tecido adeno-amigdalino. Em todas as amostras de adenóides e amígdalas que foram analisadas a detecção de *H. pylori* foi negativa, mesmo em crianças que possuíam infecção gástrica diagnosticada por serologia. Estes resultados sugerem que o tecido adeno-amigdalino não constitui um reservatório extra-gástrico para *H. pylori*, pelo menos que não constitui um reservatório permanente de *H. pylori*. Ainda considerando a importância da cavidade oral na transmissão de *H. pylori*, no Capítulo 4 foi analisada a influência da exposição de *H. pylori* à saliva e as respectivas consequências na sobrevivência e capacidade de infecção da bactéria. A culturabilidade de *H. pylori* exposta à saliva foi estudada, obervando-se que a bactéria apenas perde a culturabilidade com tempos de exposição superiores a 24 horas. O contacto com a saliva não alterou a capacidade de *H. pylori* para aderir e induzir secreção de IL-8 pelas células do hospedeiro no período de tempo que a bactéria permanece viável. Isto permitiu concluir que apenas tempos longos de exposição à saliva afectam as propriedades de *H. pylori*. Uma vez que a saliva está constantemente a ser engolida, pode-se especular que, depois de *H. pylori* entrar na cavidade oral, a bactéria pode rapidamente atingir o ambiente gástrico. Tendo em conta que a capacidade de adesão não é alterada com a exposição à saliva neste espaço de tempo, a bactéria mantém-se viável e é capaz de colonizar a mucosa gástrica. No Capítulo 5, avaliou-se a influência da exposição à água na culturabilidade de *H. pylori* e na capacidade para produzir componentes estruturais de patogenicidade tal como o "*cag* type IV secretion system" (T4SS). Foram ainda estudadas a capacidade de *H. pylori* exposta à água de aderir a células do hospedeiro e de induzir inflamação e apoptose nessas células. Quando exposta à água, *H. pylori* perde a culturabilidade e a capacidade de induzir inflamação e apoptose nas células do hospedeiro, o que pode estar relacionado com a não funcionalidade do T4SS. No entanto, depois de exposta à água, *H. pylori* mantêm ainda uma considerável capacidade de aderir às células do hopedeiro, uma propriedade importante na colonização do epitélio gástrico. Em suma, *H. pylori* exposta à água mostrou ter uma menor interacção com o hospedeiro e, do ponto de vista do microorganismo, menos inflamação e diminuição da apoptose das células do hospedeito pode ser benéfico no sentido de poder aumentar a probabilidade do estabelecimento e persistência da infecção. Assim é possível que *H. pylori* presente em reservatórios ambientais como a água, consiga recuperar a sua capacidade para infectar e colonizar a mucosa após atingir o ambiente gástrico.

Contents

List of Figures

List of Tables

List of symbols and abbreviations

AGS	Human Gastric Cancer Cell Line
ATCC	American Type Tissue Culture
ATP	Adenosine Triphosphate
BabA	Blood Group Antigen binding Adhesin
bp	Base Pairs
BSA	Bovine Serum Albumin
CAC	Citric Acid Cycle
***cag* PAI**	*cag* Pathogenicity Island
CFU	Colony Forming Units
CO_2	Carbon Dioxide
DEIA	DNA Enzime Immuno Assay
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleotide Triphosphate
EDTA	Ethylenediamine Tetraacetic Acid
EIA	Enzyme Immunoassays
ELISA	Enzyme-Linked Immunosorbent Assay
FBS	Fetal Bovine Serum
FISH	Fluorescence *in situ* Hybridization
***glm*M**	Phosphoglucosamine Mutase
H_2	Hydrogen
H2O2	Hydrogen Peroxide
HCl	Hydrochloric Acid
Hp	*Helicobacter pylori*
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IL-8	Interleukin 8
MOI	Multiplicity of Infection
NaCl	Sodium Chloride
NCTC	National Collection of Type Cultures
O_2	Oxygen
PBS	Phosphate Buffered Saline

PCR	Polymerase Chain Reaction
PMSF	Phenylmethylsulfonyl Fluoride
PNA	Peptide Nucleic Acid
PNL	Polymorphonuclear Leucocytes
PPI	Proton Pump Inhibitor
RNA	Ribonucleic Acid
rRNA	Ribosomal Ribonucleic Acid
RUT	Rapid Urease Test
SabA	Sialic Acid Binding Adhesin
T4SS	Type Four Secretion System
TMB	Tetramethylbenzidine
TSA	Tryptic Soy Agar
TUNEL	Terminal Uridine Deoxynucleotide Nick End-Labeling
UBT	Urea Breath Test
ureA	Subunit A of Urease Gene
ureB	Subunit B of Urease Gene
VBNC	Viable but Non Culturable

Chapter 1

Background and Aims

1.1 The Emergence of Helicobacter pylori

The first well-known report of gastric *Helicobacters* was by the anatomist Bizzozero in 1893 [1]. In hand-drawn color illustrations, he showed gram-negative "spirochetes" with approximately 10 wavelenghts in the gastric mucosa of dogs [2]. Some years later, Salomon was able to propagate these spiral organisms in mouse stomachs after feeding ground-up gastric mucosa of cats and dogs to his mouse colony [3]. However, reports of gastric *Helicobacter* in humans only occur in 1940 by Freedberg and Baron who found "spirochetes" in about 40% of resected human gastric specimens [4]. In the 1960's, Susumu Ito was studying the gastric mucosa appearance under the electron microscope when he found spiral organisms in his gastric samples. He published a photograph of one of these microorganisms, showing several sheathed flagella and spiral morphology [4]. Steer and Colin-Jones published in 1975 a paper where they noted that numerous spiral bacteria were present in 80% of their gastric ulcer specimens (**Figure 1.1**) [5]. They published excellent photographs of the gastric mucosa histology including spiral-shaped bacteria in the mucous layer. However they were unable to culture the microorganisms [4].

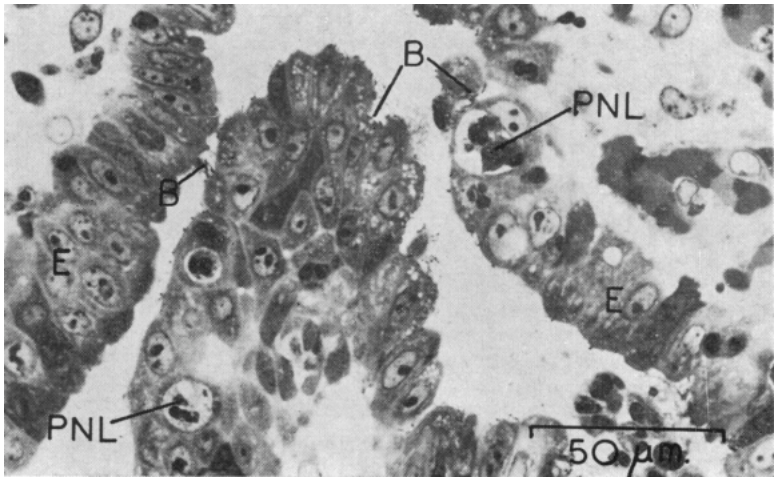

Figure 1.1 – Gastric mucosa from the gastric lesser curvature of a patient with a gastric ulcer. Bacteria (B) in close proximity with the gastric epithelium (E). Polymorphonuclear leucocytes (PNL) migrating through the gastric epithelium. Section stained with methylene blue Azur 11 [5].

The first work reporting the plate culture of *H. pylori* was only published in 1984 by two Australian scientists, Warren and Marshal [6], which were recently awarded the Nobel Prize in Physiology or Medicine. Warren and Marshall isolated *H. pylori* from biopsy specimens taken from antral mucosa of human patients submitted to gastric endoscopy. Since the bacteria were present in nearly all patients with active chronic gastritis, duodenal ulcer, and gastric ulcer, it was considered to be an important factor in the etiology of these diseases [7]. The first denomination of these new bacteria was initially *Campylobacter pyloridis*

[8] due to the morphological and physiological similarities with the *Campylobacter* genus, and afterwards corrected to *Campylobacter pylori* [9]. The current denomination of *Helicobacter pylori* occurred in 1989 [10] due to the identification of important physiological differences between this organism and other *Campylobacter* spp..

1.2 Helicobacter pylori Microbiology

1.2.1 Taxonomy

Genus *Helicobacter* belongs to the Ɛ subdivision of the Proteobacteria phylum, order *Campylobacterales*, family *Helicobacteraceae*. This family also includes the genera *Wolinella*, *Flexispira*, *Sulfuricurvum*, *Sulfurimonas*, *Thiomicrospira* and *Thiovulum* [11]. To this date, the genus *Helicobacter* consists of over 40 recognized species, with many species awaiting formal recognition.

Helicobacter species can be subdivided in two major lineages according to the colonization location, the gastric *Helicobacters* and the enterohepatic (non-gastric) *Helicobacters*. Both groups demonstrate a high level of organ specificity, such that gastric *Helicobacter* species in general are unable to colonize the intestine or liver, and vice-versa [11].

1.2.2 Morphology

Helicobacter pylori in vivo and under optimum *in vitro* conditions presents a spiral form with 2 to 4 µm long and 0.5 to 1 µm wide and have 2 to 6 unipolar sheathed flagella of approximately 3 µm in length, which often carry a distinctive bulb at the end [12]. This bacterium, when left in culture for many days or when exposed to detrimental environmental circumstances, can also assume an alternative coccoid form that range from 1 to 4 µm, passing through a U-shape during the conversion from one to another [13]. Since the spiral form is commonly found *in vivo*, is has been associated with the infectious form of the pathogen. The coccoid forms occurs when the bacterium is exposed to non-optimal conditions, such as nutrient deprivation [14], and prolonged incubation [15], suggesting that these forms could be a dormant stage of *H. pylori* and might play a role in the survival of the bacterium in a hostile environment. On the other hand, some authors defend that the conversion of the bacterium from spiral to coccoid is

a passive process that does not require protein synthesis, and the coccoid form represents the morphological manifestation of cell death [16-17]. In 2003, Saito *et al.*, have classified the coccoid forms of *H. pylori* into three groups, representing different transformation processes, living and culturable bacteria, viable but non-culturable bacteria, and dying bacteria [18]. The pathophysiological role of each form are still a subject of controversy.

1.2.3 Metabolism and Physiology

The energetic metabolism of *H. pylori* appears to be primarily that of an aerobic, respiring bacterium. Respiration provides the ability to the bacterium to conserve energy in the form of adenosine triphosphate (ATP) or perform energy-demanding processes through the generation of a transmembrane motive force. The conversion of the proton electrochemical gradient across bacterial cytoplasmic membrane into ATP is accomplished by the ATP synthase. The prime generator of the proton electrochemical gradient is the respiratory chain, where organic compounds, such as D-Glucose [19], or inorganic, such H_2 [20], are submitted to a process of oxidation. Apart from fumarate [21], there is no direct experimental evidence that *H. pylori* is able to use alternative acceptors other than oxygen, explaining the requirement for oxygen in this bacterium. Despite an obligate requirement for oxygen, the bacterium possesses several essential, highly oxygen-labile metabolic enzymes typical of anaerobic type metabolism [22]. Moreover, *H. pylori* present several mechanisms to protect from the threat of damage from oxygen per se or one of the radicals produced during the oxygen reduction [23]. For all this, *H. pylori* is a microaerophilic bacterium with optimal growth at O_2 levels of 2 to 5% and the additional need of 5 to 10% CO_2 and high humidity [11].

H. pylori exhibits a narrow host and target organ range which suggests a strong adaptation to its natural habitat, the mucus layer overlying the gastric epithelial cells. As a consequence, *H. pylori* lacks several of the biosynthetic pathways commonly found in less specialized bacteria, such as enteric bacteria [21, 24-25]. The citric acid cycle (CAC) plays a key role both in catabolic and biosynthetic pathways and is present in most bacteria. Genomic analysis of *H. pylori* CAC genes failed to identify several homologs of genes encoding enzymes necessary to the typical CAC. As a consequence, it has been suggested that *H. pylori* possesses a branched incomplete CAC [21, 26].

Urease is central to *H. pylori* metabolism and virulence. This highly active enzyme is produced in large amounts by the bacteria. It has been estimated that up to 10% of the total protein content of *H. pylori* consists of urease [27]. The urease enzyme catalyzes the hydrolysis of urea into carbon dioxide and ammonia which helps to neutralize the acid environment of the stomach, allowing *H. pylori* to colonize the gastric mucosa [28-29]. On the other hand, ammonia is the major source of nitrogen in *H. pylori*, and the metabolism of this bacterium seems to be adapted to an environment in which this compound is rarely limiting. The large amounts of ammonia generated by *H. pylori* are probably involved in bacterial pathogenesis. The ammonia produced by urease was shown to be toxic for various gastric cell lines [30].

The absence of several amino acid synthesis pathways in *H. pylori* is probably due to the adaptation of the bacteria to the stomach, which leaves the bacterium dependent on many of the amino acids from the host to their own transcriptional apparatus [31].

The metabolism and physiology of *H. pylori* are still not fully understood.

1.2.4 Genome

The genome of *H. pylori* contains ≈ 1.7 Megabase pairs with a G+C content of 39% and ≈ 1,500 predicted coding sequences [32]. *H. pylori* has an extraordinary genetic heterogeneity, although similarities between strains based on human-population origins are maintained [33]. In fact, diversity among the strains includes variation in the complement of genes, chromosomal gene order, deployment of repetitive DNA, sequence variation in conserved genes, homoplasies, status of phase-variable genes, complement of restriction-modification loci and mobile DNA [34]. The plasticity of the *H. pylori* genome derives from its natural competence for transformation by exogenous DNA, from recombination and from mutations. These properties are the origin of an extensive allelic diversity occurring even in a single host. Furthermore, *H. pylori* has a mutation rate significantly higher than that of many other bacteria. Genome analysis reveals that this bacterium apparently lacks homologues of many of the genes that contribute to DNA repair [35], and it has been suggested that competition between repair and anti-repair pathways may provide a mechanism to generate strain diversity [36]. *H. pylori* genome also contains numerous repetitive sequences of different lengths that permit intragenomic deletions or rearrangements [37-39]. In addition to the intrastrain diversification mechanisms outlined above, it has been suggested that

recombination between different strains during colonization of an individual host could also contribute for the genetic diversity [40-41]. This genetic diversification may help *H. pylori* to adapt to a new host after transmission, to different micro-niches within a single host, and to changing conditions in the host over time, for example, avoiding clearance by host defenses [42].

1.3 Helicobacter pylori Pathogenesis

H. pylori colonizes the gastric mucosa of humans, it is usually acquired in childhood and, if not treated, can persist throughout the host lifetime [43]. The infection with *H. pylori* can have different outcomes, according with the genetics of the bacterial strain and also the type of inflammatory response of the host [44]. While most of the infected individuals, carry *H. pylori* throughout their life without major complications, a proportion of them may develop more severe clinical consequences [11]. Among the bacterial factors that are involved in the colonization and infection mechanism of *H. pylori*, the adhesion molecules at the bacterial surface and the presence of the virulence factors CagA and *VacA* are considered very importance for the final outcome of the infection.

1.3.1 Adhesion

All *H. pylori* are found within 25 µm of the cell surface in the mucus layer immediately overlaying the cells [45]. In this microenvironment, the bacteria survive in two major populations: one that is free-living in the gastric mucus layer, and another, representing approximately 20% of the bacterial population, found directly adhered to the epithelial surface of the cells [46-47].

The adhesion is as a crucial step for the bacterium survival and infection of the host cells. In fact, adhesion could allow the growth of the bacteria in conditions where non-adherent bacteria die [48], and could also allow the *H. pylori* to remain in the host time enough for the existence of genetic recombination with other strains of *H. pylori* that could also be present, originating a higher genetic diversification [49]. The *H. pylori* genome contains a large array of open reading frames coding for outer membrane proteins, generally identified as adhesins. Two of the most studied adhesins are BabA and SabA, which mediate binding to glycoproteins at the surface of the gastric epithelial

cells, such as Lewis^b [47] and to sialyl-Lewis^x human blood group antigen, respectively [50]. Infection with *H. pylori* strains that contain these two adhesins has been associated with more severe diseases [51].

1.3.2 Virulence factors CagA and VacA

The infection with *H. pylori* results in chronic gastritis in all infected hosts, and most of the infected individual do not develop other complications and are free of clinical symptoms [52]. However, a proportion of individuals may develop more severe disease, such as peptic (gastric and duodenal) ulcers, gastric carcinoma, and mucosa-associated lymphoid tissue (MALT)-lymphoma [11]. This observation, together with the high genetic diversity of *H. pylori* strains, led to the notion that some strains may be more virulent than others. Early studies of the differential pathogenic properties of *H. pylori* strains indicated that increased pathogenicity was correlated with the ability of some strains to induce morphological changes, vacuolization, and other alterations in *in vitro*-cultured cells [53]. Later on, this activity was associated with the presence of the bacterial molecule CagA. CagA is a highly immunogenic protein with a molecular mass of approximately 140 kDa that is encoded by the *cag* pathogenicity island (*cag* PAI). The *cag* PAI is a genomic region of 40 Kb containing about 30 genes that encode a type IV secretion system (T4SS). CagA is present in about 60% of the western strains of *H. pylori* [54]. The T4SS is a syringe-like structure capable of penetrating the gastric epithelial cells and facilitating the translocation of CagA, peptidoglycans fragments, and possibly other bacterial factors into the host cells (**Figure 1.2**) [55-56]. CagA, once translocated into the host cell cytoplasm, is phosphorylated at tyrosine residues in EPIYA motifs [57-59] by Src and Abl family kinases [60-62]. Phosphorylated CagA interacts with diverse of host signaling molecules, including the tyrosine phosphatase SHP-2 [63]. These interactions play a role in *H. pylori*-induced actin cytoskeletal rearrangements, scattering and elongation of infected host cells in culture [64]. Unphosphorylated CagA can also elicit host cell responses such as disruptions of tight and adherent junctions, loss of cell polarity, proinflammatory and mitogenic responses [65]. Infection with *H. pylori* strains containing CagA and the T4SS leads to increased risk for the disease development [44, 66-67].

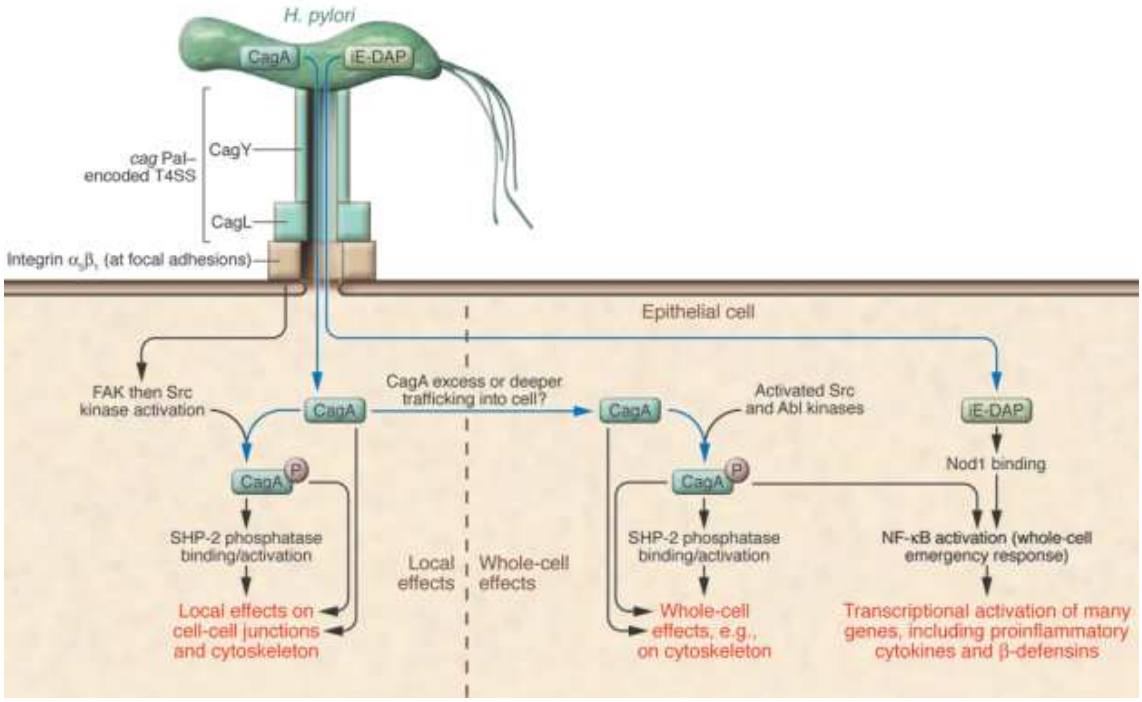

Figure 1.2 - CagA phenotypes and variation. Local and whole-cell effects of the *H. pylori cag* PAI–encoded T4SS and its major effector protein CagA [43].

Another *H. pylori* molecule associated with bacterial virulence is *vacA*, which codes for a secreted toxin, VacA [68]. VacA can induce multiple cellular activities, including cell vacuolation, membrane channel formation, disruption of endossomal/lysossomal function, apoptosis, and immunomodulation [69]. Although all *H. pylori* strains carry *vacA* gene, there is considerable variation in vacuolation activity among strains [68, 70-71]. This is due to the sequence heterogeneity within *vacA* gene in three major regions: a 5' region, encoding the signal peptide and mature protein N-terminus (s1 or s2 genotype); an intermediate region, encoding part of the p33 subunit (i1 or i2 genotype); and a mid region, encoding part of the p55 epithelial cell binding subunit (m1 or m2 genotype) [43]. The s1/i1/m1 form of VacA is fully active, and the s2/i2/m2 form is inactive, but intermediate forms exist and are common in many human populations [72]. Strains with *vacA* s1/i1/m1 genotype, encoding an active form of the VacA toxin, are strongly associated with peptic ulcer disease and with gastric carcinoma [73-74].

1.4 Diagnosis and Treatment of Helicobacter pylori Infection

1.4.1 Diagnosis

The diagnosis of *H. pylori* can be performed through several tests that have been developed since the discovery of this pathogen, each with their specific advantages and disadvantages. In research protocols, a combination of two or more methods is often applied, whereas in daily clinical practice, the use of a single test is generally adequate, and most tests are sufficiently accurate to be used for this purpose. The detection methods for *H. pylori* infection are usually divided into noninvasive tests, based on peripheral samples, such as blood, breath samples, stools, urine, or saliva for detection of antibodies, bacterial antigens, or urease activity, and invasive tests, that require gastric biopsy specimens for histology, culture or molecular detection methods.

1.4.1.1 Noninvasive methods

1.4.1.1.1 Serology

Serology detects the amount of immunoglobulin G (IgG) or (IgA) specific for *H. pylori* present in the serum, total blood or urine through an Enzyme-Linked Immunosorbent Assay (ELISA). There are several commercially available kits and the sensitivity and specificity ranges between 80% and 90%. This technique has insufficient reliability for routine screening and cannot prove ongoing infection due to immunological memory [11]. Therefore, ELISA is not suitable for assessing *H. pylori* eradication.

1.4.1.1.2 Urea Breath Test (UBT)

The urea breath test is based on the ability of *H. pylori* to break down urea, into ammonia and carbon dioxide which then is absorbed from the stomach and eliminated in the breath. In this assay, patients swallow urea labeled with radioactive carbon 14 (^{14}C-UBT) or non-radioactive carbon 13 (^{13}C-UBT). In the subsequent 30 minutes, the detection of isotope-labeled carbon dioxide in exhaled breath indicates that urea was metabolized by the urease enzyme of the bacteria, and hence that *H. pylori* is present. The ^{13}C-UBT was shown to be one of the most accurate diagnosis tests for *H. pylori*.

This test is also the most reliable to evaluate success of eradication treatment of *H. pylori*, since it detects viable bacteria, that is the actual infection. One limitation of this assay is, however the requirement of specific and expensive equipment [75-76].

1.4.1.1.3 Stool Antigen Test

Stool antigen assays offer an alternative method for the diagnosis of infection. They have been included in several clinical guidelines as a recommended noninvasive test in young dyspeptic patients [77-80]. The detection of *H. pylori* in stool samples is achieved by enzyme immunoassays (EIA) based on monoclonal or polyclonal antibodies. The sensitivity and specificity of these assays have been evaluated in several studies [81-85], with different values for the diverse commercial tests available. The reliability of these tests for evaluation of success of eradication treatment of *H. pylori* remains controversial.

1.4.1.2 Invasive Methods

1.4.1.2.1 Culture

H. pylori culture is the "gold standard" method for identification of viable forms of the bacteria [86]. *H. pylori* shares some common biochemical characteristics with the enteric *Campylobacters*, including positive catalase and oxidase reactions, nonfermentation of cardohydrates, and a requirement for microaerobic conditions for growth [87]. The culture of *H. pylori* is needed and is a prerequisite for further studies of the organism, such as strain classification, antibiotic resistance monitoring, and other comparative studies. There are two main types of media: nonselective media based on nutrient agar, such as brain heart infusion or brucella agar complemented with 5% to 10 % of sheep or horse blood [88-89], and selective media, based on supplemented nutrient agar containing antibiotics [89-91]. The optimum temperature for the growth of bacteria is 37ºC in microaerophilic conditions (5% O_2 and 10% CO_2) and it can take from 3 to 7 days (or more) incubation to obtain a positive culture [92]. The disadvantages of culture, besides the time and specificities of growth, are that it requires microbiological expertise. In samples from extra-gastric locations and from environmental sources *H. pylori* has rarely been grown using these microbiological culture techniques [93].

1.4.1.2.2 Histology

The histological identification of *H. pylori* infection is a widely used means of diagnosis. Several staining methods can be used including the modified Giemsa [94], Warthin-Starry [95], HpSS method [96], and Genta [97]. All of these staining-based methods depend on the morphology of the bacterium for identification. In situations where, there may be other microbes in the gastric mucosa, morphologic identification of *H. pylori* can be difficult. It is also known that *H. pylori* may demonstrate pleomorphism, and therefore morphology alone may not be reliable for diagnosis. Immunohistochemical techniques use anti-*H. pylori* antibodies, reacting with whole bacterial antigens or specific proteins with good correlation with the presence of the bacteria. In fact, immunohistochemistry using a polyclonal antibody against *H. pylori* has demonstrated good specificity and sensitivity and has been recommended when the density of the microorganism is low [98-99]. Histological and immunohistochemical detection of *H. pylori* has the disadvantage of the need of an experienced pathologist for observation. On the other hand, this also constitutes an advantage, since the lesional status of the gastric mucosa is evaluated.

1.4.1.2.3 Molecular Methods

1.4.1.2.3.1 Rapid Urease Test (RUT)

Rapid urease test is a rapid test for diagnosis of *H. pylori*. This test is based on the ability of the bacteria to secrete the urease enzyme that catalyzes the conversion of urea to ammonia and carbon dioxide. The RUT consists of a medium containing urea and a pH indicator, and where the gastric biopsy samples are placed. If *H. pylori* is present in the samples, the urease produced by the bacteria hydrolyzes the urea of the medium to ammonia, raising the pH of the medium and changing its colour. In the gastric environment the presence of other bacteria than *H. pylori* is rare, and the specificity and sensitivity of the RUT are 98% and 94% respectively [100]. In extra-gastric samples the use of this test must be taken with caution, because of the possibility of the presence of urease-positive other bacteria than *H. pylori,* that can lead to false positives results.

1.4.1.2.3.2 Polymerase Chain Reaction (PCR)

PCR methods are used for the detection of *H. pylori* DNA in gastric mucosa and gastric juice, as well as in feces, saliva, dental plaque, and environmental samples [101-104]. Limitations of PCR methods include the propensity for false-positive results in part due to the detection of DNA from non-*H. pylori* organisms. This is especially important in environmental samples which may contain previously uncultured organisms or non-*pylori Helicobacter* spp. False-negative results may also occur due to a low number of organisms or to the presence of PCR inhibitors in the sample, particularly in stools and environmental samples [105]. A number of target genes have been proposed as candidates for PCR detection of *H. pylori*, including the 16S rRNA gene, the *glmM* gene, the *ureA* gene, the *ureB* gene, the *vacA* gene, and the *cagA* gene [106-112]. Controversy remains regarding which primer set or sets is the potencial "gold standard" for gastric and non-gastric samples such as saliva or environmental samples. In fact, studies using very well characterized samples by means of different tests which compare different PCR primer pairs are rare [113-114].

1.4.1.2.3.3 Fluorescence in situ Hybridization (FISH)

FISH is one of the most common methods used for the detection and localization of a microorganism or particular groups of organisms within a sample. FISH detects nucleic acid sequences by a fluorescently labeled probe that hybridizes specifically to its complementary target sequence within the intact cell. The procedure includes the following steps (**Figure 1.3**): (i) fixation of the specimen; (ii) preparation of the sample, possibly including specific pretreatment steps; (iii) hybridization with the respective probes for detecting the respective target

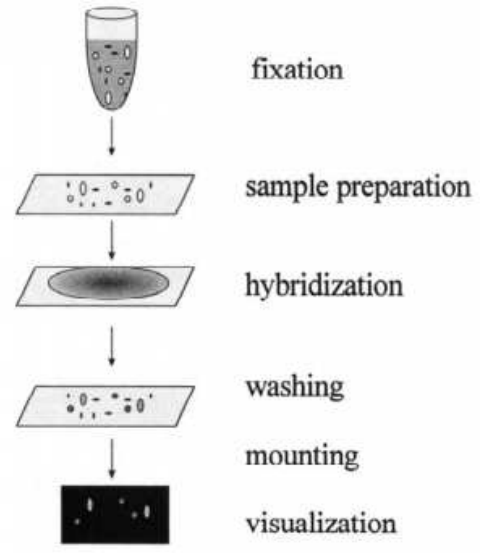

Figure 1.3 - Flow chart of a typical FISH procedure [111]

sequence; (iv) washing steps to remove unbound probes; (v) mounting, visualization and documentation of results [115].

In microbiology the most commonly used target molecule for FISH is 16S rRNA because of its genetic stability, its domain structure with conserved and variable regions, and its high copy number [116]. The choice of probes must consider specificity, sensitivity and ease of tissue penetration. A typical oligonucleotide probe is between 15 to 30 base pair in length, and is normally labeled by direct fluorescent labeling, which is the fastest, cheapest and easiest way of labeling because does not require any further steps after hybridization [115]. Traditionally, FISH methods are based on the use of conventional DNA oligonucleotide probes, containing around 20 bases. More recently, peptide nucleic acid (PNA) probes have been developed and optimized for bacterial detection. PNA molecules are pseudopeptides with DNA-binding capabilities. These compounds were first reports earlier in the 1990s in connection with a series of attempts to design nucleic acid analogues capable of hybridizing, in a sequence-specific fashion, to DNA and RNA [117].

Peptide nucleic acid molecules are DNA mimics, where the negatively charged sugar-phosphate backbone is replaced by an achiral, neutral polyamide backbone formed by repetitive units of *N*-(2-aminoethyl) glycine (**Figure 1.4**). PNA can hybridize to complementary nucleic acid targets obeying the Watson-Crick base-pairing rules [118]. Compared with traditional DNA probes and due to the uncharged backbone, PNA probes have superior hybridization characteristics, exhibiting rapid and stronger binding to complementary targets, an absence of electrostatic repulsion, it is not a substrate for the attack of proteases or endonucleases, and usually are shorter, optimum size is 15 bases, than conventional DNA probes [119-120]. The PNA FISH method can be applied in a large variety of samples such as,

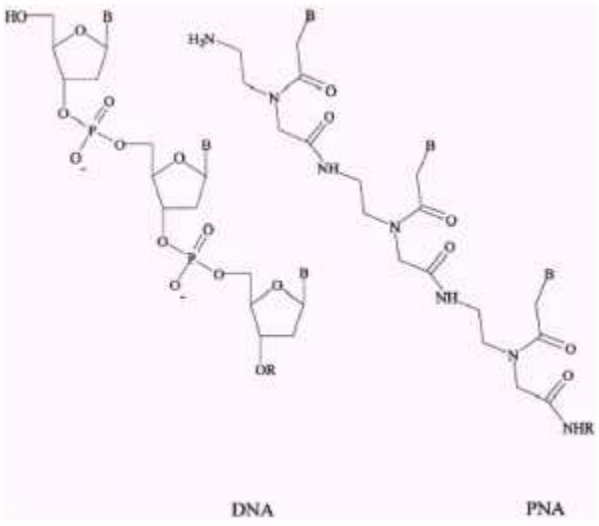

Figure 1.4 - Comparison between the DNA and PNA chemical structure [118].

slides, membrane filters or even formalin-fixed paraffin-embedded gastric biopsies [121].

1.4.2 Treatment

Nowadays, the question of whether asymptomatic patients should undergo treatment to eradicate *H. pylori* is subject to different opinions [122-123]. Some physicians advise the eradication of this pathogen upon detection, while others think that treatment should only be applied when symptoms appear. In any case, the treatment consists of a triple or quadruple therapy. Triple therapy consists in a one or two week course of treatment which involves taking two antibiotics (e.g. metrodinazole, tetracycline, amoxicillin) and either an acid suppressor (a proton pump inhibitor - PPI) or a stomach lining shield (usually bismuth subsalicylate) [124]. In the quadruple therapy, both stomach lining shield and acid suppressors are used together with two antibiotics [125]. A meta-analysis found only four studies of sufficient quality to allow comparisons between triple and quadruple therapy and concluded that there was no statistically significant difference between both therapies [126]. In adults, triple therapy reduces ulcer symptoms and prevents ulcer recurrence in more than 90% of patients [127]. However the increased bacterial resistance to antibiotics, as well as the poor patient compliance are causing an increase failure of these *H. pylori* eradication therapies [128]. Due to this resistance, new concepts in eradication therapy are emerging, namely the sequential therapy. In this form of therapy, antibiotics are administrated in a sequence rather than all together. The sequential regimen that has been well described is a 10 day treatment consisting of a proton pump inhibitor (PPI) and amoxicillin (both twice a day) administrated for the first 5 days followed by triple therapy consisting of a PPI, clarithromycin and tinidazole for the remaining 5 days [129]. In conclusion, the therapy for *H. pylori* treatment must be carefully chosen by the clinicians. Emerging sequential therapies are promising and are a potential alternative for triple therapy. Despite strategies based on traditional treatment are generally successful, the increasing need for second and third line treatments and the group of patients who fail all standard treatments remain a cause of concern [130]. Several groups are at the moment trying to develop a vaccine against *H. pylori* but there are no successful results up to the present date [131-134].

1.5 Prevalence and Routes of Transmission of Helicobacter pylori

The prevalence of *H. pylori* and the possible routes of transmission in the human population were described, together with the presentation of both epidemiological and microbiological data supporting or dismissing each individual route, in a review from Azevedo *et al* [49].

1.5.1 Prevalence across the world

H. pylori infection occurs worldwide and affects on average approximately 50% of the world population, although the incidence has been decreasing in recent years [135-137]. However in Portugal and Japan, ranked, respectively, as 29th and 8th in the Human Development Index published by the United Nations Development Program [138] the incidence is higher than 80% [49, 139]. Significant differences in prevalence have been found both within and between countries [140]. Generally, the overall prevalence is higher in countries of underdeveloped regions, such as Africa and Asia, than in the more developed countries in Western Europe and North America (**Figure 1.5**). In undeveloped countries, most of the infections seem to be acquired during childhood while in developed countries the incidence increases gradually with age. In the first case, the number of children *H. pylori* positive can reach 75% contrary to what happens in developed countries, where the prevalence is normally lower than 10% [49, 135, 141-143]. Epidemiological studies have shown that, in general, the high incidence of *H. pylori* is correlated with a deprivation in sanitation, hygiene and educational habits. Therefore lower socio-economical status, high population density in undeveloped countries are directly related to the high occurrence of *H. pylori* [144-145]. Overall, *H. pylori* prevalence is decreasing as a result of improved sanitary conditions and treatment procedures [136].

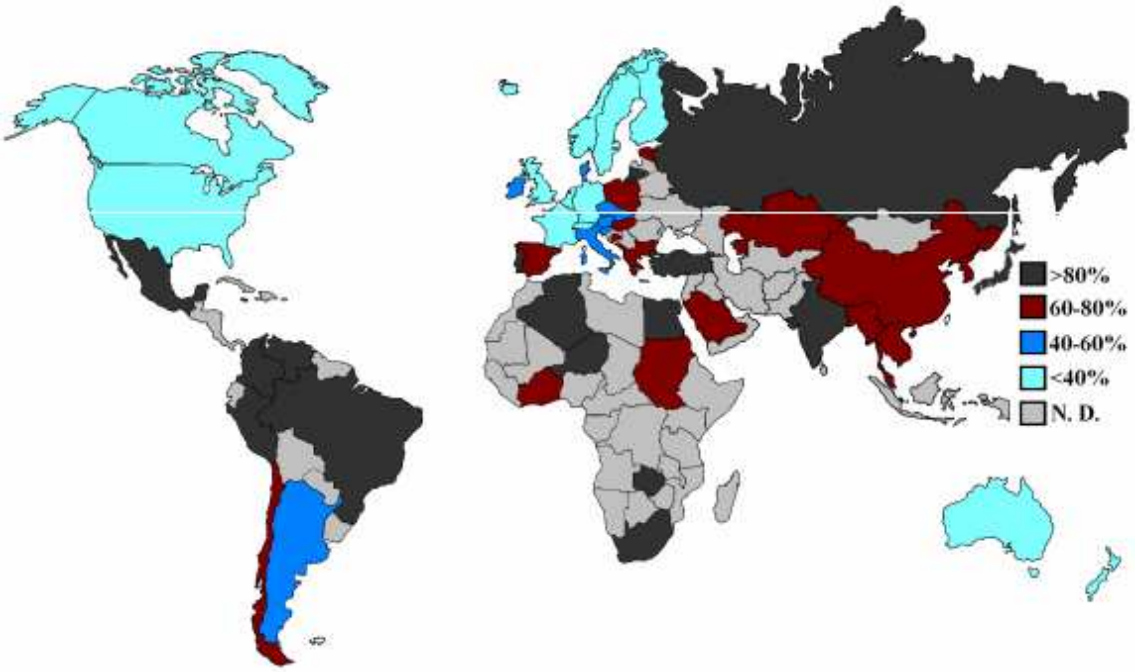

Figure 1.5 - Worldwide prevalence of *H. pylori* [48].

1.5.2 Routes of Transmission

Numerous epidemiological studies have been conducted to identify the factors influencing transmission of this pathogen. Socioeconomic status is clearly the most important determinant for the development of *H. pylori* infection, with poorer/lower social classes exhibiting much higher prevalence [140], which is also in accordance with differences found between underdeveloped and developed countries described in previous section. This factor encompasses conditions such as levels of hygiene, density of living, sanitation and educational opportunities, which have all been individually identified as markers of the bacterium presence.

Largely based on epidemiological and microbiological evidence, several routes of transmission have been conjectured (**Figure 1.6**).

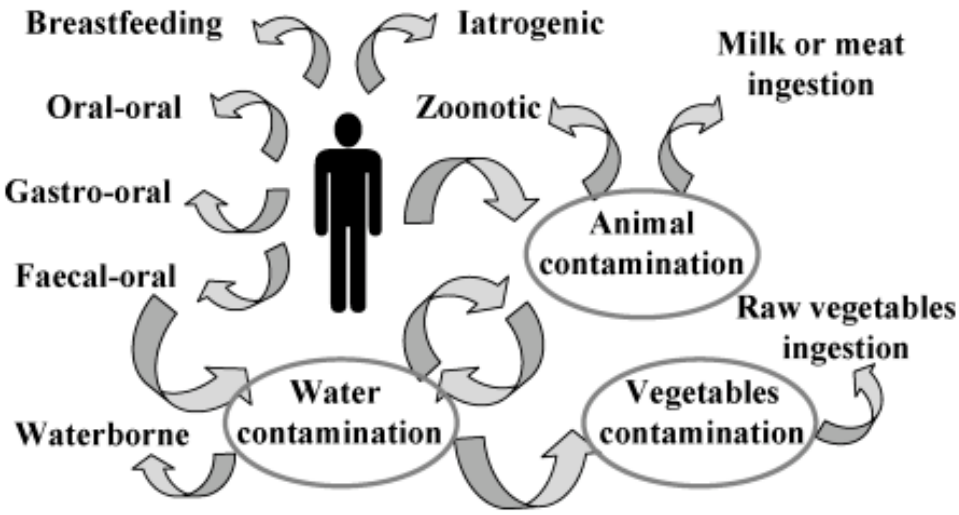

Figure 1.6 - Suggested transmission routes for *H. pylori* [48].

Person-to-person transmission is widely seen as the most probable route of infection, mainly because of the apparent failure to consistently isolate *H. pylori* in places other than the human gastro-intestinal tract and of the perception that lower transit time between different hosts would certainly be favorable for the bacterium. Furthermore, numerous epidemiological studies have consistently identified domestic overcrowding and infection of family members as a risk factor for *H. pylori* transmission. Roma-Giannikou *et al.* [146] found a strong homology of the *H. pylori* genome in infected members of the same family, and clustering of *H. pylori* infection in families has been widely reported in other studies [e.g. 147]. Although these studies support the hypothesis of person-to-person transmission, exposure of a family to an alternative common source still remains a possibility.

The most relevant pathways of person-to-person transmission encompass the gastro-oral, oral-oral and faecal-oral routes. Breastfeeding and iatrogenic transmission are also included as alternative ways for the dissemination of the pathogen. In addition, there are at least three possible vectors that have been suggested to sustain the bacterium in viable form: water, food and animals. Most authors agree that the relative importance of these routes in the transmission of the bacterium is likely to vary between developing and developed countries [148-149]. The most relevant in overall terms are now addressed in detail.

1.5.2.1 Gastro-oral transmission

It has been suggested that exposure to microscopic droplets of gastric juice during endoscope manipulation could explain an higher prevalence of infection in gastrointestinal endoscopists [150], but the gastro-oral transmission has been postulated mainly for young children, among whom vomiting and gastro-oesophageal reflux are common. In a recent epidemiological study, exposure to an infected household member with gastroenteritis and vomiting episodes was associated with a 6.3 fold increased risk of new infection [151]. It is important to realize, however, that because vomiting episodes might cause for an increased risk of the presence of *H. pylori* in the oral cavity, this type of study does not discriminate whether the transmission is gastro-oral or oral-oral.

In a study by Parsonnet *et al.* [152], vomitus from infected subjects and surrounding air were sampled for *H. pylori*. All vomitus samples were positive (often recovering the bacterium in high quantities), and even the surrounded air tested positive for 37.5% of the cases. Successful cultivation of *H. pylori* from vomitus was also obtained in two other studies [153-154]. Amazingly, there is a blatant lack of data on the survival/culturability time of the bacterium in gastric juice, and as such, it is not possible to estimate for how long the infectious state might last on these conditions and to establish comparisons with culturability times obtained for other conditions.

On the other hand, the discovery of enterohepatic *Helicobacter* species might challenge the importance of a gastro-oral (and an oral-oral) route [155]. As the name suggests, these bacteria have been identified in the intestinal tract and/or the liver of humans, other mammals, and birds, which implies a more unlikely presence for them in the oral cavity and stomach. How these bacteria are transmitted is something that has been little studied, but most works appear to support a faecal-oral route [156-158]. The question to be asked here is whether the phylogenetic proximity to *H. pylori* would imply that transmission routes are similar.

1.5.2.2 Oral-oral transmission

The oral cavity has been considered to be a suitable reservoir for *H. pylori* subsistence, and oral-oral transmission has therefore been suggested to occur with kissing or other contact with infected saliva, the use of chopsticks by Chinese immigrants or, as it

happens in some ethnic backgrounds, from mothers to their babies as they pre-masticate their food. Identical strains of the pathogen have been detected by polymerase chain reaction (PCR) in the mouth and stomach of symptomatic infected individuals [159], and in these populations detection of *H. pylori* in the oral cavity by PCR is in fact very common [160]. Nevertheless, studies conducted afterwards using similar techniques indicated that the oral cavity does not favor prolonged colonization of *H. pylori* in populations with high prevalence of infection when the individuals are asymptomatic, and concluded that colonization of the mouth is only transient and occurs after vomiting [160-161]. Similarly, isolation and cultivation of the microorganism has been sporadic and related to transitory regurgitations of the microorganism from the stomach into the mouth [160, 162]. Microbiological studies on the culturability of *H. pylori* on a buffer containing a peroxidase system with high concentrations of H_2O_2 (to simulate saliva), showed that after 1 hour at 37 ºC the bacterium started to be inhibited, but this inhibition was not noticed when the buffer system was added to real human saliva [163]. Luman *et al.* compared the genotypes of *H. pylori* isolated from patients and their spouses by PCR-restriction fragment length polymorphism and found very little similarity [164]. It is however possible that several mechanisms, such as point mutations and intragenic recombination, could enhance strain diversity once the infection is acquired.

1.5.2.3 Faecal-oral transmission

It has been suggested that the faecal-oral route for *H. pylori* transmission is very unlikely due to the contact with human bile, to which it is very sensitive, during the passage through the intestine [165-166]. One epidemiological study appears to support the view that this transmission mode is less common than gastro-oral or oral-oral, by showing that exposure to an infected household member with diarrhea elevated, but not significantly, the risk for new infection [151].

However, the fact that *H. pylori* is able to colonize the duodenum (upper part of the small intestine) in areas of gastric metaplasia, appears to be an inconsistency, and has raised some questions about the exact effect of the passage of the microorganism through the intestine [167]. Well-established detection methods based on PCR or enzyme-linked immunoassays systematically identify the presence of the microorganism [e.g. 168, 169-171], but growth of the bacterium using culture methods

has been more elusive, and achieved most of the times in individuals with accelerated gut transit time [160, 162].

1.5.2.4 Breastfeeding

The detection by PCR of *H. pylori* in breast milk has also raised the possibility of breastfeeding as a route of transmission [172], even though earlier studies stated that infants born from *H. pylori*-positive women are not more likely to acquire the infection [173]. The contamination of milk could be possible if the bacterium survived in nipples or fingers. However, most epidemiological studies appear not to find any correlation between breastfeeding and *H. pylori* acquisition [174-180]. In fact, a few of them actually mention breastfeeding as a protection practice against the microorganism [174, 177-178]. Survival studies indicate that the bacterium remains culturable in commercial pasteurized milk for 5 days at 4 °C and an inoculum concentration of $\approx 10^4$ CFU/mL [180]. It is likely, however, that this relatively long time of survival is related to the low temperatures at which the experiment was carried out.

1.5.2.5 Iatrogenic transmission

Acquisition of *H. pylori* by patients submitted to upper endoscopy, i.e. iatrogenic transmission, is supported by three out of four epidemiological studies [181-184]. *H. pylori* has been consistently detected by culture in endoscopes after their use in infected patients [185-187], but adequate disinfection procedures are thought to greatly reduce (or even eliminate) the transmission risk for this microorganism [188]. Back in 1995, Tytgat estimated a transmission frequency of approximately 4 patients per 1000 endoscopies when the infection rate in the endoscoped population was about 60% [189].

1.5.2.6 Zoonotic transmission

Including contact with animals as a possible transmission mode is an obvious reasoning, as zoonotic transmission represents one of the leading causes of illness and death from infectious disease worldwide. Most epidemiological studies appear to support the role of animals in the acquisition of *H. pylori*, but the extent of this support depends on the

animals under study. Considered vectors include cows [190], sheep [191], cockroaches [192], houseflies [193] and domestic pets [194].

In the first two cases, the suspected route of transmission is mainly by the ingestion of contaminated raw milk. Milk could become contaminated when the breast of a cow or sheep is in contact with faeces in the soil. Epidemiologic data has shown higher prevalence in shepherds and their families than in the general population [195-196]. The detection of the bacterium in animal milk is described ahead (see food ingestion section).

Inamura *et al.* [192] suggested that cockroaches, which usually live in unsanitary environments, may contaminate foods and food containment areas such as pantries. The authors studied the survival of *H. pylori* on the external surfaces (legs and body) and excreta of *H. pylori*-exposed cockroaches and found that the microorganism was culturable from the excreta of the exposed group for 24 h postchallenge, but not from the external surfaces. A similar study was also performed with houseflies [197]. In this case, *H. pylori* was recovered from external surfaces for up to 12 h and from gut and excreta for as long as 30 h postchallenge. The negative detection after 30h was attributed to the appearance of other Gram-negative bacteria that overgrew the cultures. However, when this study was repeated exposing the houseflies to *H. pylori*-contaminated human faeces instead of *H. pylori* grown on agar plates, the microorganism was not cultured from any of the locations [193].

Epidemiological studies showed controversial results in respect to the risk of the presence of domestic animals in the household [e. g. 198, 199-200]. *H. pylori* has not been found in dogs and only very rarely in cats' stomachs [201-202], and it has been suggested that the presence in animals is of human origin [201, 203]. Recent work has identified *H. pylori* by PCR in the bile of cats, thus increasing the chance of this animal as a vector [194]. It is now known that nearly each animal is colonized by its own endogenous *Helicobacter* spp. Like *H. pylori*, that has co-evolved with humans to be highly specialized in the colonization of the human GI tract [33] , these bacteria have specialized in colonizing the GI tract of their specific natural host. In the model where only one strain colonizes the stomach of a mammal, *H. pylori* would find fierce competition by these other *Helicobacter* spp. in search for essential nutrients and not subsist. With the emergence of a multiple infecting strains and species model for the same host it is more credible that *H. pylori* is also a zoonotic agent.

1.5.2.7 Water ingestion

A large number of epidemiological studies have investigated drinking water, or drinking water-related conditions, as a risk factor for *H. pylori* infection [33, 92-93, 111, 145, 199, 204-223]. Although a few studies report the absence of an association between prevalence of *H. pylori* and water quality, the majority of the other studies support a relationship between these parameters.

Concomitantly, molecular methods such as FISH, PCR and antibody assays, were able to detect the presence of the bacterium in water and water-associated biofilms from wells, rivers and water distribution networks [e. g. 224, 225-226]. However, when suspended in water, *H. pylori* has a very low culturability time when compared to other waterborne pathogens. In fact, several studies report culturability times of less than 10 hours for *H. pylori* [216, 227-228] at temperatures over 20°C which compares to culturability times of more than 40 days for *Escherichia coli* and *Salmonella typhimurium* at the same temperature. While *H. pylori* culturability usually ends after little time in water and water-exposed biofilms [229-230], Shahamat *et al.* [216] determined that total cell counts did not decrease for much longer periods (2 years at 4 °C). This raises questions on the exact physiological state of the bacterium in water and in the evaluation of methods that could be more appropriate for the detection of the infectious microorganism. For instance, a nutrient shock effect was also observed when recovering the water-stressed bacterium to high nutrient medium, showing some level of *H. pylori* adaptation to this environment [228]. Furthermore, it has been attempted to demonstrate that coccoid (cell shape associated with non-culturability) *H. pylori* induced by water is capable of colonizing the gastric mucosa and cause gastrititis in mice [231-232].

1.5.2.8 Food ingestion

At least two epidemiological studies have found a positive relationship between the consumption of uncooked vegetables and *H. pylori* transmission [204, 233]. Raw vegetables are suspected to be vulnerable to *H. pylori* colonization when contaminated water is used for washing or irrigation. It is important to bear in mind that this route assumes that *H. pylori* is also able to survive in water and has therefore all the problems

associated with this possible transmission route. No reports have been found about cultivation methods or molecular biology procedures trying to detect the microorganism from these products. Survival studies indicate that inoculated *H. pylori* (temperature: 8 °C; inoculation density $\approx 10^6$–10^7 CFU/g) dropped below detection limits at 4 days in sanitised lettuce and carrot samples, and at 5 days in sterilised carrot [144]. In a different study, survival of the microorganism lasted for up to 2 days in leaf lettuce (4 °C; $\approx 10^2$ CFU/g) [180].

Milk is another type of food implicated as a possible transmission vehicle by epidemiological studies. Constanza *et al.* correlated infection with the intake of milk products in Mexico [234]. Conversely, an epidemiological study in Italy reported an inverse correlation between the elevated consumption of milk and *H. pylori* prevalence [235]. The differences obtained in both studies might reflect variable milk microbiological quality between these two countries. Interestingly, a recent study in Poland showed that prevalence in shepherds and their families was 20-30% higher than in farmers with no contact to sheep [236]. They understandably attributed this difference to the contact with animals (zoonosis), but failed to consider a probably higher quantity ingestion of raw milk by the shepherds and their families as a variable. Previously, in 1999, Dore *et al.* found similar results in a community of Sardinian shepherds and their families [195], but the research group went on to try and detect *H. pylori* presence in sheep milk. They were able to report the recovery of viable *H. pylori* from raw milk samples on two separate occasions [191, 237], but failed to confirm the survival of the microorganism after pasteurization of the milk. Furthermore, a larger screening of 400 raw sheep milk samples performed in Turkey detected no viable *H. pylori* [238]. The pathogen has also been cultured from one sample of raw cow's milk in Japan [190], and in the same work PCR demonstrated the presence of the *ureA* gene of *H. pylori* in 13 of 18 (72.2%) raw milk samples and in 11 of 20 (55%) commercial pasteurized milk samples.

Poms and Tatini studied the survival of *H. pylori* in other commercially available food products, such as yoghurt, chicken meat and tofu [180]. The bacterium was cultured for 1, 2 and 7 days, respectively (4 °C; $\approx 10^2$ CFU/g). Differences in the culturability could be explained by the work of Jiang and Doyle [239], who, based on the effect of environmental and substrate factors on survival and growth of *H. pylori*, have stated that the microorganism usually exhibits extended survival in low acid/high moisture environments. Also, autochthonous microbiota present in the yoghurt, such as

Lactobacillus and *Bifidobacterium*, have been shown to inhibit the survival of *H. pylori* [240-241].

1.6 Rationale and Aims

Since the discovery of *H. pylori* several methods have been developed aiming at detection of the bacteria. The criteria for selecting methods for *H. pylori* diagnosis needs to be regarded according to each particular situation. For example, methods that are accurate and specific for gastric samples may not be suitable for oral or for environmental samples. Fluorescence *in situ* hybridization (FISH) has been developed for identification of several bacteria and the usual DNA probes have been replaced by new peptide nucleic acid (PNA) probes. Design of a PNA probe specific for *H. pylori* and optimization of the FISH process to be used in a large variety of samples would be an improvement for the detection and localization of *H. pylori*.

The most relevant routes of person to person transmission encompass the gastro-oral, oral-oral, and fecal-oral routes. Despite all the data regarding this way of transmission, whether the oral cavity is a reservoir for *H. pylori* and what is the behavior of the bacteria when exposed to saliva, are questions unanswered.

The majority of studies that have investigated drinking water, or drinking water-related conditions, as a risk factor for *H. pylori* infection support a relationship between these parameters. Water has therefore been appointed as a possible vector in the transmission of *H. pylori*. Although some studies have shown important information about how water affects *H. pylori* it is still not clear whether *H. pylori* retain the viability and infection capability after being exposed to water.

In view of this, the aims of this Thesis are the following:

- → To design a new peptide nucleic acid probe for the specific detection of *H. pylori* in a several types of samples.
- → To clarify whether the oral cavity, specifically the adenoids and tonsils, may constitute an extra-gastric reservoir for *H. pylori*.
- → To assess the cultivability of saliva exposed *H. pylori* and to determine whether these bacteria retain the ability to adhere to and to induce inflammation in an experimental model of human gastric epithelial cells.
- → To assess the cultivability of water exposed *H. pylori* and to determine whether these bacteria retain the ability to adhere to, to produce structural components of pathogenicity like the *cag* type IV secretion system (T4SS), and to induce

inflammation and cell cycle alterations in an experimental model of human gastric epithelial cells.

In this Thesis the results will be presented in the format of scientific papers, as they were submitted to peer reviewed international journals.

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Chapter 2

Development and application of a novel peptide nucleic acid probe for the specific detection of *Helicobacter pylori* in gastric biopsies

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In this work, a fluorescence *in situ* hybridization (FISH) method for the rapid detection of *Helicobacter pylori* is reported using a novel peptide nucleic acid (PNA) probe. Laboratory testing with several different bacterial species, including other *Helicobacter* spp., has shown that this probe is highly specific for *H. pylori* strains. In addition, the PNA FISH method has been successfully adapted for detection of the pathogen in paraffin-embedded gastric biopsies.

2.1 Introduction

Helicobacter pylori is an important human pathogen that causes chronic gastritis and is associated with the development of peptic ulcer disease, atrophic gastritis and gastric cancer (31). Infection with *H. pylori* can be diagnosed either by non-invasive testing or by invasive techniques that require upper endoscopy with collection of gastric biopsy specimens (13). Non-invasive tests are the most usual methods for routine *H. pylori* detection but they fail to provide complementary information on *H. pylori* location in the stomach and on the histopathological lesions underlying the presence of the bacteria. Therefore, there are situations where invasive techniques should be performed to provide a more complete diagnosis. Gastric biopsy specimens obtained by upper endoscopy can be analyzed for the presence of the bacterium by culture or by other molecular methods. In recent years, molecular methods, including random amplified polymorphic DNA, PCR, and fluorescence *in situ* hybridization (FISH), for the identification of several bacteria, have been imposing themselves over the more time-consuming culture methods (9,23,32).

FISH is arguably the most common method used for the detection and localization of a microorganism or particular groups of microorganisms within a sample (32). It detects nucleic acid sequences by a fluorescent labelled probe that hybridizes specifically to its complementary target sequence within the intact cell (17). So far, FISH methods have been based traditionally on the use of conventional DNA oligonucleotide probes containing around 20 bases. More recently, peptide nucleic acid (PNA) probes have been developed and optimized for bacterial detection. PNA molecules are DNA mimics, where the negatively charged sugar-phosphate backbone is replaced by an achiral, neutral polyamide backbone formed by repetitive units of *N* – (2-aminoethyl) glycine (19,20) . PNA can hybridize with complementary nucleic acid targets obeying the Watson-Crick-base pairing-rules (8). When compared with the traditional DNA probes and due to the uncharged backbone, PNA probes have superior hybridization characteristics, exhibiting rapid and stronger binding to complementary targets and absence of electrostatic repulsion (22,28). As such, the optimum length for a PNA probe is 15 base pairs (bp).

Several PNA probes have been designed and optimized for different organisms including *Campylobacter spp, Candida albicans, Mycobacterium avium* and *Legionella pneumophila* (14,15,34). We have previously developed a PNA probe targeting the 16S

rRNA of *H. pylori* (sequence: 5'-TAATCAGCACTCTAGCAA-3') that was shown to be very specific (5). However, due to the extensive genetic diversity observed within *H. pylori*, the high specificity of the probe was counterbalanced by the lack of sensitivity.

2.2 Design of the PNA oligonucleotide probe

To identify potentially useful oligonucleotides, the freely available Primrose program was used (http://www.cf.ac.uk/biosi/research/biosoft/Primrose/index.html) coupled with the 16S rRNA databases of the Ribosomal Database Project II (http://rdp.cme.msu.edu/html) version 8.1 (3,7). In accordance with the Primrose Program instructions, the selection of oligonucleotides was based on the 16S rRNA comparison of six randomly chosen *H. pylori* strains. To avoid missing possible sequences of interest, several sets of six random *H. pylori* strains were tested. Based on the large number of 15-bp length sequences obtained that could match all targets, additional criteria for the selection of the PNA FISH probe were used. These included no self-complementary structures within the probe and high specificity and sensitivity for *H. pylori*. Once the probe sequence was selected, a search was made at the National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/) to further confirm probe specificity (16). Afterwards, the desired sequence was synthesized (ATDBio, Southampton, UK) and the N terminus of the oligomer connected to Alexa fluor 546 via a double AEEA linker.

Table 2.1 - Predicted specificity and sensitivity of the probes for *H. pylori* detection. Estimation of binding affinity through fluorescence intensity was based on the work by Fuchs et al (12).

Probe[b]	Type	Specificity (%)[c]	Sensitivity (%)[d]	Fluorescence (%)	Reference(s)
HP16S-1	DNA	82	82	41–60	19
Hpy-1	DNA	86	95	0–5	5a, 8a, 23, 23a
Hprobe	PNA	100	25	6–20	4, 25a
Hpy769	PNA	85	89	6–20	This work

[a] Estimation of binding affinity through fluorescence intensity was based on the work of Fuchs et al. (10).
[b] Sequences are as follows: HP16S-1, 5′-GGA GTA TCT GGT ATT AAT CAT CG-3′; Hpy-1, 5′-CAC ACC TGA CTG ACT ATC CCG-3′; Hprobe, 5′-TAATCAGCACTCTAGCAA-3′. The sequence of Hpy769 is given in the text.
[c] Calculated as (number of *H. pylori* strains detected by the probe)/(total number of bacterial strains detected by the probe) × 100.
[d] Calculated as (number of *H. pylori* strains detected by the probe)/(total number of *H. pylori* strains in the databases) × 100.

According to the criteria mentioned above, we have chosen the following PNA oligomer sequence: 5'-GAGACTAAGCCCTCC-3'. The probe was designated Hpy769 due to the starting position of the target sequence in the 16S rRNA of the *H. pylori* NCTC 11637 strain. Searches showed that the Hpy769 probe differed by at least one bp from the 16S rRNA sequences of bacteria other than *H. pylori*, except for a few uncultured *Helicobacter* species and one strain of *Helicobacter acinonychis.* More importantly, evaluation with the NCBI Blast program showed that it is identical to 89 % of all *H. pylori* sequences, which is a significant improvement on the 25 % obtained for the PNA probe that has been already published (4), and represents a comparable value with other DNA probes used for *H. pylori* detection (21,25) (**Table 2.1**).

For the estimation of specificity and sensitivity, only sequences considered to have high quality and over 1200 bp by the RDP-II program were selected. The *H. pylori* sequences were further assessed for the existence of chimeras using the Mallard software, version 1.02 (2). At the end, 57 sequences were selected as being trustworthy. This is still a relatively low number to assess sensitivity and specificity, especially if we bear in mind that many strains also have the bias of being from similar locations and consequently possess similar 16S rRNA sequences. As such, it is likely that these values will vary as more sequences are deposited.

Another advantage of the probe is that, as it is showed in **Figure 2.1** the location of the target sequence is in a higher affinity binding area compared to Hpy-1, according to the study of Fuchs *et al.* (1998). Even if both probes were DNA based, this should result in a brighter signal and easier visualization under the microscope for Hpy769.

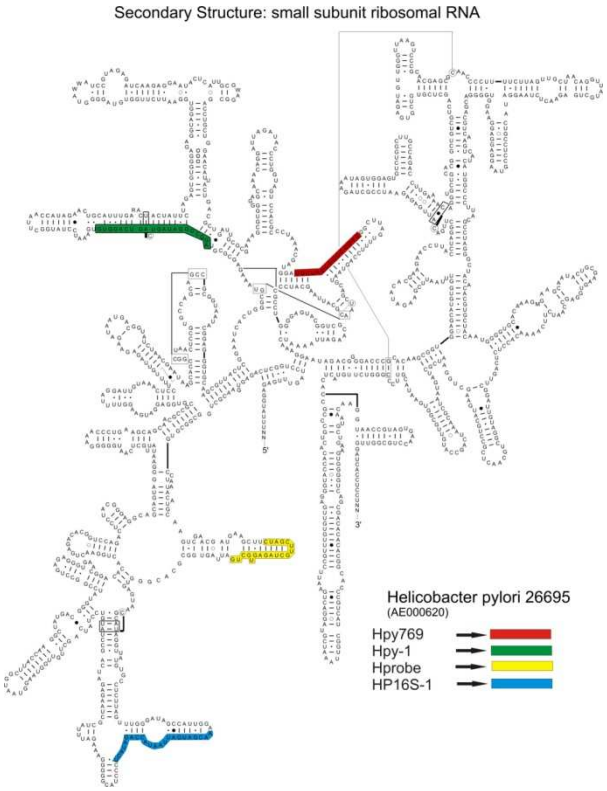

Figure 2.1 - Location of the target sequences of each probe in the *H. pylori* 22695 rRNA. The secondary structure was adapted from http://www.rna.icmb.utexas.edu/.

Because the latter is PNA based and hybridizations can therefore be performed under low salt conditions that promote the destabilization of the secondary structure of the 16S rRNA (28), enhanced signal intensity was expected.

2.3 Optimization of the hybridization conditions of the probe

The hybridization method was based on the procedure referred in Azevedo *et al* (4) with some modifications. We started by testing different hybridization temperatures, between 50ºC and 68ºC, and the best hybridization results were obtained at 59ºC (data not shown); however in certain random samples it was noticed that hybridization was not as bright as expected, which made detection difficult to be performed. This problem was solved by altering the fixation procedure to include a step of paraformaldehyde immersion followed by ethanol. The reason why this problem has not appeared for the Hprobe has not been investigated, but it might be related to altered probe characteristics due to the binding of the different type of fluorochrome (Alexa Fluor 546) used in this work.

For the final procedure, smears of *H. pylori* NCTC 11637 prepared by standard methods were immersed in 4% (wt/vol) paraformaldehyde followed by 50% (vol/vol) ethanol for 10 minutes each and allowed to air dry. The smears were then covered with 30 µl of hybridization solution (27) and a coverslip, placed in moist chambers and incubated for 90 minutes at 59ºC. Following hybridization, coverslips were removed and slides were submerged in a prewarmed (59 ºC) washing solution containing 5 mM Tris Base (Sigma), 15 mM NaCl (Sigma) and 1% (vol/vol) Triton X (pH 10; Sigma). Washing was performed at 59 ºC for 30 minutes and the slides allowed to air dry.

The smears were mounted with one drop of non-fluorescent immersion oil (Merck) and covered with coverslips. Slides were stored in the dark for a maximum of 48 hours before microscopy. Microscopy was conducted using a Zeiss Axioplan (Oberkochen, Germany) and an Olympus BX51 (Perafita, Portugal) epifluorescence microscope equipped with one filter sensitive to the signaling molecule of the PNA probe. Filters that were not able to detect the probe were used as negative controls. For each experiment, a negative control was performed where all the steps described here were carried out but where no probe was added to the hybridization solution.

2.4 Specificity and sensitivity of the probe

To test the specificity and sensitivity of the probe, several *H. pylori* strains, *Helicobacter* spp. and other bacteria were tested **Table 2.2**. All *H. pylori* strains were maintained on Columbia agar (Oxoid, Basingstoke, UK) supplemented with 5% (vol/vol) defribinated horse blood (Biomérieux, Marcy l'Etoile, France). Plates were incubated at 37 °C in a CO_2 incubator (HERAcell® 150; Thermo Electron Corporation, Waltham MA, USA) set to 10% CO_2 and 5% O_2 and single colonies were streaked onto fresh plates every two or three days. All other *Helicobacter* and *Campylobacter* species were grown on Campylobacter Selective Agar (Sigma) supplemented with 5% (vol/vol) defibrinated sheep blood (Probiológica, Sintra, Portugal) and maintained in similar conditions to *H. pylori*, except for *Campylobacter* spp. that were incubated at 42ºC; other bacteria used in this study were grown on R2A agar at room temperature (20 - 25ºC) for three days.

Table 2.2 - Results of the *H. pylori* probe specificity test

Organism	FISH outcome
H. pylori	
NCTC 11367^T	+
ATCC 700392^T	+
ATCC 700824^T	+
1342[a]	+
1198[a]	+
1320[a]	+
957[a]	+
968[a]	+
1330[a]	+
ATCC 49503^T	+
H. cinaedi	
33221-1.2[b]	−
33052-1.3[b]	−
H. mustelae	
2G1[b]	−
2H1[b]	−
H. felis 2I4[b]	−
H. muridarum 2A5[b]	−
H. mustelae CIP 103769^{Tc}	−
H. canadensis CCUG 47163^{Tc}	−
H. pametensis CIP 104249[c]	−
H. pullorum CCUG 33837^{Tc}	−
H. salomonis CIP 105607[c]	−
H. hepaticus CIP 104100[c]	−
H. cinaedi CIP 105369[c]	−
H. canis CIP 104753[c]	−
Sphingomonas capsulata	−
Staphylococcus sp.	−
Acinetobacter calcoaceticus	−
Campylobacter sp. strain 1	−
Campylobacter sp. strain 2	−
Campylobacter sp. strain 3	−

[a] Clinical isolate kindly provided by Maria Lurdes Monteiro.
[b] Isolate kindly provided by Jay Solnick.
[c] Isolate kindly provided by Francis Mégraud.

As shown in **Table 2.2**, Hpy769 hybridizes with all *H. pylori* strains whereas no hybridization was observed for the other bacterial species used. It is interesting to observe that, despite the predicted 89% sensitivity, the probe was able to detect all *H. pylori* strains used in this study. Positive detection of culture collections was already expected, as the 16S rRNA sequences deposited in the databank were known to be complementary to our probe. It could be expected that some clinical isolates would not

be detected by our probe. This was not the case, but it is worth mentioning that all clinical isolates were obtained from the same institution (strain collection of the National Institute of Health in Lisbon, Portugal), and from individuals within the same geographical region, which might imply that conserved 16S rRNA sequences between strains are more likely to occur.

All hybridized *H. pylori* strains emitted a bright red fluorescence and the three different morphological types of the bacterium (spiral, U-shape and coccoid) (1) could be clearly observed (**Figure 2.2**). There was no cross-hybridization to the rRNA of other bacteria used in this study and as such Hpy769 labeled cells of *H. pylori* could be easily distinguished from non *H. pylori* strains. This was the first FISH probe targeting *H. pylori* that was tested against such a large number of closely related species. This is particularly important because it has been reported that existing DNA probes are at times unable to discriminate between sequences with only one base pair mismatch (34).

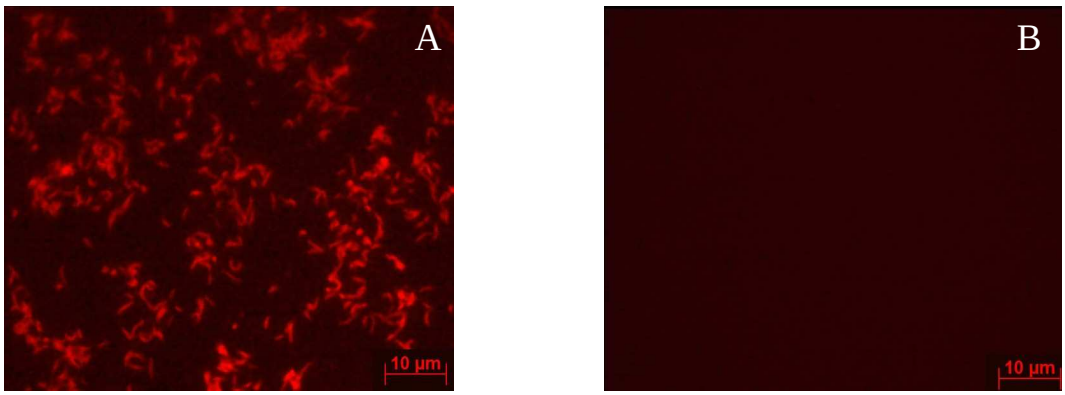

Figure 2.2 - Detection of *H. pylori* using the red fluorescent Hpy769 probe in a smear of pure culture of *H. pylori* NCTC 11637. Notice the presence of all three morphological types (A); and lack of signal in a smear of pure culture of *Helicobacter muridarum* 2A5 (B)

The identification of coccoid forms by this method assumes particular importance, because it has been recently shown that the stringent response in *H. pylori* induces, besides the morphological conversion into coccoid shape, a decrease in the total RNA production (18,33). Nevertheless, this method appears to be sensitive enough to detect these lower numbers of rRNA copies per cell.

2.5 Hybridization in gastric biopsies

After designing the probe and optimizing the FISH procedure we applied the method to fifteen histological slides of formalin-fixed, paraffin-embedded gastric biopsy specimens from five patients, four infected with *H. pylori* and one uninfected. Three μm thickness histological slides were deparaffinazed and rehydrated in xylol and ethanol. Slides were immersed twice in xylol for 15 minutes each time, and in decreasing concentrations of ethanol (100%, 95%, 80%, 70% and 50%) for 5 minutes each time and finally washed with distilled water for 10 minutes. Histological slides were then allowed to air dry. As shown in **Figure 2.3**, the presence of *H. pylori* could be easily detected using the new PNA probe, nevertheless some experience at microcopy is required. After applying the PNA FISH method we counterstained the sample with the Giemsa staining method to confirm our results (**Figure 2.4**). In short, histological slides of gastric biopsy specimens were stained with 2% (vol/vol) Giemsa Solution for 30 minutes and washed in distilled water. Slides were then immersed in 95% (vol/vol) alcohol followed by absolute alcohol.

Additional experiments have proven that PNA FISH is also able to be easily adapted to membrane filters and solid surfaces of different metallic and polymeric materials (data not shown).

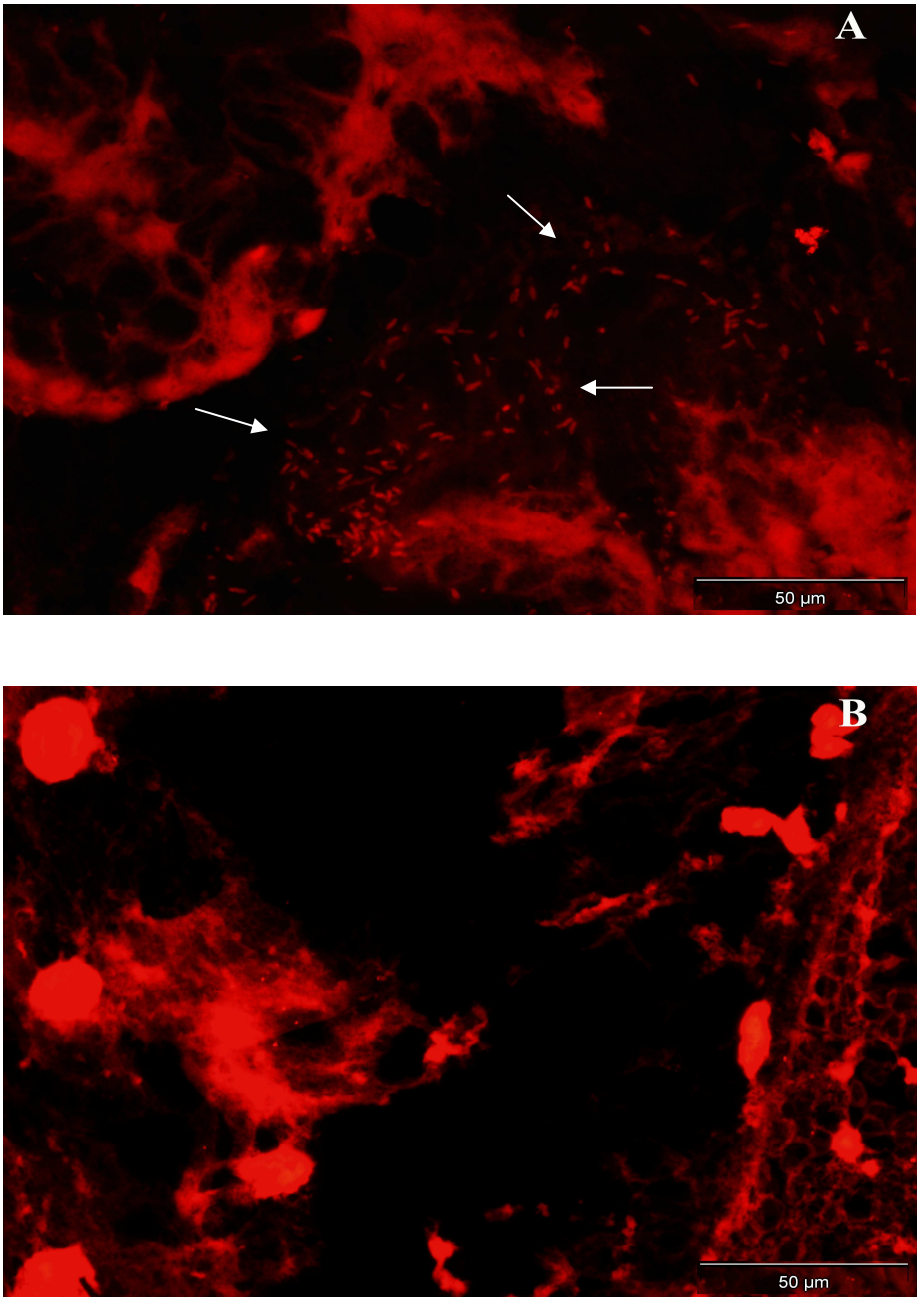

Figure 2.3 - Detection of *H. pylori* using the red fluorescent Hpy769 probe in a histological slide of a gastric biopsy specimen of an infected patient (A) and of a non-infected patient (B). The experiment was performed in parallel and images were obtained with equal exposure times.

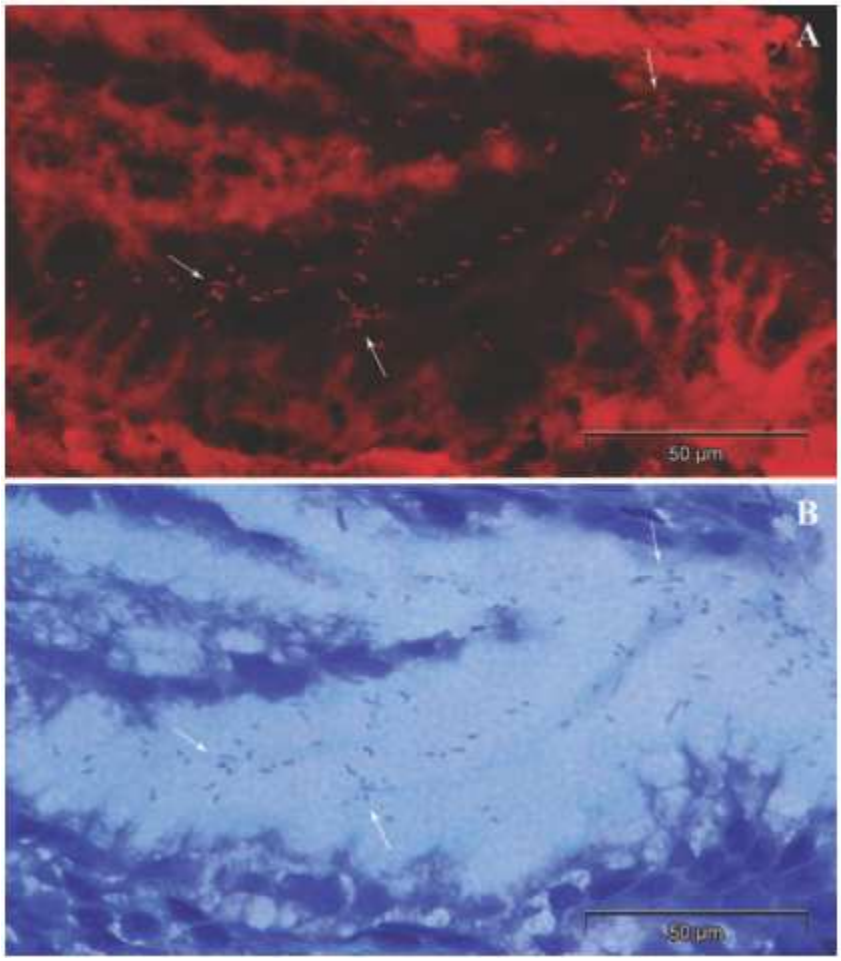

Figure 2.4 - Detection of *H. pylori* in a histological slide of a gastric biopsy specimen using the red fluorescent Hpy769 probe (A) and counterstained with the Giemsa stain (B).

2.6 Conclusions

As it was shown, the Hpy769 probe presented an improvement in the detection of the bacteria when compared with the previous PNA probe (4) and is at least as specific as DNA probes reported in the literature for *H. pylori* detection. Even though sensitivity is slightly lower than that of probe Hpy-1 based on current data, only when more strains from a range of patients of diverse geographical locations are sequenced it will be possible to have a more correct idea of the exact values. Nevertheless, the higher suitability of PNA probes to adapt to multiplex experiments (i.e. hybridization of several probes at the same time), implies that future improvements of the method can be more easily undertaken. In fact, Hpy769 was one of the first probes with PNA chemistry coupled with a fluorophore from the Alexa Fluor family. One of the advantages of these dyes, when compared with other fluorochromes that are used routinely, is that they have a very narrow emission band, allowing a better discrimination of different dyes under the microscope in multiplex experiments.

The PNA FISH procedure using the Hpy769 probe has been shown to be a very sensitive and specific method for the detection of *H. pylori* in a variety of samples such as smears, and gastric biopsies. In addition, the method is easy to implement without the requirement of special equipment or facilities, apart from an epifluorescence microscope.

The detection of the bacteria in biopsy specimens is very useful because with this technique we are able to not only detect all bacteria but also pinpoint their exact location in the gastric tissue and even identify their morphology, which is not possible with standard techniques such as culture methods or even PCR. In the future, this method can be adapted to identify *H. pylori* in other locations than the stomach, detect antibiotic resistant strains (24), study possible interactions between different *H. pylori* strains colonizing the same individual, or between *H. pylori* and other *Helicobacter* spp. that are known to inhabit the human gastrointestinal tract (11,30).

2.7 Acknowledgments

We would like to thank Maria Lurdes Monteiro, Francis Mégraud and Jay Solnick for providing the clinical isolates and *Helicobater spp* used in this study. We would also like to thank to Fátima Carneiro for providing the histological slides of the gastric

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Chapter 3

Helicobacter pylori colonization of the adenotonsillar tissue: fact or fiction?

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* Equal contribution.

Objective: The transmission of the of the gastric pathogen *Helicobacter pylori* involves the oral route. Molecular techniques have allowed the detection of *H. pylori* DNA in samples of the oral cavity, although culture of *H. pylori* from these type of samples has been sporadic. Studies have tried to demonstrate the presence of *H. pylori* in adenotonsillar tissue, with contradictory results. Our aim was to clarify whether the adenotonsillar tissue may constitute an extragastric reservoir for *H. pylori.*

Methods: Sixty-two patients proposed for adenoidectomy or tonsillectomy were enrolled. A total of 101 surgical specimens, 55 adenoid and 46 tonsils, were obtained. Patients were characterized for the presence of anti-*H. pylori* antibodies by serology. On each surgical sample rapid urease test, immunohistochemistry, FISH with a peptide nucleic acid probe for *H. pylori*, and PCR-DEIA directed to the *vacA* gene of *H. pylori* were performed.

Results: 39% of the individuals had anti-*H. pylori* antibodies. Rapid urease test was positive in samples of three patients, all with positive serology. Immunohistochemistry was positive in samples of two patients, all with negative serology. All rapid urease test or immunohistochemistry positive cases were negative by FISH. All samples tested were negative when PCR-DEIA for *H. pylori* detection was used directly in adenotonsillar tissue.

Conclusions: The adenotonsillar tissue does not constitute an extra-gastric reservoir for *H. pylori* infection, at least a permanent one, in this population of children. Moreover, techniques currently used for detecting gastric *H. pylori* colonization are not adequate to evaluate infection of the adenotonsillar tissues.

3.1 Introduction

Helicobacter pylori is considered the most frequent chronic infection of humans, with more than half of the world's population being infected [1]. *H. pylori* is a microaerophilic, gram negative bacterium that persistently colonizes the human gastric mucosa. The infection is usually acquired early in childhood and can persist for the lifetime of the host if not treated [1]. *H. pylori* infection occurs worldwide, but its prevalence is significantly different between countries [2]. Studies of risk factors for *H. pylori* infection have associated the prevalence of infection with the socioeconomic indicators, with higher prevalence in developing regions and lower prevalence in developed regions [2].

The transmission of *H. pylori* is still poorly understood, but the fecal-oral and the oral-oral are the most consensual routes of transmission [1,2]. The presence of *H. pylori* in the oral cavity has been detected in samples from supragingival plaque, dental plaque and saliva by polymerase chain reaction [2-4], but culture of the bacteria from this type of samples has been sporadic. More recently, it has also been suggested that adenotonsillar tissues may be a reservoir for *H. pylori*, and several studies evaluated the presence of *H. pylori* in adenoid and tonsil specimens [5-7]. However, results have been contradictory and, most importantly, techniques used to detect *H. pylori* in these tissues were not appropriate. Therefore, our aim was to clarify whether the adenotonsillar tissue may constitute an extragastric reservoir for *H. pylori.* We have studied 62 children proposed for adenoidectomy and/or tonsillectomy, and a total of 101 samples (55 adenoid and 46 tonsils) were characterized for the presence of *H. pylori* using different techniques.

3.2 Materials and Methods

3.2.1 Patients and surgical procedures

Sixty two consecutive patients (mean age = 7.9 ± 5.5 years and male/female ratio of 1:1.2) were recruited for this study. Thirty nine patients (62.9%) underwent adenoidectomy, sixteen patients (25.8%) underwent adenotonsillectomy and seven patients (11.3%) underwent tonsillectomy at the Hospital São Marcos, Braga, Portugal. Routine surgical indications for adenotonsillectomy were followed. All patients were

operated under general anesthesia. Blocage of gastro-pharyngeal reflux was obtained with gauze placed on the hipopharynx after oro-tracheal entubation and before trendlenburg positioning. Protocols followed in the study were in accordance with the institutional ethical standards. Informed consent was obtained from the patients' parents. Samples were delinked and unidentified from their donors.

3.2.2 Serology

Serum samples were used for detection of IgG antibodies against *H. pylori*, using the Pyloriset EIA-G III Kit (Orion Diagnostics, Espoo, Finland). The assay was performed following the manufacturer's instructions.

3.2.3 Rapid urease test

A 1 mm^3 sample from each surgical specimen was immersed in urea broth medium containing phenol red (Fluka, Buchs, Switzerland) and incubated for 24h at 37ºC. At the end of that time period, color changes in medium were registered.

3.2.4 Histology and imunohistochemistry

Surgical specimens were formalin fixed, paraffin embedded, and sections of 3 μm were cut. Slides were deparaffined in xylol and hydrated with grade ethanol concentrations, and stained with hematoxylin and eosin, modified Giemsa and with a rabbit anti-human polyclonal antibody against *H. pylori* (Cell Marque, California, USA). Briefly, antigen retrieval was performed by microwave pre-treatment in 10 mmol/L citrate buffer (pH 6.0) for 15 min. Endogenous peroxidase activity was blocked with hydrogen peroxide block solution (Labvision, UK) for 10 min. After washing in phosphate buffered saline (PBS), sections were incubated for 30 minutes with primary antibody (1:500). Staining was achieved using Dako RealTM EmvisionTM/HRP rabbit/mouse polymer, for 30 minutes at room temperature. Binding was detected using diaminobenzidine (DAB), which upon oxidation forms a stable brown end-product. Nuclei were slightly counterstained with Mayer's hematoxylin. Gastric biopsies positive for *H. pylori* infection were used as positive controls. Slides were visualized by two experienced pathologists that were blind to the patients' clinical data.

3.2.5 PNA-FISH

H. pylori was detected in the adenotonsillar samples using the Hpy769 probe, a peptide nucleic acid probe (PNA) that is highly specific and sensitive for the detection of *H. pylori* [8]. The surgical specimens were deparaffinazed and rehydrated in xylol and ethanol. Samples were immersed twice in xylol for 15 minutes, and in decreasing concentrations of ethanol for 5 minutes, and were finally washed with distilled water for 10 minutes. Samples were allowed to air dry and the fluorescence *in situ* hybridization (FISH) procedure was performed as described in Guimarães *et al* [8].

3.2.6 DNA isolation

DNA was isolated of 8 mm^3 fragments from all surgical specimens using the DNA Invisorb Spin Tissue Mini Kit (Invitek, Berlin, Germany). The extraction was performed following the manufacturer's instructions. In a subset of cases, the whole surgical specimen was fully homogenized in Tris-EDTA buffer (10mM Tris pH 7.5, 1mM EDTA) using an ultraturax homogenizer and DNA was isolated.

3.2.7 PCR and DEIA

Detection of the *vacA* gene of *H. pylori* was performed using primers VA1F and VA1XR, described previously [9]. PCR reaction mixtures were performed in a volume of 25µL, 1x PCR Buffer, 2.5mmol/L of $MgCl_2$, 0.25mmol/L dNTPs, 0.25U AmpliTaq Gold, and 25pmol of each primer. PCR was performed with 9 min pre-denaturation at 95ºC, followed by 40 cycles of 30s at 95ºC, 45s at 50ºC, and 45s at 72ºC. Final extension was performed for 10 min at 72ºC. Amplimers carrying a biotin moiety at the 5' terminus of the reverse primer were detected in a microtiter well-based DNA hybridization assay (DEIA) using a specific general probe for the *vacA* gene [10,11]. DNA isolated from *H. pylori* Tx30a (ATCC 51932, American Type Tissue Culture) and 26695 (ATCC 700392) strains was used as reference.

3.2.8 Statistical analyses

Age distribution between *H. pylori* seropositives and seronegatives was analysed by the Student's *t* test. Association between gender and *H. pylori* serology was assessed by the Pearson Chi-Square test. Logistic regression models were computed with Statview for Windows software (version 5.0; SAS Institute Inc., Cary, NC). Differences in data values were considered significant at P values lower than 0.05.

3.3 Results

The study population was constituted by 62 patients living in the North of Portugal, which were indicated to surgery due to adenotonsillar hypertrophy (47 cases), infection (5 cases) or both (10 cases). 62 blood samples and 101 surgical samples (55 adenoids, 23 left tonsils and 23 right tonsils) were obtained.

The presence of anti-*H. pylori* antibodies in serum was detected in 24 (39%) cases. Infected patients were older than uninfected patients (p=0.013) and were more frequently females (p=0.044) (**Table 3.1**). In a logistic regression analysis, including both gender and age in the model, only age remained associated with *H. pylori* seropositivity (p=0.039).

Table 3.1 - Characteristics and *H. pylori* serology in the studied individuals.

	Serology		
	Negative (n=38)	**Positive (n=24)**	**p value**
Age (Average ± SE)	6.5 ± 0.6	10.1 ± 1.5	0.013
Gender			
Female	17	17	0.044
Male	21	7	

To evaluate the presence of *H. pylori* in the adenotonsillar tissues, we have used the rapid urease test, immunohistochemistry with a polyclonal anti-*H. pylori* antibody, FISH with a specific *H. pylori* PNA probe, and PCR-DEIA for *H. pylori vacA* gene (**Table 3.2**).

Table 3.2 - *H. pylori* detection in adenotonsillar tissues by different methods

Method	**Adenoids (n=55)**	**Tonsils (n=46)**
Rapid urease test	2 (3.6%)	2 (4.3%)
Immunohistochemistry	0 (0%)	3 (6.5%)
PNA-FISH	0 (0%)	0 (0%)
PCR-DEIA	0 (0%)	0 (0%)

The rapid urease test was positive in two (3.6%) adenoid and in two (4.3%) tonsil specimens from three patients. All urease test positive samples were from patients infected with *H. pylori* as evaluated by serology.

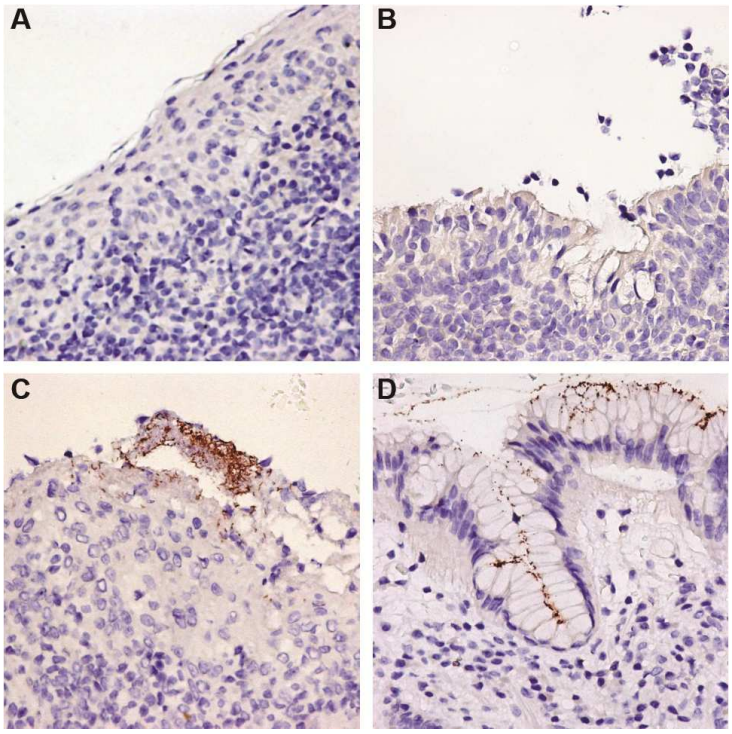

Figure 3.1 - Immunohistochemistry using a policlonal anti-*H. pylori* antibody in adenoid and tonsil surgical specimens. (A) and (B) Negative specimens; (C) Tonsil specimen showing *H. pylori*-like microorganisms; (D) *H. pylori*-infected gastric mucosa used as positive control.

Immunohistochemistry was positive in three tonsil specimens from two patients (**Figure 3.1**), and these patients were *H. pylori* serology negative. FISH using a specific PNA probe for *H. pylori* was negative in paraffin cuts consecutive to those used for immunohistochemistry (**Figure 3.2**). PNA-FISH was negative in all studied specimens.

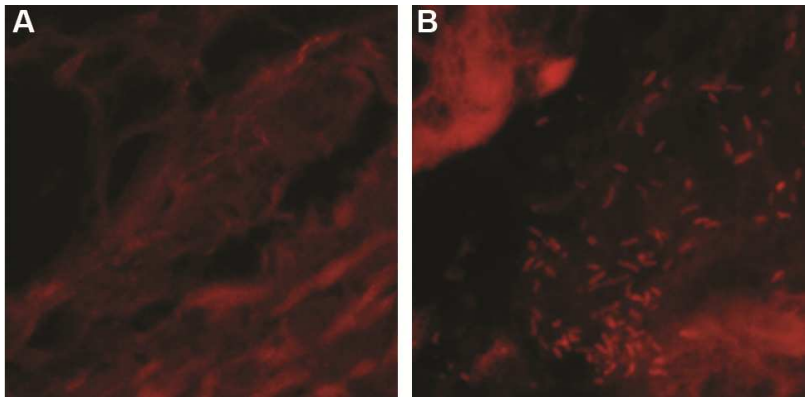

Figure 3.2 - PNA-FISH for *H. pylori* detection in adenoid and tonsil surgical specimens: (A) Negative tonsil specimen; (B) *H. pylori*-infected gastric mucosa used as positive control.

The presence of *H. pylori* in the adenotonsillar samples was also assessed by PCR, directed to the *vacA* gene, which is present in all strains of *H. pylori*. In order to improve the sensitivity of the PCR, amplimers were hybridized with a *vacA* specific probe in DEIA assays. All samples were negative for the presence of *H. pylori*.

3.4 Discussion

Data concerning the presence of *H. pylori* in adenoids and/or tonsils are inconsistent. In this study we have initially characterized all patients for the presence of serum anti-*H. pylori* IgG antibodies. In agreement with previously published data on the prevalence of *H. pylori* infection in the Portuguese population in pediatric age, 39% of the patients were *H. pylori* positive [12]. The presence of the infection was associated with older patients and, in keeping with previous studies, gender did not constitute a risk factor for *H. pylori* infection [13].

To clarify whether *H. pylori* is present in the adenotonsillar tissues of these childre, we have used a variety of approaches directly aimed at the tissue samples. Therefore, each adenoidal and tonsillar specimen were submitted to rapid urease test, immunohistochemistry, PNA-FISH and PCR-DEIA. We detected urease-positive microorganisms in four tissue specimens of three patients with positive *H. pylori* serology. However, we also demonstrated by immunohistochemistry, PNA-FISH and PCR-DEIA that these urease-producing microorganisms were not *H. pylori*. Though immunohistochemistry was positive in three tonsil specimens from two patients, these specimens were *H. pylori*-negative by PNA-FISH and PCR-DEIA, and the patients did not present antibodies anti-*H. pylori.*

All adenotonsillar specimens included in this study were negative when PNA-FISH or PCR-DEIA were used. The PNA-FISH method has been previously tested in different types of samples and with different microorganisms, and has shown a high specificity for *H. pylori* [8]. PCR is recognized as having high specificity if the target gene and the primers are carefully chosen. In this study, we have used as amplification target *H. pylori vacA* gene that, besides having no homology to other bacterial species, is present in all *H. pylori* strains. The primers used are well established and their high sensitivity and specificity has been previously demonstrated [10,11]. PCR sensitivity was increased by hybridization of amplified products with a specific *vacA* probe [11].

Considering that PNA-FISH and PCR-DEIA are the most specific and sensitive methods and that all tissue samples analyzed were negative, results obtained by rapid urease test and immunohistochemistry are false positives. Several studies have reported the presence of *H. pylori* in adenoid and/or tonsillar tissues based on the solely use of rapid urease test [6,7]. However, we and others showed that, in cases positive for the rapid urease test, the use of additional detection methods reveals that those are likely false positive results [14,15]. One very important aspect concerns the specificity of the rapid urease test and of immunohistochemistry. These are related with the probability of the presence in the sample of microorganisms, other than *H. pylori*, that could origin cross reactivity leading to false positive results. So, although these techniques have a high specificity when used in gastric samples, the specificity may be lower in samples from polimicrobial environments such as the adenotonsillar tissues [14]. The rapid urease test is not in fact a specific test for *H. pylori*, but for urease-producing microorganisms. Therefore, the positive results that we have obtained probably reflect the presence in adenotonsillar tissues of other urease-producing bacteria. Likewise, and although immunohistochemistry allows higher reproducibility of *H. pylori* detection in gastric samples when compared with conventional histological techniques [16], in polimicrobial extragastric samples antibodies can recognize bacterial epitopes other than *H. pylori*, leading to false positive results.

The negative results we have obtained by PNA-FISH and PCR-DEIA are also in accordance with previously published data using PCR and/or culture [14,15,17-20]. The use of PCR for detection of *H. pylori* in adenotonsillar tissues has sporadically given positive results [21]. However, primer choice needs to be viewed with caution, since certain primer sets used for detection of *H. pylori* produce a very high rate of false-positive results [22].

The main limitation of this study is related with the fact that the gastric infection status of the studied population was only evaluated by IgG serology, which is not specific for active gastric *H. pylori* infection. Therefore, we can not exclude the possibility that the adenoids and tonsils are infected only in cases of active or recurrent disease might be of importance to the clinical management. Furthermore, and because *H. pylori* infection is less common in the pediatric population than in adults, the lack of adenotonsillar *H. pylori* infection in children does not exclude this possibility in the adult population.

3.5 Conclusion

The adenotonsillar tissue does not seem to constitute an extragastric reservoir for *H. pylori*, or at least a permanent one, in this population of children. Furthermore, the rapid urease test and immunohistochemistry assays alone are not suitable for *H. pylori* detection in adenotonsillar samples and highlight the importance of methodology selection for detection of *H. pylori* in non-gastric samples.

3.6 Acknowledgements

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Chapter 4

Saliva influence on survival and infection of *Helicobacter pylori*

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The epidemiology of *H. pylori* has been extensively studied for many years but there is still uncertainty about the exact mode of transmission of the bacterium. Person-to-person transmission is widely seen as the most probable route of infection and several studies have identified *H. pylori* in oral samples from supragingival and dental plaque and in saliva. Saliva is present in high quantities in oral cavity and is constantly being swallowed, which may constitute a means for *H. pylori* to reach the gastric environment. However, the influence of the exposure of *H. pylori* to saliva and its consequences in the survival and infection capacity of the bacteria are largely unknown. In order to evaluate the effect of saliva in *H. pylori* we assessed the cultivability of saliva-exposed *H. pylori* and determined whether this bacterium retains the ability to adhere and induce inflammation in an experimental model of human gastric epithelial cells. Results demonstrated that contact with saliva did not alter the ability of *H. pylori* to adhere to host cells, and only with exposure times higher than 24 hours the bacteria loses their culturability and ability to induce IL-8 secretion by the host cells. In conclusion, only longer times of exposure to saliva affect the properties of *H. pylori*. Since saliva is constantly being swallowed it could allow the bacteria to rapidly reach the gastric environment in a viable form.

4.1 Introduction

Helicobacter pylori is a spiral, microaerophilic, Gram-negative bacterium that colonizes the human stomach and has been associated with the pathogenesis of chronic gastritis, peptic ulcer disease and gastric carcinoma [1]. *H. pylori* is the most prevalent chronic infection in the world and is usually acquired in childhood [2]. The epidemiology of *H. pylori* has been extensively studied for many years but there still uncertainty about the exact mode of transmission of the bacteria. Taken together epidemiological and microbiological evidence several routes of transmission have been conjectured. Person-to-person transmission is widely seen as the most probable route of infection [3], and the oral cavity may play a role in this process as well as in the reinfection of the stomach [4]. In fact, information about the presence of *H. pylori* in the oral cavity is controversial. *H. pylori* has been isolated from saliva [5-8], supragingival dental plaque [9-10], and tongue dorsum [9, 11] in some reports, whereas in others there was no detection at all of oral *H. pylori* [11-12]. Whether the oral cavity could be a permanent or transient reservoir for *H. pylori* is still a matter of controversy. Also, the association between oral and gastric *H. pylori* detection is inconsistent [5, 13-17]. While these questions remain without answer, little is known about the ability of the bacteria to survive in the oral environment, since most of the studies report detection of bacterial DNA but no information about the biological state of the bacteria is assessed. Because the oral cavity is almost constantly flushed with saliva, it would be important to know the influence of this fluid in *H. pylori.* Therefore, the aim of this work was to assess the cultivability of saliva exposed *H. pylori* and to determine whether these bacteria retain the ability to adhere to and to induce inflammation in an experimental model of human gastric epithelial cells.

4.2 Materials and Methods

***H. pylori* culture and maintenance.** Experiments were performed with *H. pylori* 26695 obtained from the American Type Culture Collection (ATCC). Bacteria were grown in tryptic Soy Agar (TSA) supplemented with 5% sheep blood (Biomérieux, Marcy L'Étoile, France) and incubated at 37°C under microaerophilic atmosphere for 48 hours.

Cell Culture and Reagents. AGS cells, derived from a human gastric carcinoma, were maintained in RPMI 1640 medium (Invitrogen), supplemented with 10% fetal bovine serum (FBS) and 1% Pen-Strep (Invitrogen) at 37°C, under a 5% CO_2 humidified atmosphere.

Saliva. Saliva was collected from 15 healthy adult volunteers that were asked to chew tampons of Salivettes® for 1 minute. Salivettes were centrifuged at 1000g for 2 minutes at 20°C. The saliva recovered was pooled together and centrifuged at 14000 rpm for 20 minutes. The supernatant was filtered through a 0.22 µm filter and stored at -20°C for future use.

Saliva exposed *H. pylori*. After being in culture for 48 hours, *H. pylori* was harvested from TSA plates and suspended in 3 mL of centrifuged and filtered saliva in a 10^9 bacteria/mL concentration. The suspensions were kept at 37°C under aerophilic conditions. Cells were exposed to saliva for 6, 15, 24 and 48 hours. As controls, *H. pylori* inocula that have not been exposed to saliva were used.

Culturability. The number of culturable bacteria in the different time points was determined by plating serially dilutions of the suspensions in TSA plates containing 5% sheep blood. Culturability was analyzed by comparing the number of colony-forming units (CFU's) of each time point.

Adhesion assay. Cells were cultured in 96-well plates at 37°C and 5% CO_2 for 48 hours in RPMI 1640, supplemented with 10% FBS. A volume of *H. pylori* suspension, corresponding to the different times of saliva-exposure, was added to the cells at a multiplicity of infection (MOI) of 100 and the plate was gently agitated for 30 minutes at 37°C. Cultures were fixed with 1% parafomaldehyde and blocked with 1% phosphate buffered saline (PBS) - bovine serum albumin (BSA) for 30 minutes. Bacterial adhesion was determined by ELISA as previously described [18] using a rabbit polyclonal anti-*Helicobacter* (Cell Marque) as primary and an anti-rabbit IgG-horseradish peroxidase as secondary antibody. Binding was visualized after incubation with tetramethylbenzidine (TMB) and with 1M HCl. Absorbance was read at 450 nm. Controls for *H. pylori* binding to wells comprised wells with no AGS cells, to which bacteria were added and

allowed to adhere to the plastic before fixation. Negative controls contained neither AGS cells nor *H. pylori*. Bacterial adhesion was expressed as percentage of adhesion to AGS cells of *H. pylori* that were not exposed to water.

IL-8 Production. AGS cells were grown in 24-well plates for 48 hours in RPMI supplemented with 10% FBS at 37°C and 5% CO_2. A volume of bacteria suspension corresponding to each saliva-exposure times were added to cells, at a MOI of 100, and incubated for 24 hours at 37°C. Afterwards, the media were recovered and centrifuged at 1200 rpm for 10 minutes. Supernatants were collected and IL-8 levels were assessed by ELISA using a commercially available kit (Quantikine, Human CXCL8/IL8, R&D Systems).

Statistical analyses. Data were analyzed with Student's t test using the Statview for Windows software (version 5.0; SAS Institute Inc., Cary, NC), and were expressed as mean values of, unless otherwise stated, three independent experiments ± standard deviations. Differences in data values were considered significant at P values lower than 0.05.

4.3 Results

4.3.1 *H. pylori* culturability after saliva exposure

The culturability of the *H. pylori* was evaluated after 0, 6, 15, 24 and 48 hours of saliva exposure (**Figure 4.1**). Results showed a decrease in culturability with increasing times of exposure to saliva, and after 48 hours of saliva-exposure *H. pylori* was no longer culturable.

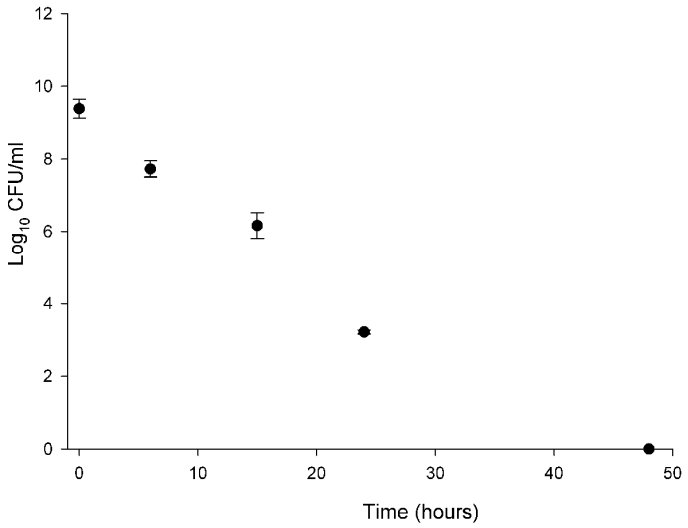

Figure 4.1 - Effect of saliva exposure on *H. pylori* culturability. After saliva exposure, bacteria suspensions were platted in TSA and incubated for 7 days at 37ºC in microaerophilic conditions. Each experiment was performed in triplicate.

As the subsequent studies on virulence factors were performed for all time points as well, we were able to observe the modulation of virulence properties as the bacteria transitioned from the culturable to the non-culturable state.

4.3.2 Saliva-exposure influence on the adhesion of *H. pylori* to host cells

Adhesion to the host cells is a fundamental step in the colonization of the gastric mucosa by *H. pylori* [19]. To assess whether the ability of *H. pylori* to adhere to gastric epithelial cells is altered by the contact of the bacterium with saliva, we performed an adhesion assay in an ELISA format [18]. Results have showed that the exposure of *H.*

pylori to saliva did not lead to statistically significantly alterations in the ability of bacteria to adhere to AGS cells, even after 48 hours of saliva exposure (**Figure 4.2**).

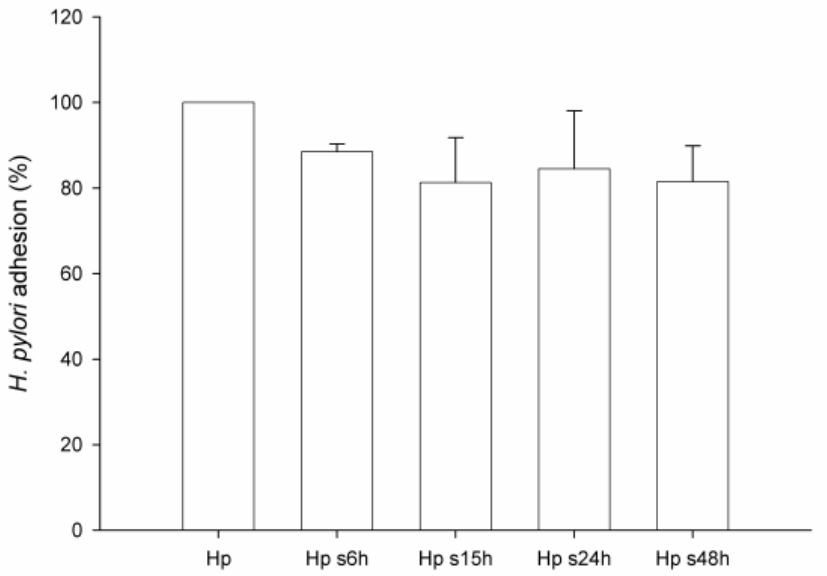

Figure 4.2 - Effect of saliva exposure on *H. pylori* adhesion to host epithelial cells. AGS cells were infected with *H. pylori* 26695 inocula that have been exposed to saliva for 6 (Hp s6h), 15 (Hp s15h), 24 (Hp w24h), and 48 (Hp w48h) hours, at a MOI of 100.

4.3.3 Influence of saliva on *H. pylori* induction of IL-8 secretion by host epithelial cells

H. pylori infection leads to an inflammatory response by the host, which includes secretion of the pro-inflammatory cytokine IL-8 by epithelial cells [20]. To evaluate if the exposure to saliva affects the capability of the bacteria to induce inflammation, we assessed the secretion levels of IL-8 from AGS cells infected with *H. pylori* inocula that were exposed to saliva for different time periods (**Figure 4.3**).

The results revealed that the bacterium retains the ability to induce IL-8 production even after 24 hours of exposure to saliva. However, after 48 hours *H. pylori* is no longer able to induce IL-8 secretion by the AGS cells. These observations are in keeping with the results obtained with bacteria culturability after exposure to saliva.

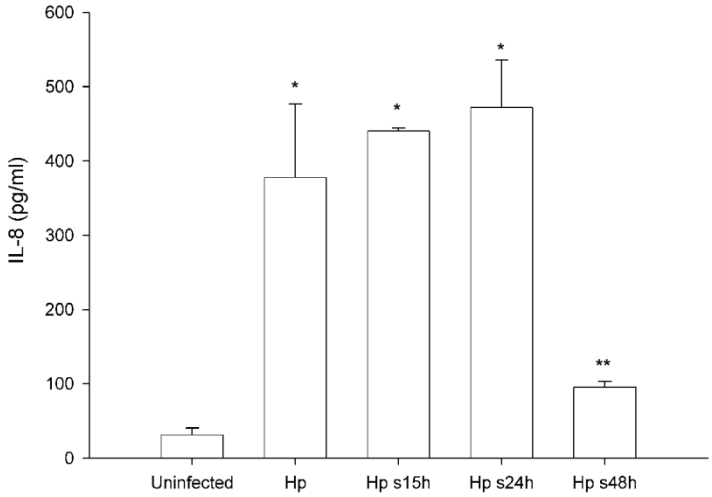

Figure 4.3 - Effect of saliva exposure on *H. pylori* induction of IL-8 secretion by host epithelial cells. AGS cells were infected with *H. pylori* 26695 inocula that have been exposed to saliva for 15 (Hp s15h), 24 (Hp s24h), and 48 (Hp s48h) hours, at a MOI of 100. As control, *H. pylori* 26695 that were not exposed to water were used (Hp). IL-8 production was evaluated by ELISA. Graphics represent mean ± SD and are representative of three independent experiments. *, significantly different from uninfected cells; **, significantly different from non-exposed *H. pylori* ($p<0.05$).

4.4 Discussion

The oral cavity assumes an important role in *H. pylori* transmission, as it constitutes a portal of entry for *H. pylori*, and as it may serve as a reservoir for the infection [17, 21-25]. Saliva is present in high quantities in the oral cavity and is constantly being swallowed, possibly representing the way for *H. pylori* to reach the gastric environment [23]. It has been shown that *H. pylori* binds to saliva [26-27]. Yet, the influence of the exposure of *H. pylori* to saliva and its consequence in the survival and in the infection capacity of the bacteria are largely unknown.

In this study, we have shown that the cultivability of *H. pylori* after being exposed to saliva was maintained until exposures times of 24 hours. Because saliva is constantly being shed, this survival time could be enough for bacteria to access the gastric environment in a viable form. Nevertheless, and although longer times of exposure to saliva result in a non-culturable *H. pylori* form, *H. pylori* may remain viable and able to colonize the gastric mucosa of the host, as it has been shown in mice [28-30].

To be capable to colonize the gastric mucosa, and in the way from the oral cavity to the gastric environment, *H. pylori* has to be released from the major secreted mucin components of saliva [31-32] and to adhere to gastric mucin components. It is known that dynamic interactions between *H. pylori* and both oral and gastric mucins occur via a range of bacterial adhesins, pH conditions and different environmental niches [23]. In keeping with these observations, our results showed that the adhesion of *H. pylori* to the host gastric cells was not altered by the previous contact of the bacteria with saliva. Also in accordance with those data, the contact with saliva for 24 hours did not modify the ability of *H. pylori* to induce IL-8 secretion by host epithelial cells. This suggests that the infection process elicited by *H. pylori* and leading to inflammation of the gastric mucosa with the production of proinflammatory molecules [20] is not altered by the contact of bacteria with saliva. Although longer times of saliva exposure may result in the absence of IL-8 secretion by the host, which is in accordance with the lack of cultivability of *H. pylori*, viable but non-culturable *H. pylori* may still be able to recover their capacities *in vivo* and induce gastric inflammation, as it has been observed in animal models [28]. These results are similar to the ones obtained when *H. pylori* was exposed to water (unpublished data). In fact, in both cases despite the loss of culturability and ability to induce IL-8 secretion in AGS cells the bacteria retains the ability to adhere to the cells. The maintenance of the adhesion capacity suggests a

modulation of bacterium physiology after being exposed to stressful environments, such as saliva or water, and might allow the bacterium to colonize the host cells. Even if the bacterium is not able cause a lifelong infection, it could remain in the host time enough to contribute to a higher genetic diversity of other *H. pylori* that could be present in the human stomachs.

Altogether, our data suggest that exposure to saliva in the oral cavity does not immediately lead to a decrease in *H. pylori* viability. Since a large quantity of saliva is swallowed every day, giving multiple opportunities for the bacteria to reach the stomach, 24 hours could be more than enough for the bacteria to reach the stomach and start the infection mechanism.

4.5 Acknowledgements

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Chapter 5

Water-induced modulation of *Helicobacter pylori* virulence properties

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While the influence of water in *H. pylori* culturability and membrane integrity has been extensively studied, there is little data concerning the effect of this environment on virulence properties. For that, we studied the culturability of water exposed *H. pylori* and determined whether there was any relation with the bacterium ability to adhere, produce structural components of pathogenicity, and induce inflammation and alterations in apoptosis in an experimental model of human gastric epithelial cells. Results have demonstrated that *H. pylori* partially retained the ability to adhere to epithelial cells even after complete loss of culturability. However, the microorganism is no longer effective in eliciting *in vitro* host cell inflammation and apoptosis, possibly due to the non-functionality of the *cag* type IV secretion system (T4SS). These *H. pylori*-induced host cell responses, which are lost together with culturability, are known to increase epithelial cell turnover and consequently could have a deleterious effect on the initial *H. pylori* colonization process.

The fact that adhesion is maintained by *H. pylori* in detriment of other factors involved in later infection stages appears to point to a modulation of the physiology of the pathogen after water-exposure, and might provide the microorganism with the necessary means to, at least transiently, colonize the human stomach.

5.1 Introduction

Helicobacter pylori is an important human pathogen that causes chronic gastritis and is associated with the development of more severe diseases such as peptic ulcer disease and gastric cancer (Blaser and Atherton, 2004). Since the isolation of *H. pylori*, numerous studies have been published addressing the prevalence and epidemiology of infection (Brown, 2000; Kikuchi and Dore, 2005; Magalhaes Queiroz and Luzza, 2006), its relationship with disease, the identification and characterization of virulence factors and their role in pathogenesis (Prinz et al., 2003; Blaser and Atherton, 2004; Figueiredo et al., 2005). However, the scientific community is still struggling to understand how *H. pylori* is transmitted (Azevedo et al., 2009).

The most widely accepted routes of transmission are the oral-oral, fecal-oral and gastric-oral routes. Nevertheless, a growing amount of data report the identification of *H. pylori* in external environmental reservoirs such as food, domestic animals and, most significantly, in water (Dore et al., 2001; Park et al., 2001; Fujimura et al., 2002; Watson et al., 2004). In fact, several epidemiological studies have concluded that drinking water source, or drinking water-related conditions, was a risk factor for *H. pylori* acquisition (Karita et al., 2003; Krumbiegel et al., 2004; Fujimura et al., 2008). Molecular methods such as fluorescence *in situ* hybridization (FISH) and polymerase chain reaction (PCR) were able to detect the presence of *H. pylori* in water and water-associated biofilms from wells, rivers and water distribution networks (Flanigan and Rodgers, 2003; Fujimura et al., 2004; Bragança et al., 2005). However, the demonstration that *H. pylori* can be detected in water does not imply that the microorganism can colonize the human host. In fact, while it has been shown that water-exposed *H. pylori* total cell counts did not decrease for a period of 2 years at 4ºC (Shahamat et al., 1993), the complete loss of culturability of the microorganism takes less than 10 hours at temperatures over 20°C (Adams et al., 2003; Azevedo et al., 2004). This transition to the non-culturable state is typically accompanied by a morphological transition from spiral to coccoid cells. Depending on the authors, the latter state has been considered a manifestation of cell death (Kusters et al., 1997), or a cellular adaptation to less than optimum environments (Azevedo et al., 2007b). In the determination of the physiological state of these non-culturable cells that are still able to retain their structure for a much longer period lies the key to our understanding of the exact role of water on *H. pylori* transmission. More specifically, it is important to

address the effect of water exposure on several *H. pylori* mechanisms that are, under favorable conditions, able to induce a response in host cells. At the moment, and apart a couple of studies that concluded that water-induced coccoid forms of *H. pylori* can colonize the gastric mucosa and cause gastritis in mice (Cellini et al., 1994; She et al., 2003), there is still lack of information regarding the capacity of water exposed bacteria to induce a response in host cells.

In this study we assessed the culturability of water exposed *H. pylori* and determined whether this bacterium retains the capacity to adhere and to elicit host cell responses, such as inflammation and apoptosis, using an experimental model of human gastric epithelial cells. Since these host cell responses may be related with structural components of bacterial pathogenicity, we have also evaluated the capacity of water exposed *H. pylori* to assemble a functional *cag* type IV secretion system (T4SS).

5.2 Results

5.2.1 *H. pylori* culturability after water exposure

The culturability of the *H. pylori* was evaluated after 0, 2, 6, 24 and 48 hours of water exposure. Based on previous studies (Adams et al., 2003; Azevedo et al., 2004), we anticipated that the longest time points would be sufficient to turn the bacterium into the non-culturable state. The obtained results confirmed our expectations, as the culturability of *H. pylori* progressively decreased, and after 24h of water-exposure *H. pylori* was no longer culturable (**Figure 5.1**). As the subsequent studies were performed for all time points as well, we were able to observe the modulation of virulence properties of *H. pylori* as bacteria transitioned from the culturable to the non-culturable state.

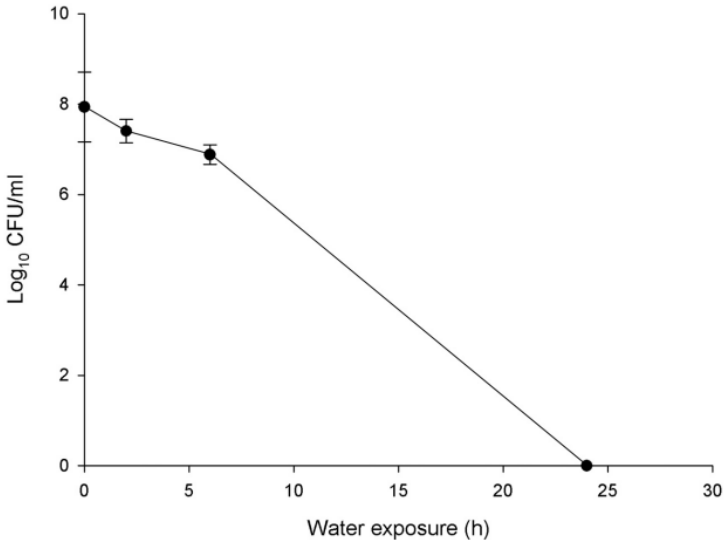

Figure 5.1 - Effect of water exposure on *H. pylori* culturability. After water exposure, bacteria suspension was platted in TSA plates and incubated for 7 days at 37ºC in microaerophilic conditions. The CFU's formed were counted to assess the culturability. Each experiment was performed in triplicate.

5.2.2 Influence of water exposure on the adhesion of *H. pylori* to host cells

Adhesion is the first step that *H. pylori* needs to undertake in order to successfully colonize the gastric mucosa. To assess whether the ability of *H. pylori* to adhere to gastric epithelial cells is altered by the contact of the bacterium with water, we performed an adhesion assay in an ELISA format using the human gastric epithelial AGS cell line. Whereas exposure of *H. pylori* to water for only 2 hours led to a statistically significant decrease of its ability to adhere to AGS cells ($P<0.05$), adhesion levels remained constant for bacteria that were exposed to water for longer time periods (**Figure 5.2**). Compared to non-exposed *H. pylori*, the decrease in adhesion of water-exposed bacteria was about 40%. Nevertheless, the observation that water-exposed *H. pylori* are still capable of adhering to cells suggests that in these conditions the bacterium may still exert effects in host gastric cells.

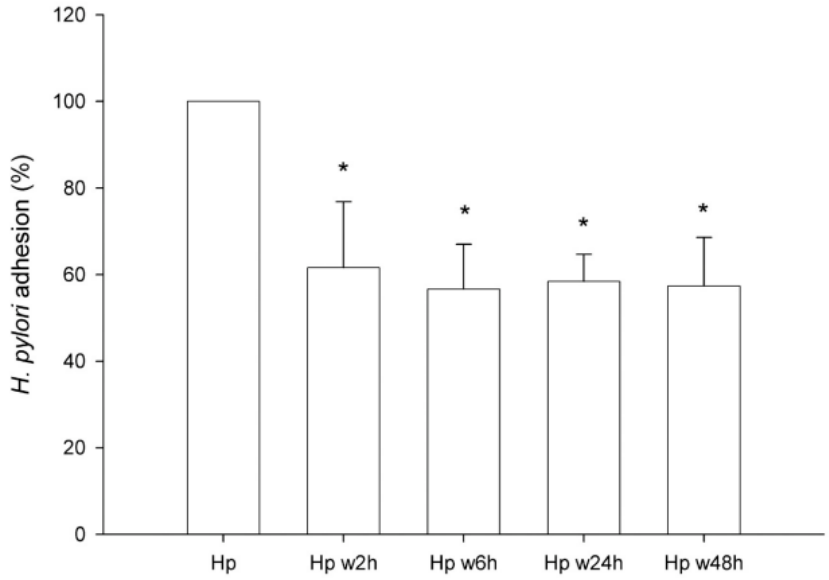

Figure 5.2 - Effect of water exposure on *H. pylori* adhesion to host epithelial cells. AGS cells were infected with *H. pylori* 26695 inocula that have been exposed to water for 2 (Hp w2h), 6 (Hp w6h), 24 (Hp w24h), and 48 (Hp w48h) hours, at a MOI of 100. As control, *H. pylori* 26695 that were not exposed to water were used (Hp). Cells were washed to remove non-adherent bacteria and adhesion was evaluated by ELISA. Data are expressed as percentage of control. Graphics represent mean ± SD and are representative of three independent experiments. *, significantly different from non-exposed *H. pylori* ($p<0.05$).

5.2.3 Influence of water exposure on *H. pylori* induction of IL-8 secretion by host cells

When in close contact with the gastric mucosa, *H. pylori* leads to increased production by the epithelium of the proinflammatory cytokine interleukin-8 (IL-8) (Shimoyama and Crabtree, 1998). As water-exposed *H. pylori* was able to adhere to epithelial cells, we studied the capability of *H. pylori* to induce inflammation by evaluating the secretion levels of IL-8 from AGS cells infected with *H. pylori* inocula that were exposed to water for different time periods (**Figure 5.3**). Results show that *H. pylori* with 2 and 6 hours of water exposure still have the ability to induce IL-8 secretion in a similar way to that of unexposed bacteria. However, after 24 hours of exposure *H. pylori* is no longer able to induce IL-8 production by AGS cells. As such, in this case the inflammation induced by the bacterium appears to be more related to the culturability status of *H. pylori* than with the ability of this microorganism to adhere to epithelial cells, In fact, although adhesion to host cells is immediately decreased after

contact with water, after short time-periods in water (up to 6 hours), the *H. pylori* that adhere are still able to induce inflammation in those cells.

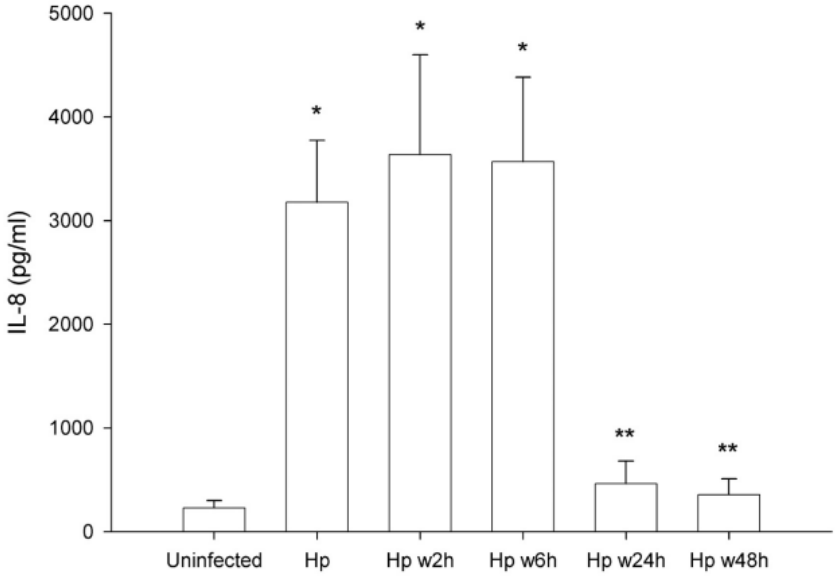

Figure 5.3 - Effect of water exposure on *H. pylori* induction of IL-8 secretion by host epithelial cells. AGS cells were infected with *H. pylori* 26695 inocula that have been exposed to water for 2 (Hp w2h), 6 (Hp w6h), 24 (Hp w24h), and 48 (Hp w48h) hours, at a MOI of 100. As control, *H. pylori* 26695 that were not exposed to water were used (Hp). IL-8 production was evaluated by ELISA. Graphics represent mean ± SD and are representative of three independent experiments. *, significantly different from uninfected cells; **, significantly different from non-exposed *H. pylori* ($p<0.05$).

5.2.4 Influence of water exposure on *H. pylori* deregulation of host cell apoptosis

H. pylori infection has been shown to modify epithelial cell apoptosis (Moss et al., 2001; Cover et al., 2003). To elucidate whether water exposed *H. pylori* are able to induce such impairment, AGS cells were infected with bacteria previously exposed to water, and cell apoptosis was evaluated. As expected, non-exposed *H. pylori* increased AGS cell apoptosis (**Figure 5.4**). In contrast, water exposed *H. pylori* induced significantly lower levels of apoptosis than non-exposed bacteria ($P<0.01$ for all water exposure times). Furthermore, no statistically significant differences were observed between the apoptosis in uninfected cells and those infected with water-exposed *H. pylori* ($P>0.05$), except for cells infected with *H. pylori* exposed to water for 24h ($P<0.05$). These experiments indicate that water exposure, although allowing *H. pylori* to adhere, limits the influence of the bacteria on host cell apoptosis.

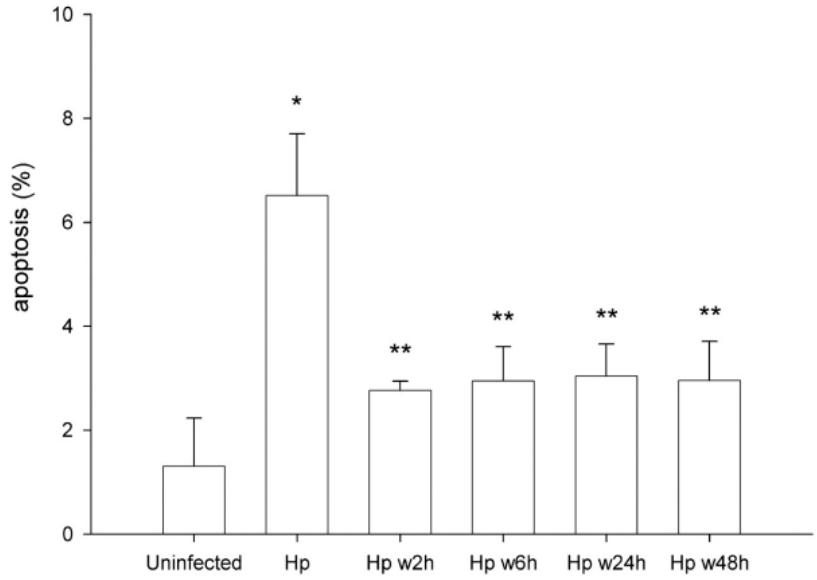

Figure 5.4 - Effect of water exposure of *H. pylori* on apoptosis of host epithelial cells. AGS cells were infected with *H. pylori* 26695 that have been exposed to water for 2 (Hp w2h), 6 (Hp w6h), 24 (Hp w24h), and 48 (Hp w48h) hours, at a MOI of 100. As control, *H. pylori* 26695 that were not exposed to water were used (Hp). Apoptosis was detected at single cell level using the TUNEL assay. Graphics represent mean ± SD and are representative of at least two independent experiments. *, significantly different from uninfected cells; **, significantly different from non-exposed *H. pylori* ($p<0.05$).

5.2.5 Influence of water exposure on the *H. pylori* structural component of pathogenicity *cag* T4SS

One important virulence characteristic of *H. pylori* is the presence in some strains of a *cag* T4SS. The T4SS is a molecular syringe that allows the injection of bacterial effectors into the host cytoplasm, altering host cellular processes including induction of inflammation and deregulation of apoptosis (Segal et al., 1999; Moss et al., 2001; Viala et al., 2004). Because after water exposure *H. pylori* was still able to adhere to epithelial cells, and in view of the fact that host cellular processes mediated by the *cag* T4SS were influenced by water-exposed bacteria, our next experiment aimed at elucidating if these results could be explained by the lack of a functional T4SS in water-exposed *H. pylori*. To assess the functionality of the T4SS, we evaluated by western blot CagA tyrosine phosphorylation in AGS cells after infection with *H. pylori* 26695 inocula that were exposed to water for different time-periods (**Figure 5.5A**). CagA is one of the *cag* T4SS effectors that, after injection into the host cytoplasm, can undergo tyrosine phosphorylation by host protein kinases (Odenbreit et al., 2000; Backert and Selbach,

2008). CagA phosphorylation only occurs inside the host cell and is an indirect measure of the T4SS functionality. As a positive control for this experiment, *H. pylori* that was not exposed to water was used (**Figure 5.5A**). In parallel, and to control for the amount of proteins present in bacterial suspensions that were incubated in water, western blot analysis for *H. pylori* CagA and Urease B were performed (**Figure 5.5B**).

While water-exposed *H. pylori* remained culturable for at least 6 hours, CagA tyrosine phosphorylation was not observed in any of the co-cultures of water-exposed bacteria. After just 2 hours in water, *H. pylori* was no longer able to translocate CagA into the host cells. This was not due to lower CagA levels present in bacteria that were incubated in water, since for at least 48 hours, water exposure did not affect the levels of either CagA or Urease B that remained similar to those of non-exposed *H. pylori*. These data suggest that water-exposed bacteria are not able to produce a functional *cag* T4SS, and consequently are not able to translocate CagA into the host cells. Taken together with our previous experiments, our results suggest that after being in water for periods longer than 6 hours *H. pylori* is still able to adhere to host cells, but is not effective in eliciting *in vitro* host cell inflammation and apoptosis, possibly due to the non-functionality of the *cag* T4SS.

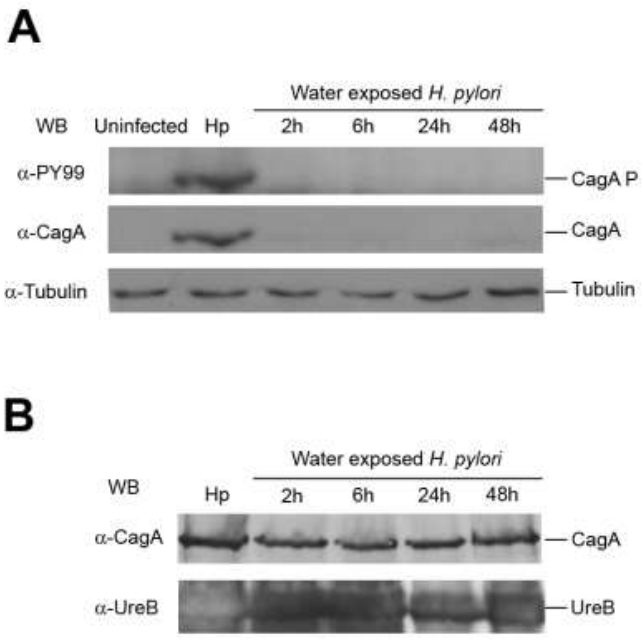

Figure 5.5 - Effect of water-exposure on *H. pylori* cag T4SS formation. (A) AGS cells were infected with *H. pylori* 26695 that have been exposed to water for 2 (Hp w2h), 6 (Hp w6h), 24 (Hp w24h), and 48 (Hp w48h) hours, at a MOI of 100. As control, *H. pylori* 26695 that were not exposed to water were used (Hp). CagA tyrosine phosphorylation levels were evaluated by western blot using an anti-PY-99 antibody

against tyrosine phosphorylated motifs, and after membrane stripping, CagA was detected by re-probing with an anti-CagA antibody. Tubulin was used as equal protein loading control for co-cultures. (B) Protein lysates of *H. pylori* 26695 suspensions of each timepoint of water exposure were used as parallel controls of the amount of bacterial CagA and Urease B proteins present. *H. pylori* 26695 that were not exposed to water (Hp) were also used as control.

5.3 Discussion

Epidemiologial evidence has pointed to environmental water as a risk factor for *H. pylori* infection among humans (Klein et al., 1991; Goodman et al., 1996; Karita et al., 2003). In order to elucidate if there are mechanisms that might allow water-exposed *H. pylori* to colonize the human stomach, several properties related with the survival and pathogenicity of *H. pylori* when exposed to water were studied. Our results showed that after being exposed to water for 24 hours at 25 ºC, *H. pylori* was no longer culturable. Studies have reported that when exposed to water *H. pylori* enter a viable but non culturable state as a response to unfavourable environmental conditions (Azevedo et al., 2007b), which means that despite *H. pylori* cannot be recovered by plating techniques, bacterial cells might remain viable.

Adhesion is one of the most important pathogenic determinants of *H. pylori*, since attachment to the host cells allows bacterial maintenance and gastric colonization. Our results showed that water exposed *H. pylori* have a decreased adhesion capacity when compared to *H. pylori* that have not been in contact with water. Nevertheless, water exposed bacteria still retain a significant adhesion capacity, and this capacity does not significantly change with the time of water exposure. Our findings together with the discovery that *H. pylori* would only grow under conditions mimicking the stomach if adhered to the surface of epithelial cells (Tan et al., 2009), could be a means for allowing *H. pylori* to remain in the host time enough for the occurrence of genetic recombination with other *H. pylori* strains that could be present in the same host, originating a higher genetic diversification (Azevedo et al., 2007a). This genetic diversification may help *H. pylori* to adapt to a new host after transmission (Dorer et al., 2009).

Inflammation of the gastric mucosa is a universal consequence of *H. pylori* interaction with the host (Shimoyama and Crabtree, 1998). Although water exposed *H. pylori* still retained a considerable capacity to adhere to gastric cells, at 24 hours of exposure *H.*

pylori was not able to influence IL-8 secretion. This is concurrent with the absence of NF-κB activation and lack of IL-8 production in epithelial cells observed after morphologic transition from bacilar into coccoid forms, and in which *H. pylori* peptidoglycan structure is modified (Chaput et al., 2006). In our experiments, bacteria that were exposed to water for short time-periods still triggered signaling leading to IL-8 production, which could represent bacteria with an as yet unmodified peptidoglycan. Whether water exposure leads to altered peptidoglycan structure and to which extent these bacterial cell wall modifications allow these bacterial forms to temporarily escape detection by the host immune system remains to be elucidated.

The infection with *H. pylori* leads to increased host epithelial cell turnover with an increase in both apopotosis and proliferation rates (Peek et al., 1997; Moss et al., 2001). Water-exposed bacteria were not able to induce alterations in the apoptotic index of host cells. As gastric epithelial cells have a rapid turnover, the lack of influence of water-exposed *H. pylori* in epithelial cell apoptosis may be an advantage for colonization and persistence in the host. In addition, the lack of ability to induce inflammation may also contribute to decreased host cell proliferation (Lynch et al., 1999), therefore slowing cell turnover.

Several lines of evidence have pointed to the importance of the *cag* T4SS in *H. pylori*-mediated host inflammation and apoptosis (Segal et al., 1999; Moss et al., 2001). In co-cultures of water exposed *H. pylori* with gastric cells we could not detect tyrosine phosphorylation of the T4SS effector CagA. Since CagA phosphorylation only occurs inside the host cell, and since water exposed *H. pylori* preserved unaltered endogenous levels of CagA, our findings strongly suggest that the *cag* T4SS after water exposure becomes non-functional. The absence of a functional T4SS may underlie the lack of influence of water exposed *H. pylori* in host cell IL-8 secretion and apoptosis. It has been shown that activation of NF-κB leading to IL-8 secretion may be influenced by CagA (Brandt et al., 2005), but also stimulated by the T4SS itself. Indeed, it has been shown that *H. pylori* uses the T4SS to deliver fragments of peptidoglycan that are sensed by the host NOD1 receptor, resulting in NF-κB activation and IL-8 production (Viala et al., 2004). In animal models, it has been show that *H. pylori* exposed to sterile tap water can colonize mice and induce gastric inflammation (She et al., 2003). Whether water exposed *H. pylori* are still able to recover the functionality of the T4SS *in vivo*, remains to be elucidated.

Altogether, our results suggest that water-exposed *H. pylori* retain important properties that might allow the bacterium to colonize the host cells. Maintenance of these virulence properties despite loss of culturability might explain why, in earlier studies, viable but not culturable *H. pylori* were able to infect mice (Wang et al., 1997; She et al., 2003). It is therefore possible that bacterial cells exposed to stress environments recover their capacity to colonize and infect when reaching the gastric environment. Nevertheless, water-exposed *H. pylori* have a decreased interaction with the host and from the standpoint of the microorganism, attenuation of inflammation and of cell apoptosis may be beneficial in the sense that it may improve the likelihood for the establishment and persistence of the infection. Even in the case where the microorganism itself is not able to cause a lifelong infection, the decreased cell apoptosis might allow *H. pylori* to remain in the host for enough time to contribute to a higher genetic diversity of other *H. pylori* strains eventually already present in the human stomach. This would allow for the conservation of the genetic diversity in the *H. pylori* population, a characteristic that has undoubtedly been crucial in the co-evolution and maintenance of a persistent equilibrium between this microorganism and the human population.

5.4 Experimental Procedures

Bacterial strains and growth conditions. Experiments were performed with *H. pylori* 26695 obtained from the American Type Culture Collection (ATCC). Bacteria were grown in tryptic Soy Agar (TSA) supplemented with 5% sheep blood (Biomérieux, Marcy L'Étoile, France) and incubated at 37ºC under microaerophilic atmosphere for 48 hours.

Water exposed *H. pylori*. After being in culture for 48 hours, *H. pylori* was harvested from TSA plates and suspended in 5 mL of autoclaved tap water in a 10^9 bacteria/mL concentration. Suspensions were kept at 25ºC under aerophilic conditions. Bacteria were exposed to water for 2, 6, 24 and 48 hours. As controls, *H. pylori* inocula that have not been exposed to water were used.

Culturability. The number of culturable bacteria in the different time points was determined by plating serial dilutions of the suspensions in TSA plates containing 5% sheep blood. Culturability was analyzed by comparing the number of colony-forming units (CFU's) of each time point.

Cell line maintenance and and bacterial co-cultures. AGS cells, derived from a human gastric carcinoma, were maintained in RPMI 1640 medium (Invitrogen), supplemented with 10% fetal bovine serum (FBS) and 1% Pen-Strep (Invitrogen) at 37ºC, under a 5% CO2 humidified atmosphere. All co-culture experiments of *H. pylori* with AGS cells were performed at a multiplicity of infection (MOI) of 100. Co-cultures were maintained at 37ºC, under a 5% CO2 humidified atmosphere.

Adhesion assay. *H. pylori* suspension corresponding to the different times of water-exposure was added to AGS cells and the plate was gently agitated for 30 minutes at 37ºC. Cultures were fixed with 1% parafomaldehyde and blocked with 1% phosphate buffered saline (PBS) - bovine serum albumin (BSA) for 30 minutes. Bacterial adhesion was determined by ELISA as previously described (McGuckin et al., 2007) using a rabbit polyclonal anti-*Helicobacter pylori* (Cell Marque) and an anti-rabbit IgG-horseraddish peroxidase as secondary antibody. Binding was visualized after incubation with tetramethylbenzidine and with 1M HCl. Absorbance was read at 450 nm. Controls

for *H pylori* binding to wells comprised wells with no AGS cells, to which bacteria were added and allowed to adhere to the plastic before fixation. Negative controls contained neither AGS cells nor *H. pylori*. Bacterial adhesion was expressed as percentage of adhesion to AGS cells of *H. pylori* that were not exposed to water.

IL-8 production. AGS cells were grown in 6-well plates for 48 hours in RPMI supplemented with 10% FBS at 37ºC and 5% CO_2. Bacteria suspensions corresponding to each water-exposure time-period were added to cells and incubated for 24 hours at 37ºC. IL-8 levels were detected in co-culture supernatants by ELISA using the QUANTIKINE, Human CXCL8/IL8 kit (R&D Systems, Minneapolis, MN).

Apoptosis assay. AGS cells were grown in 6-well plates for 48 hours in RPMI supplemented with 10% FBS at 37ºC and 5% CO_2. A volume of bacteria suspension corresponding to each water exposure time-period was added to cells, and incubated for 24 hours at 37ºC. Apoptotic cell death was determined by the terminal uridine deoxynucleotide nick end-labeling (TUNEL) assay (In Situ Cell Death Detection Kit; Roche Diagnostics). Apoptotic cells were detected using a Leica DM IRE2 fluorescence microscope.

Western blot analysis. Co-cultures and AGS uninfected control cells were lysed in cold lysis buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% Triton X-100, 1% NP-40, 3 mM sodium vanadate, 20 mM NaF, 1 mM PMSF, 10 µg/ml aprotinin and 10 µg/ml leupeptin) and lysates were separated by 6% SDS-polyacrylamide gel electrophoresis (PAGE). Proteins were transferred onto Hybond nitrocellulose membranes (Amersham), which were blocked with 4% BSA or with 5% non-fat milk in PBS with 0.5% Twen-20. Membranes were incubated with a mouse monoclonal antibody against tyrosine phosphorylated residues (α-PY-99, Santa Cruz Biotechnology) and, after stripping, re-probed with a mouse monoclonal anti-CagA antibody (Santa Cruz Biotechnology). Goat anti-rabbit (Santa Cruz Biotechnology) or rabbit anti-mouse horseradish peroxidase-conjugated secondary antibodies (Amersham) were used, followed by ECL detection (Amersham). As loading control, membranes were also incubated with a mouse monoclonal anti-α-tubulin antibody (Sigma).
Protein lysates of *H. pylori* suspensions of each timepoint of water exposure were used as parallel controls of the amount of bacterial proteins present. Twenty µg of proteins of

each sample were separated by 6% SDS-PAGE and transferred onto Hybond nitrocellulose membranes. Membranes were blocked with 5% non-fat milk in PBS with 0.5% Twen-20 and incubated overnight with mouse monoclonal anti-CagA or with rabbit polyclonal anti-Urease B (Santa Cruz Biotechnology) antibodies.

Statistical analyses. Data were analyzed with Student's t test using the Statview for Windows software (version 5.0; SAS Institute Inc., Cary, NC), and were expressed as mean values of, unless otherwise stated, three independent experiments ± standard deviations. Differences in data values were considered significant at P values lower than 0.05.

5.5 Acknowledgements

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Chapter 6

Final Conclusions and Future Perspectives

The routes of transmission are perhaps one of the most controversial areas in *H. pylori* research and this lack of knowledge assumes particular importance in Portugal, a country where *H. pylori* prevalence is among the highest in the European Community. The most widely accepted routes of transmission are the fecal-oral, the oral-oral and the gastric-oral routes. There is also a growing amount of data reporting the identification of *H. pylori* in external environmental reservoirs, most significantly in water. Despite all the information available, there are more questions than answers about these routes of transmission. Taking this in to account, the main goal of this Thesis was to establish the role that the oral cavity and water routes play in *H. pylori* transmission.

The methodologies used for detection are of utmost importance when studying *H. pylori*. In this Thesis, a PNA probe (Hpy769) that can be used for FISH was developed. This probe can be applied in gastric or extra-gastric samples and presents several improvements and advantages when compared to other *H. pylori* detection methods (Chapter 2). The PNA FISH procedure using the Hpy769 was shown to be a very sensitive and specific method for the detection of *H. pylori* in a variety of samples, such as bacterial smears and paraffin-embedded gastric biopsy specimens. The detection of the bacteria in biopsy specimens has the potencial to become very useful since this technique allows not only to detect *H. pylori*, but also to pinpoint their exact location and even to identify their morphology, which is not possible by techniques such as culture methods or PCR. In addition, the method is easy to implement without requirement for special equipment or facilities, apart from an epifluorescence microscope.

Considering that the possible routes of transmission of *H. pylori* include the oral cavity as a means of entry of the bacteria, the existence of an oral reservoir, namely at the level of the tonsils and of the adenoids was evaluated. The adenotonsillar tissue had been previously suggested as a putative extra-gastric reservoir of *H. pylori*, although contradictory results arose from those initial publications. Conflicting results stem mainly from the use of detections methods for *H. pylori* that may not be adequate. In Chapter 3, a population of children from the North of Portugal was analyzed, and the presence of *H. pylori* in adenotonsillar tissue was evaluated using several detection methods. The use of a panel of detection methods allowed to demonstrate the importance of methodology selection for detection of *H. pylori* in non-gastric samples. Although techniques like the rapid urease test and immunohistochemistry have a high specificity when used in gastric samples, the specificity in samples from polimicrobial

environments, such as the case of adenotonsillar tissue, seems to be lower originating false positive results. This observation may underlie positive results obtained in other studies, in which *H. pylori* positivity in adenoid and tonsillar tissues was assumed by the use of one of these techniques alone or by combination of both. In the group of adenotonsillar samples studied in Chapter 3, positive results were obtained when using these techniques. However, these results were not confirmed when most specific and sensitive methods, such as PNA-FISH and PCR-DEIA were used. This confirmed the lower specificity of the rapid urease test and of immunohistochemistry in extra-gastric samples like the adenotonsillar tissues. Since in all cases of adenoid and tonsillar specimens *H. pylori* detection was negative, even in children that had a gastric infection assessed by serology, one can conclude that the adenotonsillar tissue does not constitute an extra-gastric reservoir for *H. pylori*, or at least a permanent one. It remains to be elucidated whether in an adult population this observation holds true. The analysis of samples from the adenotonsillar tissue of an adult population with confirmed gastric *H. pylori* infection could help in the elucidation of this issue.

Taking into account that the oral cavity is a means of entry of *H. pylori*, the influence of the contact of *H. pylori* with saliva in bacterial survival and infection potential was studied in Chapter 4. Exposure to saliva for periods of up to 24 hours, although diminuished, did not impair bacterial culturability. Furthermore, the ability of *H. pylori* to adhere to the host cells was not altered by saliva, and this exposure also did not modify the ability to induce IL-8 secretion by the host cells. Saliva is constantly being shed and swallowed. One can speculate that 24 hours could be enough time for *H. pylori*, after entering the mouth, to reach the gastric environment. Furthermore, and since adhesion properties are not altered by the contact with saliva in this time period, bacteria are viable and able to colonize the gastric mucosa. This is also in keeping with the observation that for such time of contact with saliva *H. pylori* can induce inflammation. Longer times of saliva exposure however, result in the absence of IL-8 secretion by the host cells, which is in accordance with the lack of culturability of *H. pylori*. Nonetheless, and as it has been shown in animal models, viable but non-culturable *H. pylori* are still be able to recover their infectious capacities, colonize the mucosa and induce inflammation after reaching the gastric environment.

Epidemiological data evidenced water as a risk factor for *H. pylori* infection among humans. Despite all the studies, there is a lack of information regarding the capacity of water exposed bacteria to induce a response in host cells. In Chapter 5, properties

related with the survival and pathogenicity of *H. pylori* when exposed to water were studied, in order to elucidate if there are mechanisms that might allow water exposed *H. pylori* to colonize the human stomach. When exposed to water, *H. pylori* loses the cultivability, the ability to induce host cell inflammation and apoptosis. These observations can be attributed to the fact that the T4SS became non-functional, since the CagA protein is no longer translocated to the host cells. On the other hand, water exposed bacteria retain a significant ability to adhere to gastric cells. Although the exposure to water induces alterations in *H. pylori,* the capacity to adhere and to possibly decrease host gastric epithelial cell turnover may facilitate, at least temporarily, host colonization. It is tempting to speculate that bacteria in these conditions may exchange DNA with other strains present in the same stomach, contributing to a higher genetic variety, and resulting in better conditions for survival and gastric adaptation. It is therefore possible that *H. pylori* that were exposed to stress environments recover their capacity to colonize and infect when reaching the gastric environment.

In the future, a more detailed study about the physiological alterations of *H. pylori*, namely in the T4SS, induced by either water or saliva could give us important information to better understand what happens to bacteria in these stressful conditions. Another important aspect to be addressed in the future is whether *H. pylori* from extra-gastric and environmental environments is able to recover their properties to colonize and infect the human host once in the gastric environment.

Chapter 7

Scientific Output

7.1 Accepted and Submitted papers in peer reviewed international journals

Guimaraes, N., Azevedo, N. F., Figueiredo, C., Keevil, C. W. & Vieira, M. J. Development and application of a novel peptide nucleic acid probe for the specific detection of *Helicobacter pylori* in gastric biopsies. Journal of Clinical Microbiology 45, 3089-94 (2007).

Azevedo, N. F., Guimaraes, N., Figueiredo, C., Keevil, C. W. & Vieira, M. J. A new model for the transmission of *Helicobacter pylori*: role of environmental reservoirs as gene pools to increase strain diversity. Critical Reviews in Microbiology 33, 157-69 (2007).

Vilarinho, S., Guimarães N., Ferreira R., *et al.* "*Helicobacter pylori* colonization of the adenotonsillar tissue: fact or fiction?" (submitted)

Guimarães, N., Azevedo, N., Vieira, M. J., Figueiredo, C. "Water-induced modulation of *Helicobacter pylori* virulence properties" (submitted)

Guimarães, N., Azevedo, N., Vieira, M. J., Figueiredo, C. "Saliva influence on survival and infection of *Helicobacter pylori*" (submitted)

7.2 Oral and Poster presentations in international conferences and meetings

N. Guimarães; N. Azevedo; C. Figueiredo; C. W. Keevil and M. J. Vieira. "Development and application of a novel peptide nucleic acid probe for the specific detection of *Helicobacter pylori*". XIX International Workshop on *Helicobacter* and related bacteria in chronic digestive inflammation, Poland, 2006.

N. Guimarães; N. Azevedo; C. Figueiredo; C. W. Keevil and M. J. Vieira. A multi-route way of transmission for *Helicobacter pylori*. XX International Workshop on *Helicobacter* and related bacteria in chronic digestive inflammation", Turkey, 2007

N. Guimarães; N. Azevedo; M. J. Vieira and C. Figueiredo. "Water-exposed *Helicobacter pylori* presents decreased virulence properties". XXIst International Workshop on *Helicobacter* and related bacteria in chronic digestive inflammation and gastric cancer, Latvia, 2008