Liposome-based therapy: An alternative approach to treat *Helicobacter pylori* infection in high prevalence communities such as Aklavik, NWT

by

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Abstract

Helicobacter pylori infects about half of the world population causing chronic gastritis, peptic ulcer or gastric cancer. The Aboriginal people in Aklavik, NWT, Canada are concerned about gastric cancer because of the high prevalence of *H. pylori* infection (58%) in their community. *H. pylori* colonization of the acidic stomach environment is dependent on the production of urease enzyme and bacterial adhesins such as BabA. Toxins produced by *H. pylori,* CagA and VacA, contribute to the development of stomach diseases. Standard therapies often fail due to antimicrobial resistance and lack of compliance. Liposomes are lipid vehicles that have wide therapeutic applications in enhancing the delivery of drugs and genes into different cells.

This thesis characterizes *H. pylori* isolated from residents of Aklavik for major virulence genes, describes the prevalence of gastric biopsy histopathology outcomes, and estimates the effect of *H. pylori* virulence genotype on the prevalence of gastric biopsy histopathology outcomes. In addition, liposome-based treatment approaches are investigated as alternative treatment approaches for *H. pylori* eradication.

This thesis is the first report on the frequencies of *H. pylori* genotype and histopathology outcomes for the community of Aklavik, NWT, and identifies the association of virulence genes *cagA* and *vacA* subtypes with gastritis and/or intestinal metaplasia. Cationic liposome-enhanced delivery of urease antisense DNA inhibited urease expression by 40%, which may interfere with *H. pylori* survival in acidic conditions. Alterations in liposome composition to improve

liposome stability did not improve delivery of antisense DNA to *H. pylori*. However, cationic liposomes themselves were previously reported to have antimicrobial activity against some bacteria and protozoans at concentrations that are nontoxic to mammalian cells. This thesis is the first demonstration of the enhanced effect of liposomes on the delivery of urease antisense DNA to *H. pylori* resulting in impaired urease expression and activity, and also the first report on the antimicrobial activity of stearylamine-containing cationic liposomes against *H. pylori*. The novel findings presented in this thesis provide the potential for alternative treatment approaches for the eradication of *H. pylori* infections, important strategies for communities where there is a high prevalence of *H. pylori* infection.

Preface

This thesis is an original work by Maysoon Mahmoud. The research project, of which this thesis is a part, received research ethics approval from the University of Alberta Research Ethics Board under the Project Title "The Aklavik digestive health study: *Helicobacter pylori* infection in the Northwest Territories" Study ID Pro00001717 on February 23 2007, as well as approval from the Aurora Research Institute (Northwest Territories research licensing agency), the Aklavik Health Committee, the Hamlet of Aklavik council, the Aklavik Community Corporation (Inuvialuit governance) and the Ehdiitat Gwich'in Council. Ethics approval was continued under the Project Title "Addressing community concerns about risks from *H. pylori* infection in the circumpolar north" Study ID Pro00007868 approved October 21 2010.

Research conducted in Chapter 2 forms part of a research collaboration, specifically the Aklavik *H. pylori* Project, with the Canadian North *Helicobacter pylori* (CAN*Help*) Working Group led by Dr. Karen Goodman, with Dr. Monika Keelan as the microbiology lead at the University of Alberta. Dr. Safwat Girgis carried out the assessment of gastric biopsies. Maysoon Mahmoud performed the genotype analyses, described the distribution of genotypes and histopathology outcomes, and assessed genotype association with histopathology data. This work will be incorporated into future publications that include a more extensive genotype profile and increased sample size achieved by combining the data from several Arctic communities.

The data generated and analyzed in Chapters 3 and 4, the summarizing discussion and proposed future directions for research in Chapter 5 are my original work, as well as the literature review in Chapter 1.

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Table of Contents

List of Tables

List of Figures

List of Abbreviations

AGS	human adenocarcinoma epithelial cell line
AlpA	adherence associated lipoprotein
BabA	blood group antigen binding adhesion
c-Src	carboxy-terminal tyrosine-protein kinase
CagA	cytotoxin-associated gene encoded protein A
*cag*PAI	cytotoxin associated gene pathogenicity island
CAN*Help*	Canadian North *Helicobacter pylori*
DOTAP®	1,2-bis (oleoyloxy)-3-(trimethylammonio) propane
DupA	duodenal ulcer promoting gene A
EPIYA	glutamic acid-proline-isoleucine-tyrosine-alanine
Fyn	proto-oncogene tyrosine-protein kinase encoded by *fyn* gene
Grb2	growth factor receptor-bound protein 2
Hfq	host factor Q
HopZ	*H. pylori* outer membrane protein Z
i	Intermediate
IceA	induced by contact with the epithelium
LUV	lage unilamellar vesicles
Lyn	tyrosin protein kinase encoded by *lyn* gene
m	Mid
MALT	mucosa-associated lymphoid tissue lymphoma
MHC	major histocompatibility complex
MLV	multilamellar vesicles
MUV	medium unilamellar vesicles
MVV	multivesicular vesicles
MRSA	methicillin-resistant *Staphylococcus aureus*
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
OipA	outer membrane inflammatory protein
PPI	proton pump inhibitor
s	Signal

SabA	sialic acid-binding adhesion
SFKs	Src (tyrosine-protein kinase) family kinases
siRNA	short interfering RNA
STAT 1	signal transduction and transcription regulated genes
SUV	small unilamellar vesicles
TFSS	type IV secretion system
Th1	T-helper cell type 1
UBA	urease B gene antisense DNA
UBT	urea breath test
ULV	unilamellar vesicles
VacA	vacuolating cytotoxin A
Yes	proto-oncogene tyrosin-protein kinase encoded by *yes1* gene

Chapter 1

Introduction

1.1 *Helicobacter pylori*

1.1.1 Characteristics and History

Helicobacter pylori is a microaerophilic Gram negative bacterium that infects the stomach (1). It is one of the most common bacterial infections as it is found worldwide but is more frequently observed in less developed regions (2). The infection is thought to be acquired in early childhood and persists for life if untreated (3). The colonization of the healthy stomach with *H. pylori* produces a condition of chronic stomach inflammation (gastritis). The infection may be asymptomatic or it may proceed to symptomatic active gastritis or peptic ulceration. *H. pylori* has also been implicated in the etiology of gastric adenocarcinoma, and gastric mucosa-associated lymphoid tissue (MALT) lymphoma (4).

The basic morphology of *H. pylori* shows the bacterium as a spiral-shaped Gram negative bacterium (1). It is a flagellated bacterium with 5 to 7 polar flagella. The spiral shape along with the polar flagella were linked to its high bacterial motility. In addition to the spiral shape of the bacteria, *H. pylori* may form coccoid cells as it ages. Fresh cultures are mainly spiral bacteria, however, after 3 to 4 days of culture, coccoid cells dominate leading to a dramatic decrease in culturability (5). It is controversial whether or not these cells are viable, nonviable but dormant, or just dead. They are formed when cells are exposed to unfavorable growth conditions and may be responsible for infection transmission and treatment failures. The changes that happen to *H. pylori* as it ages were found to be independent of the method used to induce coccoid formation (6).

Studies on *H. pylori* started in 1970s when bacteria were found to be frequently associated with the surface of gastric epithelium with no possibility for being contaminants at the time biopsies were taken (7). However, bacteriological studies at that time mistakenly identified those bacteria as *Pseudomonas aeruginosa* (8). Barry J. Marshall and Robin Warren discovered *H. pylori* and its

role in gastritis and peptic ulcer disease. They have been awarded the 2005 Nobel Prize in Physiology or Medicine. When Warren and Marshall announced their discovery, there was a strong belief that peptic ulcer disease was caused by stress and lifestyle factors. Warren and Marshall connected *H. pylori* to more than 90% of duodenal ulcers and up to 80% of gastric ulcers. At that time, the clinical community criticized their findings. They had to prove their discovery with experimental and clinical evidence. In 1985, Marshall underwent gastric biopsy in order to prove that he was not infected with *H. pylori*, then he deliberately infected himself to show that *H. pylori* was the cause of his acute gastric illness (9,10). In 1982, *H. pylori* was first cultured from human stomach biopsies by Marshall (11,12). *H. pylori* culture was possible by a lucky accident, in which the cultures were left in the incubator over the long Easter weekend and so the plates were not examined until the fifth day after biopsy was cultured. Previous trials were unsuccessful because research biopsies were discarded after 48 h due to the overgrowth of contaminating commensal flora. Successful culture was done from a clean gastric biopsy with minimum commensal flora on blood agar under microaerobic techniques similar to those used for *Campylobacter* (13). *H. pylori* was originally called *Campylobacter pyloridis*, then the name was changed to *Campylobacter pylori* and finally to *Helicobacter pylori* as the organism has specific morphologic, structural and genetic features that place it in a distinct genus (14).

1.1.2 Clinical outcomes

H. pylori colonization leads to infiltration of the gastric mucosa in both antrum and corpus with neutrophilic and mononuclear (T helper) cells which results in active gastritis (15). Acute gastritis refers to the acute phase of infection, which may be associated with transient dyspeptic symptoms, such as feeling of fullness, nausea, vomiting, and inflammation of both distal and proximal stomach mucosa (pangastritis). The acute phase is often associated with reduced acid secretion (16). Persistent colonization results in chronic gastritis where the distribution of gastritis depends mainly on the level of acid secretion. If the acid secretion is normal (2.5 -

3 mmol/h) (17), *H. pylori* tends to colonize the gastric antrum due to the presence of few acid-secretory parietal cells leading to antrum-predominant gastritis (18). On the other hand, if the acid secretion is reduced, due to the use of acid suppressor drugs, *H. pylori* tends to colonize both antrum and corpus leading to corpus-predominant pangastritis (19). Acid secretion protects the corpus mucosa from *H. pylori* induced gastritis as high acidity maintains ammonia produced by *H. pylori* in the ionized form which cannot penetrate the epithelial cells and so protects the cells from the cytotoxic effect of ammonia (17).

Peptic ulcer disease may be either gastric or duodenal ulcers and are defined as defects in the mucosal lining with a diameter of at least 0.5 cm penetrating through the muscularis mucosa (20). Gastric ulcers are predominant along the lesser curvature of the stomach while duodenal ulcers usually occur in the duodenal bulb, which is highly exposed to gastric acid (21). In Western countries, duodenal ulcers are more common than gastric ulcers; however, in other countries gastric ulcers are more common (21). Duodenal ulcers are common in middle age (between 20 and 50 years of age), while gastric ulcers are usually seen over the age of 50 years (22). In 1995, *H. pylori* was considered to be a risk factor for peptic ulcer disease as approximately 81% of peptic ulcers were associated with the presence of *H. pylori* infection (23).

Chronic *H. pylori* infection may lead to shrinkage and destruction of gastric glands; a condition known as gastric atrophy (24). Intestinal metaplasia is a condition in which gastric columnar cells are replaced by intestinal-type cells, like mucin-containing goblet cells and absorptive cells. These cells are lacking in healthy gastric mucosa and are considered to be important predictors of gastric cancer risk (25). Moreover, the risk increases by 5-fold when the intestinal metaplasia involves the lesser curvature of the corpus (26).

1.1.3 Routes of transmission

The acquisition of *H. pylori* infection is proposed to usually occur in early childhood. It is not exactly known at what age the infection may occur but many researchers suggest that it may happen before the age of 5 years (27). The age at

which humans get exposed to *H. pylori* may influence the route of its transmission. To date, the exact mechanisms by which *H. pylori* is transmitted are unknown. Many transmission routes were proposed: gastric-oral, oral-oral, faecal-oral, zoonotic and waterborne (28).

The gastric-oral route of transmission may happen when a person becomes in contact with contaminated vomit. A history of vomiting in siblings was reported to be an independent risk factor for *H. pylori* infection. The study suggested that the gastro-oral route is the main route of *H. pylori* transmission among urban children (29).

The oral-oral route of transmission has been considered to be the most common route of *H. pylori* infection in developed countries where there is usually a high level of personal hygiene making the faecal-oral route an uncommon method of transmission (30). In the literature, there are conflicting data regarding the association between the presence of *H. pylori* in oral cavity and gastric colonization. In some studies, *H. pylori* were detected in oral cavity with close relationship to gastric colonization (31). However, another study reported that *H. pylori* can colonize the mouth cavity without stomach colonization (32). Eradication of *H. pylori* from the mouth is more difficult than from the stomach suggesting that the mouth may act as a source for reinfection (31).

The faecal-oral transmission of *H. pylori* is due to consumption of contaminated food. The contamination of food may occur due to irrigating vegetables with sewage-contaminated water (33) or from feces-contaminated hands as a result of lack of proper hand hygiene. However, *H. pylori* exists in food in viable but nonculturable form (34). *H. pylori* was successfully cultured from stool samples and the first report was back in 1992 from a stool sample of a 24-year old infected Gambian man (35). One year later, *H. pylori* DNA was first detected by PCR in stool samples from patients with gastritis (36). Others also reported the culture of *H. pylori* from feces (37,38), indicating the possibility of such a route of transmission.

H. pylori was detected in sheep stomach without causing any gastritis and it was cultured from sheep milk and gastric tissue, which suggests that sheep may be

a natural host for *H. pylori (39)*. Quagial *et al.,* were able to detect *H. pylori* DNA in 34.5% of 400 raw milk samples collected in Italy but they could not recover any *H. pylori* strain (40). In addition, *H. pylori* antigens were detected in the milk and faeces of cows, which may suggest that *H. pylori* may be a zoonotic infection (41). However, another study reported a rapid reduction in *H. pylori* count upon exposure to lipase hydrolyzed cow milk or saline solution supplemented with C4 to C10 fatty acids of milk fat. While raw milk may be a source of infection, hydrolyzing milk may be a method to prevent infection or transmission of infection to others by the faecal-oral route. However, since the fatty acids are absorbed by the intestine, the bacteriostatic effect of free fatty acids on *H. pylori* would be decreased, thereby limit their application in infection control (42).

Contaminated water remains a controversial route of *H. pylori* transmission. High levels of *H. pylori* infection were found in areas that have limited access to a clean water source, which raises concerns about the role of drinking water in *H. pylori* transmission. *H. pylori* DNA was detected in drinking water and sewage by PCR (43-45), and in drinking water biofilms by real-time PCR (46). However, *H. pylori* persists in cultures and in the environment in a viable but nonculturable state in which there is a transition in morphology from a bacillary to a coccoid shape (47). Researchers were not able to revert the coccoid form back to the bacilliary form (28). The survival of *H. pylori* in drinking water biofilms may be influenced by the presence of other bacteria that are indigenous to drinking water. It was reported that both *Mycobacterium chelonae* and *Sphingomonas spp.* maintain the cultivability of *H. pylori* for at least 24 hours. *H. pylori* can also survive for up to 4 days in tap water with a continuous decrease in colony forming units. The nonculturable coccoid form was detected by electron microscopy in tap water that was stored at 4°C for 7 days (48). In addition, *H. pylori* can persist for several months in river water at 4°C in a coccoid form (49), which may support the idea of waterborne infection. In the literature, there are only two reports about culturing *H. pylori* from drinking water; one from an open wastewater canal on the US-Mexico border in Ciudad Juárez, Mexico (50), and the other one in Iraq (51).

1.1.4 Diagnostic methods

H. pylori infection can be diagnosed using a number of invasive and non-invasive methods (**Table 1.1**). The invasive methods require endoscopic collection of gastric biopsies and include histology, rapid urease test, culture and polymerase chain reaction; the non-invasive methods include urea breath test (UBT), serology and stool antigen test because the collected samples are breath, blood and stool (52).

The invasive methods involve the use of an endoscope to visualize how much stomach tissue is affected and to obtain tissue samples from the stomach lining (biopsies). However, all biopsy-based methods may be liable to sampling error due to the patchy nature of *H. pylori* infections (53). The specificity and sensitivity of histology have greatly improved by the use of special stains such as Warthin-Starry, Giemsa, toluidine blue, acridine orange, McMullen, Genta, Dieterle, and Romanouski stains (52). The rapid urea test is a useful screen test when considering biopsies collected from both antrum and body (54). The test rapidly detects the presence of urease-producing organisms in gastric biopsies, and this most commonly suggests the presence of *H pylori.* Confirmation may be determined by histopathology or culture (52).

Culture is a highly specific method; however, it may have poor sensitivity if inadequate transport medium is used (55). The ideal transport medium should support *H. pylori* growth and at the same time suppress other contaminating microorganisms (56). Transport media may contain brain heart infusion, yeast extract, horse serum, and glycerol supplemented with antibiotic mixture containing vancomycin, amphotericin, cefsulodin, trimethoprim, β-cyclodextrin, polymixin B. The brain heart infusion, yeast extract and horse serum support *H. pylori* growth. The glycerol protects *H. pylori* from the effect of accidental freezing while the antibiotic mixture suppresses the growth of contaminating microflora. Biopsies should be cultured on suitable media for up to 19 days as some isolates are very slow growing or may be suppressed due to previous antibiotic intake (57). *H. pylori* can be cultured on different solid media containing blood or blood products (lysed blood or serum). Brucella agar, Columbia agar or brain heart infusion/yeast extract agar have been extensively used to culture *H. pylori*. A variable amount of 5 to 10%

blood improves the growth of *H. pylori*. Horse serum may also be used instead of blood to improve the growth of *H. pylori*. Egg yolk emulsion agar without blood has also been used as a medium for *H. pylori* growth (58). Cultures are incubated under microaerobic conditions (5% O_2, 5-10% CO_2, and balance N_2) at 37°C. Recovered *H. pylori* isolates can be identified using typical colony morphology (circular, convex and translucent), Gram stain (negative spiral rods) and positive urease, catalase, and oxidase tests. PCR can detect few bacteria in small samples without need for special treatment or transport (52). PCR detection of *H. pylori* DNA, such as 23S rRNA (59) or 16S rRNA (51) confirms its identifcation.

The [^{13}C]-urea breath test (UBT) is a non-invasive test that is now commonly used for the diagnosis of *H. pylori* infection before treatment. The high specificity and sensitivity of UBT (~95%) makes it a reliable method for diagnosis (60). Although the ELISA (enzyme-linked immunosorbent assay) serology test is a cheap and readily available method, it is the least accurate and does not differentiate between past and active infection (61). ELISA detects the serum antibody level (IgG, IgA or IgM) to *H. pylori* antigens (62). The stool antigen test detects *H. pylori* antigens in stool by enzyme immunoassay. The accuracy of the stool antigen test in the diagnosis of *H. pylori* infection, especially in children, has been confirmed (63-65). In spite of the high specificity and sensitivity of stool antigen test, it is not a common method for *H. pylori* diagnosis. It is proposed that the use of this test should be encouraged more as it is an inexpensive, accurate and non-invasive method (63,66).

1.1.5 Risk factors

Many risk factors have been studied for possible association with either increased or decreased risk for *H. pylori* infections (67). Among factors that were reported to be associated with increased risk, low socioeconomic status and household crowding were the most important risk factors. A positive association for both was reported in Bangladesh (68,69), Mexico (70), Turkey (71), China (72) and Russia (73). Other risk factors include the number of siblings (33), infected

parents (74), migration from areas where there is high prevalence (75), cigarette smoking (71), consumption of raw vegetables (76), diet (77).

There are other factors that were reported to be associated with a decreased risk for *H. pylori* infection. In Turkey, living in fruit growing regions, regular alcohol consumption and drinking bottled water were associated with a decreased risk for infection (71). Another study reported that consumption of chili peppers and concurrent parasitic infestation may protect against *H. pylori* infection (78).

1.1.6 Prevalence

The global prevalence of *H. pylori* infection is estimated to be more than 50% (79). The prevalence is higher in developing countries and Aboriginal populations than developed countries.

1.1.6.1 Prevalence in developed countries

The prevalence of *H. pylori* in developed countries varies from 7-44% and is continuously declining (**Table 1.2**). The prevalence in Canada is relatively low. A study that was done in an urban Ontario population of 1036 adults (50-80 years old) reported a seroprevalence rate of 23.1% in 2007 (75). The Nova Scotia study that included 316 non-patient adults reported an overall seroprevalence rate of 38%, with age being the only identifiable risk factor for the infection (80). In children, the prevalence is much lower than adults. A cross-sectional analysis that was published in 2008 found a 7.1% prevalence rate for *H. pylori* infection among Canadian children with a mean age of 10.3 years (81). In the US, the overall prevalence of *H. pylori* infection among adults was reported to be 32.5%. For adults (age 20-29 years), the prevalence rate was 16.7% and increased to 56.9% for those at least 70 years of age or older. When considering the ethnicity of the participants, a higher prevalence was observed among non-Hispanic blacks (52.7%) and Mexican Americans (61.6%) than among non-hispanic whites (26.2%) (82). Another study in the US reported lower prevalence among Caucasians (9%) (83). In Europe, the prevalence rate was previously reported to be from 30-70% with the highest rate of infection was observed in Eastern Europe (60). However, a more recent study observed lower prevalence rate (13.3-29.9%) in Europe (84,85). A 10

years follow-up study in Russia reported a decline in overall prevalence of *H. pylori* from 44% in 1995 to 13% in 2005 (86).

1.1.6.2 Prevalence in developing countries

The prevalence of *H. pylori* infection in developing countries is higher than in developed countries and may exceed 90%. The highest prevalence rate was observed among adults in Iran, Bangladesh and Egypt (**Table 1.3**). In developing countries, *H. pylori* infection is generally markedly more prevalent at younger ages than in developed countries, even before age 2. In Bangladesh, the prevalence of the infection was 50% in children under 2 years (87). In Asian countries that have recently become industrialized, the seroprevalence rates were high but still considerably lower than less developed countries. In addition, the infection rate has started to decline among younger populations to a similar infection rate as what is currently observed in developed countries (88). Among East Asian countries, the overall prevalence rate was 63.4% in China (77), 51.3% in Japan (89), 59.6% in South Korea (90) and 54.7% in Taiwan (91).

1.1.6.3 Prevalence in indigenous populations

The WHO defines indigenous populations as "communities that live within, or are attached to, geographically distinct traditional habitats or ancestral territories, and who identify themselves as being part of a distinct cultural group, descended from groups present in the area before modern states were created and current borders defined. They generally maintain cultural and social identities, and social, economic, cultural and political institutions, separate from the mainstream or dominant society or culture." (92). They constitute about 5% of the world population in more than 70 countries (93). The Aboriginal people in Canada are the indigenous people who are identified as descendants of the first inhabitants of North America (94). The 2011 Canada census identified 1,400,685 people as Aboriginal, representing 4.3% of the total Canadian population. They are comprised of the First Nations, Inuit and Métis peoples (95). The overall prevalence of *H. pylori* is high (50-80%) among Aboriginal populations and is similar to that observed in developing countries. In 1999, 95% of residents of the Wasagamack First Nation

people in Northern Manitoba were found to be seropositive for *H. pylori (96)*. Another study that same year on Inuit communities in Chesterfield Inlet and Repulse Bay reported the seroprevalence rate to be 51% (97). In 2008, 58% of residents of Aklavik, NWT tested positive for *H. pylori* infection by UBT (98). A study on First Nation populations in Northwest Ontario in 2013 reported prevalence rate of 37.9% for *H. pylori* infection based on histology (99). High prevalence rates (47-80%) have also been observed in northern Aboriginal communities outside Canada such as Greenland, Russia and Alaska (100).

1.1.6.3.1 The Aboriginal community of Aklavik

1.1.6.3.1.1 Demographics

In 2012, the total population of Aklavik was 628, according to the NWT Bureau of Statistics, of which 52% were males and 48% were females (101). The majority of Aklavik residents are of Aboriginal origin (91.6%, 575/628) (101) and are mainly Dene First Nation and Inuit. Generally, the western Canadian Inuit are Inuvialuit and their communities are located in the NWT (98). Over 12 years (2001-2012), the average annual % growth was -0.4% (**Figure 1.1**). In 2012, the age of about two third of the population was between 15 and 59 years, 21.6% were under the age of 15 years while 12.2% were over 60 years (**Figure 1.2**). Although the average life expectancy at 65 years was 81.1% in Canada (2007-2009), it was only 75.1% in Yukon, NWT and Nunavut (102). The percentage of households with 6 or more peoples declined from 27.3% in 1981 to only 4.4% in 2011.

In Aklavik, the major traditional activities are hunting, fishing and trapping. In addition, there are other minor activities including art and craft making (101). Similar to other small communities in NWT, Aklavik suffers from poor housing, water and sanitation, as well as unemployment (103). Education levels are low when compared to other communities across Canada; the 2006 high school graduation rate was 76% across Canada but it was only 38.6% in Aklavik (101). Although Aklavik has an Anglican school that offers education to students kindergarden to grade 12, Aboriginal youth have problems completing their formal education due to lack of continuity with informal traditional education. Moreover,

children may miss days of school when they participate in traditional activities such as hunting trips (104). In 2009, the employment rate was 21% among people who have less than high school diploma and 59.7% among people who have a high school diploma or higher. The employment rate was 32.7% among Aboriginals and 72.9% among non-Aboriginals (101). The higher employment rate observed among non-Aboriginals may be due to the fact that they settle in the community working as school teachers and nurses.

1.1.6.3.1.2 Gastric cancer concern

Residents of Aklavik have long been concerned about the perceived increased incidence of gastric cancer among their families, and were aware of the association between *H. pylori* infection and gastric cancer (98). Between 1992 and 2000, the age-adjusted gastric cancer incidence rate among men in Northwest Territories was 2 times higher than the rate in men across Canada (103). In addition, gastric cancer was the second most frequently diagnosed cancer in Inuit men and the third most frequently diagnosed cancer in Dene First Nations men, although it ranks the 10th in men across Canada (103). Moreover, Aklavik is a home for number of immigrants from Alaska where high rates of gastric cancer have been previously reported (98,107).

1.1.6.3.1.3 Aklavik *H. pylori* project

The Aklavik *H. pylori* project started in 2007 to address community concerns regarding *H. pylori* infection. The project was initially developed by the Canadian North *Helicobacter pylori* (CAN*Help*) Working Group, to investigate the health burdens associated with *H. pylori* infections in Aklavik. The CAN*Help* Working Group is a collaborative team of health care professionals, Aboriginal government representatives, the Aklavik Health Committee, and University of Alberta researchers. The main goals of the project are to identify the level of *H. pylori* infection within Aklavik, to improve clinical management and reduce health risks. In 2008, 58% of residents of Aklavik tested positive for *H. pylori* infection by UBT (98). Gastric biopsies were taken from 194 research participants, 66% of which were positive for *H. pylori* infection by histology. Among *H. pylori* positive

participants, the prevalence rates were 94% and 100% for acute and chronic gastritis respectively indicating that the local concern about *H. pylori* infection is warranted (105).

The Aklavik *H. pylori* project is a community driven research project that includes community partnerships. The project has a knowledge dissemination component that shares knowledge with community members. The benefits from such component include direct access to the community, generating new research questions or modifying others based on community needs. In addition, community partners have a better understanding of the scientific process and researchers have a better knowledge of community needs creating an atmosphere of cooperation that finally maximizes community benefits (108).

1.1.7 Virulence factors

Although *H. pylori* infection is highly prevalent worldwide, the majority of infected individuals are asymptomatic. Persistent colonization results in chronic gastritis in almost all infected individuals. However, 80-90% of them will never develop any symptoms. It is estimated that only 10-15% of infected people are at risk to develop peptic ulcer disease, 1-2% are at risk to develop gastric cancer, and very small proportion (< 0.1%) are at risk to develop MALT lymphoma (23,109-111). The clinical outcome depends on various host, pathogen and environmental factors (112). Host immune gene polymorphisms such as interleukin-1 gene cluster and gastric acid secretion determine the pathogen's ability to colonize the stomach and establish a clinical condition (3,113). It is proposed that the specific characteristics of *H. pylori* strains may play a role in clinical outcomes of infection. *H. pylori* virulence factors such as the cytotoxin-associated gene encoded protein (CagA), the vacuolating cytotoxin (VacA), the blood group antigen binding adhesin (BabA) and urease aid in gastric colonization and subsequently seem to modulate the host's immune system resulting in a condition of inflammation and development of active disease (114-116). Several environmental factors such as socioeconomic status, ethnicity, smoking and alcohol consumption may play a role in clinical outcomes of the infection by affecting the host immune response (117). In the past

few years, many *H. pylori* virulence factors have been described that explain the ability of this pathogen to survive and colonize in the extremely hostile acidic condition of the stomach. VacA, CagA, BabA, and urease have been reported to be major virulence factors of *H. pylori* (118).

1.1.7.1 *cytotoxin associated gene* pathogenicity island (*cag*PAI)

The *cag*PAI is a 40-kb DNA segment that was horizontally acquired into the *H. pylori* genome (**Figure 1.3**) (119). It contains 27 to 31 genes (the number of genes may vary in different strains), including the *cagA* gene at one end, and 18 genes encoding proteins that serve as building blocks for a type IV secretion system (TFSS), which is a syringe-like structure capable of penetrating gastric epithelial cells and injecting the CagA protein into host cells (**Table 1.4**) (119). *H. pylori* strains are classified into two main categories; type I and type II, based on whether or not they express the CagA and the VacA proteins (120). Type I strains can express both proteins and were frequently isolated from patients with duodenitis, duodenal ulcers, and gastric tumors which suggests that CagA and VacA are important virulence factors (120,121).

1.1.7.1.1 Phosphorylation of CagA

When CagA is delivered from *H. pylori* into the gastric epithelial cells, it undergoes tyrosine phosphorylation by Src (tyrosine-protein kinase) family kinases (SFKs) kinases such as c-Src (carboxy-terminal tyrosine-protein kinase), Fyn (proto-oncogene tyrosine-protein kinase encoded by *fyn* gene), Lyn (tyrosin protein kinase encoded by *lyn* gene), and Yes (proto-oncogene tyrosin-protein kinase encoded by *yes1* gene) (122). SFKs are constitutively activated in gastric epithelial cells and so phosphorylation of CagA by SFKs can occur in the absence of any external stimuli (123).

1.1.7.1.2 Site of CagA phosphorylation

CagA is phosphorylated at the carboxy (c)-terminal variable region on tyrosine phosphorylation motifs containing the glutamic acid-proline-isoleucine-tyrosine-alanine (EPIYA) amino acid sequence (123). The EPIYA motif is present

in multiple copies in the C-terminal polymorphic region of CagA and therefore called the EPIYA repeat region. There is a high diversity in the nucleotide sequence of *cagA* in the EPIYA-repeat segment. The EPIYA motifs are defined as EPIYA-A, -B -C, and -D, according to the amino acid sequence that surrounds the EPIYA sequence (**Table 1.5**) (124). The CagA protein species nearly always contain EPIYA-A and EPIYA-B sites, followed by one to five repeats of EPIYA-C in Western-type *H. pylori* isolates or EPIYA-D site in East Asian-type isolates (**Figure 1.4**). SFKs can only phosphorylate CagA on one or two EPIYA-C motifs, but never on 3 motifs at the same time (125). It is proposed that as the number of C repeats increases, the virulence of the CagA increases (126). Also, East Asian CagA containing EPIYA-D was associated with more inflammation and atrophy than Western CagA (127).

1.1.7.1.3 Actions of phosphorylated CagA

Phosphorylated CagA alters the signal transduction pathway and induces a growth factor like response leading to cytoskeletal rearrangement in gastric epithelial cells (**Figure 1.5**) (122). It interacts with SHP-2, a host cytoplasmic non-receptor tyrosine phosphatase, resulting in a conformational change in SHP-2 and inhibition of its phosphatase activity, leading to "a hummingbird" phenotype, which is a morphological change observed in AGS cells (human adenocarcinoma epithelial cell line) (128). Phosphorylated CagA can also cause negative feedback regulation of CagA phosphorylation by interacting with the c-Src kinase, which results in inhibition of Src kinase activity, thus attenuating the tyrosine phosphorylation of CagA. This inhibition mechanism is likely to contribute to a balance between CagA toxicity and the host to avoid excessive CagA-associated toxicity (129).

1.1.7.1.4 Phosphorylation independent actions of CagA

In addition to the phosphorylated CagA actions, CagA possesses phosphorylation-independent activities that are involved in cell growth, motility and proinflammatory responses associated with development of chronic gastritis and gastric cancer (130). CagA interacts with the c-Met (a proto-oncogene that

encodes for a protein known as hepatocyte growth factor receptor), causing activation of β-catenin and NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) signalling, which promotes proliferation and inflammation (130). Non-phosphorylated CagA has been reported to bind to Grb2 (growth factor receptor-bound protein 2), which is an adaptor protein. The CagA-Grb2 interaction is involved in a phenomenon known as "scattering" phenotype, which is increased cell motility. Upon complex formation with Grb2, CagA promotes proliferation of gastric epithelial cells through activation of the Ras pathway (131,132).

1.1.7.1.5 CagA and development of gastric cancer

Development of gastric cancer is a prolonged process that requires many steps involving both quantitative and qualitative alterations in the expression of oncogenes and tumor suppressor genes (114). Long-term exposure to CagA may have a role in the development of gastric cancer. This role may occur as a result of the interaction of CagA and SHP-2. The CagA-SHP-2 complex is primarily detectable in atrophic gastric mucosa, which may proceed to intestinal metaplasia (129). The interaction between CagA and SHP-2 has certain effects on SHP-2 function which are similar to those caused by certain mutations in *PTPN11* (tyrosine-protein-phosphatase non-receptor type 11), the gene encoding SHP-2, which have been associated with the development of human cancers such as acute myeloid leukemia and neuroblastoma (132,133). In addition, chronic infection with CagA-positive *H. pylori* may lead to chronic inflammation which promotes gastric cancer by disturbing the balance in IL-6-signaling pathways (134). Balanced IL-6 signaling is required to maintain gastric homeostasis. In absence of such balance, increased STAT 1 (signal transduction and transcription) regulated genes may occur which lead to the development of antral tumors with inflammation and ulceration in experimental mice models (135). Although many published studies found a significant correlation between infection with CagA positive *H. pylori* and occurrence of gastric cancer (136), other studies failed to detect such relationship (137).

1.1.7.2 Vacuolating cytotoxin (VacA)

The VacA cytotoxin belongs to the AB group of toxins in the same category as cholera toxin, which are made up of two subunits; an A-subunit, responsible for enzymatic activity, and a B-subunit, responsible for recognizing and binding to receptors on the surface of target cells (138). VacA is considered to be a multifunction cytotoxin as it induces *in vitro* cytoplasmic vacuolation in epithelial cells, disrupts mitochondrial functions and stimulates apoptosis (139). This toxin was found to cause gastroduodenal damage in a mouse model and to increase gastric ulcer risk in *H pylori*–infected Mongolian gerbils (140).

VacA is encoded by *vacA* gene, which is present in the majority of *H. pylori* strains, but it does not always induce vacuolation (115). This suggests that genetic polymorphism within *vacA* may affect the ability of the strain to induce vacuolation (141). Two major polymorphic regions have been identified within *vacA*: the signal region (*s1* or *s2*) and the midregion (*m1* or *m2*) (142). *vacA* type *s1/m1* strains were reported to be the most virulent strains, those with *s1/m2* were found to be less virulent strains while the least virulent stains were those with *s2/m2* type (143-145). A new *vacA* polymorphic site, designated as the intermediate (*i*) region, was recently identified with the possibility of two sequence types (*i1* and *i2*). It was reported that the *i1* type was strongly associated with gastric adenocarcinoma (146,147). This association is controversial as another study reported that *vacA s/i/m* genotypes failed as a marker for clinical outcomes in the East Asian and Southeast Asian countries where gastric cancer is a major clinical problem (148).

1.1.7.3 Blood group antigen binding adhesin (BabA)

H. pylori colonises the gastric mucosa by penetrating the mucus layer lining the gastric epithelium and adhering to the gastric mucosa (149). BabA is thought to contribute to the ability of *H. pylori* to adhere to the gastric mucosa (150). Adherent bacteria are proposed to have a better growth profile and colonization ability as they are closer to the gastric epithelium and so they are closer to the source of nutrition (151). In addition, adherent bacteria are more protected from being washed out by the gastric peristaltic movement and from the high acidity of the gastric fluid.

Furthermore, adherent bacteria are better able to deliver their virulence products, such as CagA and VacA to the host, which ultimately will lead to more inflammation to the host and better survival of the bacteria (151). Inflammation and toxins cause more tissue damage leading to disruption of epithelial cell junctions and leakage from blood vessels, which provides more nutrients to *H. pylori* (152).

BabA adherence is mediated through the fucosylated Lewis b (Le^b) blood-group antigen (153). Fucosylation was also reported in *H. pylori* and is mediated by fucosyltransferases; enzymes, which catalyze the transfer of a fucose group from donor guanosine-diphosphate fucose to acceptor molecules such as oligosaccharides, glycoproteins and glycolipids (154). Fucosylation is more common in eukaryotes than prokaryotes. Several bacterial fucose transferases have been identified in *E.coli*, *Salmonella enterica, Yersinia enterocolitica* and *H. pylori.* However, of those enzymes, only *H. pylori* fucosyltransferases have been extensively studied and functionally characterized (155). Fucosylation results in molecular mimicry between *H. pylori* lipopolysaccharide and host glycoconjugates, which helps *H. pylori* to evade host immune responses (156). *H. pylori* strains were classified according to Le antigen expressions into type I strains (< 5%) which can express Le^a and Le^b, and type II strains which can express Le^x and Le^y. It was reported that *H. pylori* can alter its Le antigen phenotype to match the Le antigen phenotype of its host (157).

BabA has the ability to adapt to the fucosylated blood group antigens that are most prevalent in the local population. Strains expressing BabA are classified into two main types; generalist stains and specialist stains depending upon the blood group antigens they bind to. In Europe and the United States where all blood group phenotypes exist, the *H. pylori* strains (generalist strains) bind to blood group A, B, and O determinants (158). However, in populations such as the indigenous South American native population, which only has the blood group O phenotype, the *H. pylori* strains (specialist strains) bind only to the blood group O determinants (159). Three different *bab* alleles have been identified *babA1*, *babA2*, and *babB*. *babA1* lacks the translational initiation codon while both *babA2* and *babB* encode homologus proteins which have polymorphic midregion sequences. Only BabA,

not BabB, mediates Le^b binding (153,160). *H. pylori* strains expressing BabA together with the CagA and VacA s1 are associated with severe gastric diseases such as peptic ulcer and gastric adenocarcinoma (116,151). However, other studies reported high prevalence of *babA2* gene with no correlation to clinical outcomes (161,162). Since Le^b is not expressed in all humans, other adhesins may be involved in *H. pylori* adhesion (**Table 1.5**) such SabA (sialic acid-binding adhesin) and SabB (163), HopZ (*H. pylori* outer membrane protein) (164), and AlpA and AlpB (adherence associated lipoprotein) (165).

1.1.7.4 Urease

Urease is a large molecular weight (550 kDa) metalloenzyme, which constitutes about 10% of total *H. pylori* protein (166). Urease is encoded by urease genes which are localized to a 34 kb portion of *H. pylori* chromosome. The urease genes are composed of structural (*ureA*, *ureB*), regulatory (*ureC*, *ureD*) and accessory (*ureI*, *ureE*, *ureF*, *ureG*, *ureH*) genes (167). The two structural genes, *ureA* and *ureB*, are highly conserved in different *H. pylori* strains. UreB subunit is the active site of the enzyme; the site where the substrate binds leading to catalysis (**Figure 1.6**) (168).

Urease is a vital enzyme for *H. pylori* colonization. It catalyzes the hydrolysis of urea to form carbon dioxide (CO_2) and ammonia (NH_3), which neutralizes the gastric acid, thereby allowing *H. pylori* to grow. Both ammonia and bicarbonate produced by *H. pylori* play a role in *H. pylori* pathogenesis. Ammonia is thought to have a cytotoxic effect on gastric epithelial cells by impairing mitochondrial respiration and energy metabolism (169), whereas bicarbonate is thought to suppress peroxynitrite, a nitric oxide metabolite that has a bactericidal effect (170). In addition, urease itself has a potent immunogenic effect as it stimulates the inflammatory response by recruitment of leukocytes and triggering of the oxidative burst in neutrophils (171). Moreover, *H. pylori* urease B subunit binds to CD_{74} (class II MHC) on gastric epithelial cells leading to NF-κB activation and IL-8 Production (172).

Urease is essential for *H. pylori* colonization. A urease-negative mutant of *H. pylori,* constructed by allelic exchange mutagenesis, lacked the ability to colonize the stomach of nude mice (173). Similar results were observed for gnotobiotic piglets infected with urease negative *H. pylori,* which illustrate the importance of urease for *H. pylori* colonization (174). Urease inhibitors, such as acetohydroxamic acid, have been studied for possible use in treating *H. pylori* infections (175). A follow up study incorporated acetohydroxamic acid into gellan based floating beads to achieve controlled and sustained drug release (176). However, *in vivo* studies have not yet been reported.

1.2 Genetic transformation of *H. pylori*

H. pylori has acquired a great proportion of its genetic diversity through the acquisition of DNA sequences from other related organisms. Gene transfer results in a continuously changing genome in which new DNA sequences are either introduced into or deleted from the chromosome. Gene transfer adapts *H. pylori* to new environments and greatly enhances its pathogenicity. The *cag*PAI is an example of such adaptation as it was horizontally acquired into the *H. pylori* genome (177).

1.2.1 Natural transformation

H. pylori has high level of genetic diversity and plasticity as most wild-type strains are naturally competent for transformation. Naturally competent bacteria are able to take up foreign DNA (plasmid or chromosomal DNA). Plasmids are self-replicating DNA and so *H. pylori* can replicate plasmids once they are inside cells. However, chromosomal DNA must be integrated into the chromosome to be replicated. *H. pylori* is naturally competent for both plasmid and chromosomal DNA (178). It was proposed that the natural competence occurs through T4SS as *H. pylori* has two independent T4SS; the classical T4SS which is encoded by the genes in the *cag* pathogenicity island and the ComB system (179). More recently, it was found that certain *H. pylori* strains can harbor up to four T4SSs in their genome (cag-T4SS, ComB, tfs3, tfs4) and so indigenous plasmids can be efficiently transferred between *H. pylori* strains either by natural transformation or

conjugation (180). *H. pylori* natural transformation occurs through the actions of proteins encoded by the *comB* locus which consists of four genes with partially overlapping open reading frames, *orf2*, *comB1*, *comB2* and *comB3* (181). However, for a better correlation with the *Arogobacterium tumefaciens* VirB components, the first T4SS to be described, it was proposed that *comB1*, *comB2*, and *comB3* should be re-named as *comB8*, *comB9*, and *comB10*, respectively. In addition, *orf2* is now named as *comB7 (182)*. ComB dependent natural transformation does not have the same efficiency in different strains. Furthermore, the system can be saturated and depends on the length, symmetry and whether the DNA is single or double stranded (183).

1.2.2 Conjugation

Conjugation refers to the horizontal transfer of genetic materials between cells by direct cell-to-cell contact through the formation of pili. Conjugation was first described in *H. pylori* back in 1998 when the transfer of a plasmid carrying kanamycin resistance cassette was observed between *H. pylori* cells even when the culture treated with DNase I (184). On the other hand, the transfer was completely abolished by the presence of both DNase I and a membrane separating the cells (184). Later on, the genes essential for plasmid conjugation were identified, conjugal transfer protein gene (*traG*) and relaxase gene (*rlx1*), and it was found that they act independent of T4SS (185). More recently, it was reported that ComB T4SS can also accomplish conjugative DNA transfer. Two conjugative DNA transfer were identified, one mechanism depends on relaxase (plasmid mobilization) and the other mechanism is completely independent of relaxase, and any T4SS (180).

1.2.3 Electroporation

Electroporation involves the application of high-intensity electric fields of short duration to increase the permeability of biomembranes reversibly and to allow DNA entry. The amplitude and duration of the electric charge are important, and optimal values may differ from one type of bacteria to another (186). Segal was able to transform *H. pylori* by electroporation at a transformation frequency more

than 10^5 transformants per µg DNA by using electric voltage which is higher than that used to transform *E. coli* (187).

Plasmids are classified into conjugative (transmissible on their own), mobilizable (transmissible in the presence of a helper plasmid) or non-mobilizable. Mobilizable plasmids have genes that code for *oriT* (origin of transfer), relaxases and coupling proteins but lack genes that code for mobilization (188). Electroporation was found to be the only way to transform plasmid-free *H. pylori* with mobilizable or non-mobilizable plasmids. On the other hand, plasmid-containing strains can be transformed by natural competence (189). Liposomes were studied as a possible means to improve the electroporation efficiency of *E. coli*. DOTAP® [1,2-bis (oleoyloxy)-3-(trimethylammonio) propane], a cationic liposome, was found to be successful in inducing *E.coli* transformation without electroporation by enhancing DNA delivery and protecting DNA from degradation by DNases (190). The use of DOTAP® to improve the electroporation efficiency or to induce transformation in *H. pylori* was not previously investigated.

1.3 Gene silencing

Gene silencing is the process by which a gene is switched off without gene manipulation and may occur by transcriptional gene silencing or post-transcriptional gene silencing. Transcriptional gene silencing refers to gene silencing occurring through repression of transcription (191). siRNAs (short interfering RNA) targeting gene promoters can result in gene silencing in human cells. However, it is unclear whether the antisense strand of the siRNAs binds directly to DNA or to the RNA transcript (192). During the 1990s, a number of gene-silencing phenomena that occur at the post-transcriptional level were discovered in plants, fungi, animals, ciliates and bacteria, opening the gate for a new concept of post-transcriptional RNA silencing (193).

1.3.1 Gene silencing in bacteria

1.3.1.1 Naturally occurring gene silencing

Bacteria have the ability to survive in extreme environmental conditions including wide variations in pH values, temperatures and osmotic pressure. An important feature of pathogenic bacteria is their ability to adapt and thrive in multiple environments. Noncoding RNA is reported to play an important role in the regulation of expression of bacterial virulence factors (194). Interfering RNAs may be either *cis*- or *trans*- encoded.

The *cis*-encoded RNAs are encoded at the same genetic location, but on the opposite strand to the RNAs they act upon, and therefore contain perfect complementarity nucleotide sequences to their targets (195). The majority of *cis*-encoded RNAs were discovered in plasmids, transposons and bacteriophages (196). More recently, a few of them were found to be associated with bacterial chromosomes such as those discovered in *E. coli* and *B. subtilis*. The main biological functions of *cis*-encoded RNAs associated with plasmids are replication, conjugation and ion transport control (197). Transposons-encoded RNAs were linked to transposition control, while phage-encoded RNAs were linked to the lysis/lysogency switch and superinfection override (195). A *cis*-encoded antisense small RNA that regulates the expression of *H. pylori ureB* gene was recently discovered. The sRNA-mediated downregulation of urease facilitates gastric colonization over a wide range of intragastric pH (198).

The *trans*-encoded RNAs are encoded at a chromosomal location distinct from the RNAs they act upon and generally do not exhibit perfect base-pairing potential with their targets. They are more abundant in Gram negative bacteria than Gram positive bacteria (199). In addition, there is a distinct variation in the mode of action between the *trans*-encoded RNAs from these two groups of bacteria (200). While the majority of Gram negative *trans*-encoded RNAs need Hfq (host factor Q) protein for their stability or RNA interaction, the function of that protein in Gram positive bacteria is not clear (201).

1.3.1.2 Experimental gene silencing in bacteria

RNA interference has been used as a possible means by which the sensitivity to antimicrobials can be enhanced. Antisense peptide nucleic acid (PNA) targeting the CmeABC multidrug efflux transporter in *Campylobacter jejuni* rendered *C. jejuni* isolates more susceptible to ciprofloxacin and erythromycin (202). Another study investigated the possibility of downregulating YidC; a membrane protein that translocates and inserts other proteins into the membrane. It is a conserved protein and has been reported to be essential for *E. coli* growth. Eugenol (a phenylpropene) and carvacrol (monoterpenoid phenol) can be extracted from several essential oils of plant origin and have antibacterial activity against a number of Gram negative and Gram positive pathogenic bacteria (203). Enhanced antibacterial activity of eugenol and carvacrol was reported against *E. coli* treated with *YidC* antisense RNA (204). Antisense DNA targeting the *oprM* gene of multidrug resistant *P. aeruginosa* increased its susceptibility to pipracillin due to impaired multidrug efflux system (205).

RNA interference alone was also investigated as antimicrobial agent by interfering with the expression of vital proteins. Targeting methicillin-resistant *Staphylococcus aureus* (MRSA) coagulase with siRNA, delivered by natural competence, inhibited both mRNA expression and the activity of MRSA coagulase *in vitro*. The treatment was effective in reducing the bacterial load in a murine model of haematogenous pulmonary infection (206). An antisense RNA targeting alkyl hydroxyperoxide reductase (*AhpC*) gene of *H. pylori* achieved 72% knochdown of AhpC expression (207). Since gene silencing was possible in both Gram positive and Gram negative bacteria, including *H. pylori*, it may be a useful strategy to downregulate *H. pylori* essential genes and interfere with its survival in gastric mucosa.

1.4 Treatment

Since its discovery in 1982, eradication was the ultimate goal for the treatment. Several antibiotics and treatment regimens were tried but 100% eradication rate has never been achieved. Treatment may be unsuccessful due to

antimicrobial resistance, (208) re-infection (209), patient compliance and the high cost of currently used drugs (210). The three treatment regimens that are commonly used for the treatment are triple (3 drugs) therapy, sequential therapy and quadruple (4 drugs) therapy.

1.4.1 Triple therapy

In triple therapy, a proton pump inhibitor (PPI) (lansoprazole, omeprazole, pantoprazole, rabeprazole, or esomeprazole) with amoxicillin and clarithromycin or metronidazole are taken for 7 to 10 days. Most recent guidelines do not support the use of the triple therapy as first line treatment (52). Treatment failures with the triple therapy have been reported in many countries due to increased resistance to clarithromycin and metronidazole. Moreover, treatment failure may be observed independent of antimicrobial resistance, such as with amoxicillin where treatments fail even though *H. pylori* is susceptible to amoxicillin (211). A meta-analysis that was published in 1999 including more than 53,000 patients showed that the eradication rate is <80 % (212). More recent studies showed even lower eradication rates. A 10-year follow up study in Turkey showed a decline in the eradication rate with triple therapy from 79.4% to 68.8% (213). In Aklavik NWT, where there is high prevalence of *H. pylori* infection, triple therapy had a 59% eradication rate (214). *H. pylori* resistance rates to clarithromycin have been reported to range from 2.5-48.2%. The highest prevalence was observed in Turkey, while the lowest prevalence was observed in Brazil (**Table 1.7**). The resistance to metronidazole is higher than clarithromycin and is continuously increasing. The resistance rates have been reported to range from 19-64% where the highest prevalence was observed in Iran and China. Resistance to amoxicillin was reported in China and Iran at low rates (0.3 and 2.5% respectively) (215,216) as *H. pylori* lacks β-lactamase activity, the main mechanism for amoxicillin resistance (217). However, a recent study reported a 12.2% resistance rate among *H. pylori* isolated from Israeli children although the resistance rate was much lower among adults (2.3%) (218).

1.4.2 Sequential therapy

Sequential therapy is a 10-day course that includes a 5-day treatment with PPI + amoxicillin, followed by another 5-day treatment with PPI + clarithromycin + tinidazole or metronidazole. Tinidazole is chemically similar to metronidazole and has the same toxicity and side effects. A study that was done in Italy reported no difference in efficacy between the two drugs (219). Amoxicillin acts by inhibiting cell wall synthesis, weakens bacterial cell walls and enhances the uptake of other antibiotics into *H. pylori* (220). Many studies compared the eradication rate of sequential therapy to that of standard triple therapy. Most of these studies reported better eradication rates with sequential therapy (91.3%), and recommended the use of sequential therapy instead of triple therapy as first line option for *H. pylori* treatment (221). In Aklavik, NWT the eradication rate with sequential therapy was 73% (214). A recent study in Korea reported 75.9% eradication rate with sequential therapy and 58.7% with triple therapy (222). The study recommended considering other treatment regimens like the quadruple therapy because sequential therapy did not offer a satisfactory eradication rate (222). Another study reported no significant difference between eradication rates achieved by triple and sequential therapies due to antimicrobial resistance (223). All the above studies reported no significant difference in side effects between the two regimens. Sequential therapy has now replaced triple therapy as a first line option for *H. pylori* treatment with quadruple therapy reserved in case of treatment failure (224,225).

1.4.3 Quadruple therapy

Quadruple therapy is composed of a combination of bismuth subcitrate potassium, metronidazole, tetracycline, and omeprazole (226). It is considered to be an acceptable second line choice for *H. pylori* treatment in areas where there is high level of clarithromycin resistance (>15-20%) (227). European investigators conducted an industry-sponsored, open-label, randomized phase III trial involving 438 patients in 39 different sites in Europe with *H. pylori* infection (228). Patients received either quadruple therapy for 10 days or standard triple therapy for 7 days.

Successful eradication of *H. pylori* infection was confirmed by urea breath tests. The eradication rate was higher in the quadruple therapy group than in the standard triple therapy group (80% vs. 55%; $p < 0.0001$) (228). A recently published meta-analysis showed high efficacy for 10 days quadruple therapy when compared to 7 days triple therapy. However, the 7 or 10 days quadruple therapy or triple therapy yielded similar eradication rates. Compliance and side effects were similar in both therapies (229).

1.4.4 Vaccination

1.4.4.1 Challenges in vaccine development

Because of the high prevalence of *H. pylori* infection in developing countries and the continuously declining eradication rates, mass vaccination may be an ideal strategy for controlling *H. pylori* infection. Efforts to develop a vaccine began in the 1990s (230,231). However, the progress in that field is relatively slow as there is lack of certainty regarding whether a vaccine is needed, especially with the decline in *H. pylori* prevalence in developed countries, although there are remote areas in developed countries where there is high prevalence of *H. pylori* infection similar to that observed in developing countries. A vaccine is also controversial because a number of researchers have linked *H. pylori* eradication with the increased incidence of some extra gastric conditions such as gastroesophageal reflux disease, Barrett esophagus, and esophageal adenocarcinoma (232).

Several efforts were directed towards the development of a prophylactic and therapeutic vaccine against *H. pylori*. The prophylactic vaccine would be very useful to protect children from getting infected, especially in areas where there is high prevalence of *H. pylori* infection. On the other hand, a therapeutic vaccine would be ideal for those who are infected to eradicate *H. pylori* and protect them from reinfection. To date, most of the trials in that field have had limited success. Complete sterilizing immunity in experimental animals was rarely achieved. This may be due to the fact that neither the optimum antigenic composition nor the major protective mechanisms in the human body were identified. The immune response that is required for *H. pylori* vaccination remains poorly understood. Previous

studies in animal models reported that the production of antibody in the stomach (IgA) did not protect against *H. pylori* infection (233,234). In contrast, major histocompatibility complex (MHC) Class II T-cells such as T-helper 1 (Th1), Th2 and Th17 cells are important in mediating immunity in mice (235). However, chronic infection by *H. pylori* is associated with robust immune and inflammatory responses, which are rarely successful in eradicating the organism. Moreover, there is no complete agreement regarding the best adjuvant or route of administration (236).

1.4.4.2 Antigenic composition of vaccines

A number of antigens have been selected to be putative components of a vaccine. They include the flagellar antigens FlaA and FlaB which are important for bacterial motility (237), as well as several adhesins, such as BabA, SabA and AlpA, which are important for bacterial attachment to the gastric mucosa (238). Others include subunits of urease (UreA and UreB), which are vital for *H. pylori* survival in an acidic medium (230,231), toxins such as CagA, VacA and NAP (neutrophile activating protein) (239), or outer inflammatory protein (OipA), which are considered to be important *H. pylori* virulence factors (240).

1.4.4.3 Vaccine trials

In the literature, there are several vaccine trails in experimental animal models; however, there are limited trials in human volunteers (**Table 1.8**). Mori *et al.* were able to incorporated FlaA into a chimeric flagellin in which both terminal segments of *H. pylori* flagellin were replaced by Toll like receptor (TLR 5)-activating flagellin from *E.coli*. The designed chimeric protein was able to significantly increase serum IgG and IgA antibody response in mice without an adjuvant and so was considered as self-adjuvanting vaccine (237). In 2011, Moss *et al.* developed a multi-epitope vaccine in a DNA-prime/peptide boost approach that was administrated intranasally or intramuscularly to *H. pylori* infected mice (241). The vaccine induced IFN-γ production, and led to a sterilizing immunity 32 weeks after administration in 5 of 19 mice (241). Another promising study done at that year investigated the use of a measles virus vaccine encoding the *H. pylori*

neutrophil activating protein. The designed vaccine was able to induce a robust antibody- and cell-mediated immune response in mice against *H. pylori* (242). Guo L *et al.* constructed a fusion protein of cholera toxin B subunit and an UreA epitope of *H. pylori* to be used as a vaccine. The fusion protein had good immunogenicity and could induce a significant increase in the level of specific IgG *($p < 0.001$),* but no animal studies were performed to assess the prophylactic and therapeutic effect of the vaccine (243). The first clinical trial in healthy human volunteers was published in 2008. Fifty eight volunteers were immunized orally with *Salmonella enterica* serovar Typhi (Ty21a) that expressed *H pylori* urease, or with Ty21a only (control group), and then challenged with $2x10^5$ *cag*PAI (-) *H pylori*. Although the vaccine developed T cell-mediated immune response, it was ineffective, as all volunteers developed *H. pylori*-induced gastritis (244). Another study published that year reported safety and immunogenicity for an intramuscular injection of *H. pylori* vaccine containing CagA, VacA and NAP to fifty seven human volunteers, but the efficacy of the vaccine was not tested (245). A recent randomised, double blinded, placebo-controlled vaccine trial demonstrated safety, efficacy and immunogenicity of an oral recombinant vaccine in children in china. The vaccine utilized urease B subunit fused with heat-labile enterotoxin B subunit and showed 72% efficacy following one year follow up (246).

In conclusion, some progress has been developed in the field of vaccine production in the last few years. Several new approaches have been attempted and more work has been directed towards understanding the mechanisms of immunity against *H. pylori,* which may support the development of a new vaccine.

1.4.5 Novel treatment approaches

The increased reports of treatment failure and antibiotic resistance attract attention for novel treatment approaches. One strategy is to improve the stability and delivery of currently used antibiotics by incorporating them into liposomes. Jain *et al.,* were able to design polyelectrolyte coated multilayered liposomes (nanocapsules) loaded with metronidazole and amoxicillin (247). The designed liposomes were able to prolong drug release in simulated gastric fluid and eradicate

H. pylori from infected Balb/c mice (247). Another group of researchers was able to incorporate amoxicillin trihydrate and ranitidine bismuth citrate into double liposomes to be used as a dual drug delivery system against *H. pylori.* Double liposomes were prepared in two steps; in the first step inner liposomes containing one drug were prepared by thin film hydration and sonication, then double liposomes were prepared by hydrating a thin film of lipids containing the other drug with a suspension of inner liposomes (248). A recently published study designed nanolipobeads based on a dual drug delivery system for *H. pylori* targeting (249). Targeted liposomes have also been designed to improve the delivery of ampicillin and metronidazole to *H. pylori* and not to other bacteria in the stomach or gastrointestinal tract. Targeting was achieved by incorporating a neoglycolipid with a structure similar to Le^b that can bind the BabA protein (250). However, the previously mentioned antibiotic-loaded liposome-based drug delivery systems may not be sufficient to eradicate multidrug resistant *H. pylori* especially if the susceptibility cannot be improved by enhancing drug delivery to *H. pylori* (251). For that reason, more research should be directed towards developing novel therapies that do not rely on current antibiotics or their derivatives. Two articles have investigated the possibility of using novel treatments. The first article was published in 2004 and investigated the incorporation of acetohydroxamic acid, a urease inhibitor, into lipobeads to be used for *H. pylori* treatment (175). The second article studied the antibacterial activities of liposomal linolenic acid against antibiotic resistant *H. pylori*. The liposomal formulation did not incorporate any current antibiotics and was effective in killing both spiral and coccoid forms of a metronidazole resistant *H. pylori* strain (252).

1.5 Liposomes

Liposomes are nanosized artificial spherical vesicles that consist of phospholipid bilayers. Since their first discovery by Alec D. Bangham, they have attracted attention as potential carriers for many bioactive molecules (253). When phospholipids are dispersed in water, they tend to form bilayers where the polar head groups are attracted to water and the non-polar tails are repelled from water.

The polar nature of the liposomal core allows polar molecules to be encapsulated while amphiphilic and lipophilic molecules can be solubilized within the phospholipid bilayers. Liposomes differ from micelles, which are formed from ionized lipids and consist of only a single layer of lipids (**Figure 1.7**). Micelles cannot be used as carriers for polar molecules as their non-polar tails are clustered together at the center and therefore do not allow any polar compounds in the interior (254).

1.5.1 Classification

Liposomes can be classified into three major types based on the number of lamellae and vesicles: unilamellar vesicles (ULV), multilamellar vesicles (MLV) and multivesicular vesicles (MVV). The unilamellar vesicles may be small (20-40 nm), medium (40-80 nm) or large (80-1000 nm) in size. The MLV have more than one bilayer, while the MVV vesicles have more than one vesicle (**Figure 1.8**) (255).

Liposomes can be also classified according to their composition and application into four major types: conventional, long-circulating, immuno- and cationic liposomes (256). Conventional liposomes are composed of only phospholipids (neutral or negatively charged) and/or cholesterol. Conventional liposomes have short systemic circulation times, as they are rapidly taken up by the reticuloendothelium system, and therefore are used as vehicles to deliver drugs to macrophages (257). Long-circulating liposomes are prepared by coating conventional liposomes by hydrophilic polymer such as polyethylene glycol (PEG). Surface PEGylation creates a steric barrier that prevents interaction with cellular components in biological environments thus prolongs systemic circulation time (257). Immunoliposomes have specific antibodies that can target liposomes to particular cell types. It is possible to prepare PEGylated immunoliposomes that can be targeted and at the same time have long half-life (258). Cationic liposomes are positively charged and can be prepared by incorporating cationic lipids during their preparation. Two main cationic lipids are commonly used; 1,2-bis(oleoyloxy)-3-(trimethylammonio) propane (DOTAP®) and 3β[N-(N',N'-dimethylaminoethane)-carbamoyl cholesterol (DC-Chol) (259). In addition to those lipids, cationic

liposomes can be also prepared by incorporating the cationic detergent, stearylamine, during liposome preparation (260).

1.5.2 Composition

1.5.2.1 Phospholipids

Liposomes vary greatly in their lipid composition and the choice of the lipids depends mainly on the final application of the preparation. Glycerol-containing phospholipids are the most common component of liposomes and they may represent greater than 50% of the total weight of liposomes. The glycerol moiety has three OH groups; the OH at C_3 is esterified with phosphoric acid forming the polar part the compound while the OH at C_1 and C_2 are esterified with a long chain fatty acid forming the non-polar part of the compound (**Figure 1.9**) (255). The addition of organic moieties to the remaining OH group of phosphoric acid creates a variety of phospholipids such as phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidylglycerol (PG), and phosphatidylcholine (PC) (**Figure 1.9**). The long chain fatty acids used for liposomes preparation may be saturated (with hydrocarbon chains completely filled with hydrogens) or unsaturated (with one or more double bonds in hydrocarbon chains) (**Figure 1.9**). In general, saturated fatty acids give more stable liposomes (261).

Liposomes can be made using a single type or a mixture of phospholipids. The lipid composition of liposomes will affect the surface charge and the final application of the preparation. Conventional liposomes are made of neutral or anionic phospholipids while cationic liposomes are made of cationic and zwitterionic lipids (256).

1.5.2.2 Cholesterol

Sterols are important components of most natural membranes as they fill the gaps that may occur between lipids due to imperfect packing or due to presence of proteins giving more rigid structure to the membrane. Moreover, sterols act as membrane dynamic regulators by controlling membrane fluidity, elasticity and

permeability (262). Cholesterol is the most widely used sterol in liposome preparation. Cholesterol cannot form a lipid bilayer structure by itself but it can be incorporated with phospholipids in very high concentrations during liposome preparation where it fills the empty spaces between phospholipid molecules. The OH group at C_3 acts as a polar head while the hydrocarbon chain at C_{17} acts as a non-polar chain (**Figure 1.10**). Cholesterol is used in the manufacture of liposomes to increase their stability by increasing or decreasing the fluidity of the lipid bilayer. At temperatures lower than the transition temperature of lipids, cholesterol increases fluidity while at temperatures higher than the transition temperature, it decreases membrane fluidity and reduces the permeability of the lipid membrane to solutes (263).

1.5.3 Lipid distribution in biological membranes

Lipid bilayer is the basic structure of all biological membranes. In eukaryotic cells, membranes are formed from three classes of lipids (phospholipids, sterols and sphingolipids) (264). Mammalian membranes contain a high proportion (40-80%) of phospholipids. However, the phospholipid content and composition vary greatly between different cell membranes due to functional variations. PC is the major phospholipid and accounts for 22-45.2% of total membrane phospholipid content (265). On the other hand, mammalian cells contain only one type of sterol, cholesterol, but hundreds of different phospholipids and sphingolipids (266).

In bacteria, the total phospholipid content of cell membranes varies more greatly than in mammalian membrane. The typical composition of phospholipids in bacteria is PS, PE, PG and cardiolipin (CL), which are also found in eukaryotes (267). In *H. pylori*, cell membranes are composed of 6% neutral lipids, 20.6% glycolipids, and 73.4% phospholipids (268). The major phospholipids are PE, CL, and PG. PS was also detected, but as a minor phospholipid. Three kinds of cholesterol glucosides were detected in membranes of *Helicobacter* species: cholesteryl-6-O-acyl-alpha-D-glucopyranoside, cholesteryl-alpha-D-glucopyranoside, and cholesteryl-6-O-phosphatidyl-alpha-D-glucopyranoside (269). Although

cholesterol glucosides were rarely detected in animals and bacteria, they were detected in 13 out of 15 *Helicobacter* species examined.

1.5.4 Preparation methods

When preparing liposomes, the lipids must be first dissolved in an organic solvent, such as chloroform or chloroform/methanol mixture, to ensure uniform distribution of the lipids. The solvent is then evaporated to yield a lipid film, which is hydrated by adding an aqueous medium followed by agitation. The products of hydration are large MLVs that can be further manipulated by one of the following methods to produce small ULVs (270).

1.5.4.1 Sonication

Small ULVs can be prepared by sonicating aqueous phospholipid dispersions using either a probe or bath sonicator. This method is considered to be the one with the highest energy input into lipid dispersions, so it is not suitable for preparing liposomes containing heat-labile molecules (protein, DNA) or drugs. In addition, liposomes prepared by sonication have very limited trapping efficiency (the percentage of solute that is entrapped) due to the small size of liposomes and may be contaminated by metal from a probe sonicator (271).

1.5.4.2 Freeze-thaw sonication

The main advantage in using repetitive freeze thaw cycles during liposome preparation is the homogenization of their lipid content, which enhances the trapping efficiency due to the breaking of MLVs and the formation of more homogeneous ULVs. However, the freeze-thaw method alone does not produce a liposome population of uniform size. In addition, fragmentation and fusion caused by freeze thaw cycling may not have the same homogenization efficiency for different lipid mixtures. Accordingly, this method is usually employed with other preparation methods such as sonication or extrusion (272).

1.5.4.3 Membrane extrusion

Liposomes were first prepared by extrusion through a polycarbonate filter in 1979 by Olson *et al.* (273). Extrusion is done by a liposome extrusion system, which is pressurized using nitrogen gas, producing liposomes with a uniform size distribution. The working temperature should be higher than the phase transition temperature of the lipids used to prepare liposomes to avoid lipid crystallization. The phase transition temperature is defined as "the temperature required to induce a change in the lipid physical state from the ordered gel phase, where the hydrocarbon chains are fully extended and closely packed, to the disordered liquid crystalline phase, where the hydrocarbon chains are randomly oriented" (274). The main advantages of the extrusion technique are that it can be applied to a wide variety of lipid mixtures, and it works directly from MLVs as extrusion peels mutilamellae to form ULVs. In addition, there is no need to remove organic solvents or detergents from final preparations and there is no metal contamination from the probe. The main drawbacks are lower working volumes and difficulties achieving high temperatures. High temperatures may be needed when working with lipids that have high transition temperatures to avoid lipid crystallization (275).

1.5.4.4 Detergent removal

A detergent can be used to solubilize lipids at concentration that is equal to its critical micelle concentration. The detergent is then removed by dialysis causing phospholipids to replace the detergent so the micelles become richer in phospholipids, which finally combine to form large ULVs. The main advantages of this method are excellent reproducibility and the production of liposomes with a uniform size distribution. The main disadvantage is the difficulty in achieving complete removal of the detergent, which may be toxic (276).

1.5.4.5 Reverse phase evaporation/sonication

In this method, lipids are dissolved in organic solvent and then mixed with the aqueous phase, followed by brief sonication to form a water-in-oil emulsion. Liposomes are formed when the organic solvent is removed by continued rotatory evaporation under reduced pressure to evaporate the organic solvent only without

evaporating lipids or aqueous solutions. With this method, a high trapping efficiency can be obtained but the materials to be encapsulated may be incompatible with the organic solvent. The brief sonication may also result in heat denaturation of some proteins or DNA that are loaded inside these liposomes (276).

1.5.5 Application of liposomes

1.5.5.1 Liposomal delivery of molecules

Liposomes have attracted attention as useful drug delivery systems to enhance the bioavailability of certain drugs and reduce their toxicity. Liposomes can be used to enhance the solubility of both lipophilic and hydrophilic drugs due to their unique structure that has both aqueous and non-aqueous compartments (277). The solubility of Amphotericin B and Minoxidil was greatly improved by incorporating them in liposomal formulations. Hydrophilic drugs, such as Doxorubicin or Acyclovir can be also encapsulated in liposomes at concentrations exceeding their aqueous solubility (275).

The fact that conventional liposomes are rapidly taken by the phagocytic cells can be used to target certain drugs to cells of the immune system. Liposomes have been used to target Amphotericin B, immunomodulators, and immunosuppressors to phagocytic cells (278). Moreover, immunoliposomes can be used to target certain drugs, such as anticancer agents, to particular organs. Liposome-based drug delivery systems can be also used to avoid delivering drugs to certain sites and thereby reduce their toxicity. For example, liposomes were used to reduce the nephrotoxicity of Amphotericin B and the cardiotoxicity of Doxorubicin as they have limited access to kidney and heart (275).

Multivesicular liposomes (MVL) have been studied as a sustained release drug delivery system. IFN α-2b was successfully encapsulated in MVL and slowly released the drug into the systemic circulation (279). Tamoxifen citrate, an anti-estrogen compound, was encapsulated in liposomes that provided 50% of drug release within 3 h and 95% of drug release within 30 h (280).

Liposomal antibiotics have been extensively studied to improve drug delivery, pharmacokinetics and reduce toxicity. Most studies focused on

aminoglycosides, quinolones, polypeptides, and betalactames (281). It is also possible to incorporate more than one drug in liposomes, which would improve patient compliance. Jain *et al.,* were able to design polyelectrolyte-coated multilayered liposomes (nanocapsules) loaded with metronidazole and amoxicillin (247). Another group of researchers was able to incorporate amoxicillin trihydrate and ranitidine bismuth citrate into double liposomes to be used as a dual drug delivery system against *H. pylori* (248).

1.5.5.2 Liposomal delivery of nucleic acid

Cationic liposomes can be used as a delivery system for nucleic acid-based therapies such as antisense oligonucleotides and siRNA. The first report of using liposomes for nucleic acid delivery was in 1982 when Nicoula *et al.,* reported the *in vivo* expression of rat insulin gene following intravenous injection of a plasmid carrying the insulin gene encapsulated in liposomes (282). The main advantages associated with the use of cationic liposomes when compared to viral vectors included the ease of their manufacture, handling and preparation; their ability to deliver large lipid/nucleic acid complexes and their low immunogenic response (283). In addition, cationic liposomes protect the nucleic acid from degradation and increase its stability. Although toxicity was previously reported for cationic liposomes, loading of negatively charged nucleic acid in liposomes causes charge neutralization and reduces their toxicity. The disadvantages that might be seen with liposomes are the high cost of purified lipids and rapid clearance of conventional liposomes. In addition, leakage of encapsulated molecules may occur as a result of temperature change, depending on lipid composition, which may affect the rigidity of liposomes (255).

1.5.5.3 Liposomes as antimicrobial agents

Cationic liposomes have antimicrobial activity against bacteria, fungi and protozoan (284-286). Cationic liposomes may react with phospholipids in cytoplasmic membranes, causing membrane distortion and protoplast lysis under osmotic stress. Moreover, cationic liposomes may be adsorbed on the surface of microbial cells causing a change in overall charge from negative to positive (287).

The positive charge on microbial cells has been linked to the biocidal action as proteins might be aggregated and/or precipitated (285). Liposomes bearing phosphatidylcholine and stearylamine (**Figure 1.10**) killed Leishmania *in vitro* and *in vivo*, without having any adverse effect to the host (286).

Free fatty acids such as lauric acid, myristoleic acid, linoleic acid, and linolenic acid have antibacterial activities against wide range of bacteria including *H. pylori*. Since these lipid molecules are naturally present everywhere, they are less harmful than conventional antibiotics. In addition, free fatty acids may induce drug resistance in *H. pylori* at a much lower rate than conventional antibiotics. Incorporation of free fatty acids into liposomes may have potent antimicrobial effects (288). A recent study has reported antibacterial activity for liposomal linolenic acids against antibiotic-resistant *H. pylori*. The prepared liposomes were able to disrupt membranes of both *H. pylori* spiral and coccoid forms (252).

1.6 Rationale of the study

Aklavik is a small hamlet in the Northwest Territories where the prevalence of *H. pylori* infection is high. *H. pylori* produces several virulence factors that facilitate bacterial colonization and survival in the hostile gastric environment. Characterization of Aklavik *H. pylori* isolates was not done before and so there is a gap in our knowledge regarding the genotypic features of these isolates. Typing of Aklavik *H. pylori* isolates for *cagA*, *cagE,* EPIYA, *vacA* and *babA2* will give insight into the major virulence characteristics of those isolates. In addition, the identification of virulence isolates that are associated with more severe clinical outcomes will help to identify isolates that must be eradicated. Moreover, unique genotypic feaures that are commonly identified among *H. pylori* isolates can be used to target novel treatment approaches. It is controversial whether these virulence genes are associated with gastric biopsy histopathology outcomes, but will be investigated for the Aklavik community.

In Aklavik, NWT, 41% treatment failure was observed with standard triple therapy (214). In fact, first, second and third line antibiotic-based therapies have

been used to treat *H. pylori* infection, but a 100% eradication rate has never been achieved (52). Treatment of *H. pylori* infection may fail due to antimicrobial resistance, (208) re-infection (209), patient compliance and the high cost of currently used drugs (210). New *H. pylori* treatment strategies are needed to provide alternative therapies when standard therapies fail. Treatment targeted specifically to *H. pylori* is preferred to avoid disruption of commensal flora.

Urease is an extracellular enzyme of *H. pylori* that catalyzes the hydrolysis of urea to form carbon dioxide (CO_2) and ammonia (NH_3), which neutralizes the gastric acid, thereby allowing *H. pylori* to grow (289). In the absence of urease, *H. pylori* can only survive in a pH range of 4.0-8.5 (290). A urease-negative mutant of *H. pylori,* constructed by allelic exchange mutagenesis, lacked the ability to colonize the stomach of nude mice, which illustrates the importance of urease for *H. pylori* colonization (291). Similar results were observed for gnotobiotic piglets infected with urease negative *H. pylori* (292). Urease inhibitors, such as acetohydroxamic acid, have been studied for possible use in treating *H. pylori* infections (293). However, strategies to knockout urease gene expression have not been used to interfere with *H. pylori* survival in the stomach.

Liposomes are typically composed of cholesterol and phosphatidylcholine but they may also be composed of synthetic lipids such as DOTAP® to form cationic liposomes. Stearylamine can also impart a positive charge to liposome formulations to facilitate loading with DNA (294). Cationic liposomes have been studied as a possible means to improve the electroporation efficiency of *E. coli*. DOTAP® liposomes successfully induced *E.coli* transformation without electroporation (190). Although, liposomes have been used to deliver antimicrobial agents to *H. pylori* (247,295), it is unknown whether cationic liposomes can enhance the delivery of a plasmid carrying a mutant urease gene into *H. pylori* isolates (including those from Aklavik) to knockout urease expression.

Gene silencing using antisense DNA or short interfering RNA (siRNA) is a rapidly developing field with potential therapeutic applications. Although a *cis*-encoded antisense small RNA that negatively regulates *ureAB* expression was recently discovered in *H. pylori* (198), it is unknown whether gene silencing may

be an effective strategy for urease suppression and thereby provides an alternate strategy for its eradication. Liposome-mediated delivery of siRNAs into eukaryotic cells has been described (296), but no investigations have been carried out in bacterial cells. It is unknown whether cationic liposomes can enhance the delivery of interfering molecules (DNA or RNA) into *H. pylori*.

Cationic liposomes themselves have antimicrobial activity against bacteria, fungi and protozoan (284-286). Cationic liposomes may react with phospholipids in cytoplasmic membranes, causing membrane distortion and protoplast lysis under osmotic stress. Moreover, cationic liposomes may be adsorbed on the surface of microbial cells causing a change in overall charge from negative to positive (287). The positive charge on microbial cells has been linked to the biocidal action, as proteins might be aggregated and/or precipitated (285). Liposomes bearing phosphatidylcholine and stearylamine killed Leishmania *in vitro* and *in vivo*, without having any adverse effect to the host (286). It is unknown whether stearylamine-containing cationic liposomes have antimicrobial activity against *H. pylori*.

Like other infectious diseases, the treatment of *H. pylori* infections, faces a lot of challenges. There is a gap in our standing to the characteristics of *H. pylori* isolates from communities where the prevalence is high, such as Aklavik, NWT. Understanding of the genotypic features of Aklavik *H. pylori* isolates and their association with clinical outcomes may identify isolates that are more virulent and should be eradicated. In addition, characterization of these isolates will help in designing new treatment approaches that are more effective and more targeted to *H. pylori*.

1.6.1 Hypotheses

1) *H. pylori* genotype can predict the histopathology outcomes of infection among residents of Aklavik, NWT.
2) Liposome-mediated delivery of antisense DNA or siRNA against the urease gene of *H. pylori* will specifically disrupt the ability of *H. pylori* isolates

(including those from Aklavik) to produce urease and thereby impair *H. pylori* survival in the stomach.

3) Stearylamine-containing cationic liposomes will inhibit the growth of *H. pylori.*

1.6.2 Objectives

1) To genotype Aklavik *H. pylori* isolates for the major virulence factors and to estimate associations of the findings with gastric histopathology outcomes. (Chapter 2)
2) To design a plasmid carrying a mutant urease gene (Chapter 3)
3) To investigate the ability of DOTAP® liposomes to deliver plasmid DNA carrying a mutant urease gene and to mediate transformation and urease knockout of *H. pylori.* (Chapter 3)
4) To design antisense DNA and siRNAs targeting the *ureAB* genes of an Aklavik *H. pylori* isolate with high urease activity and evaluate their ability to knockdown urease expression in the absence and presence of DOTAP®. (Chapter 3)
5) To modify lipid composition of cationic liposomes to achieve better delivery of the interfering molecules. (Chapter 4)
6) To investigate the antimicrobial activity of stearylamine-containing liposomes against *H. pylori*. (Chapter 4)

Table 1.1 Diagnostic methods for *H. pylori* infections

Type of method	Diagnostic method	Sensitivity (%)	Specificity (%)	Advantages	Limitations	Reference
Non-invasive methods	Urea breath test	95.9 (children) > 90	95.7 (children) > 90	Simple, accurate, safe	Less specificity in children ≤ 6 years	(297) (117)
	Stool antigen test	92.2	94.4	Simple, low cost, no need for new equipment, samples are easy to obtain	Results affected by GIT disorders, bleeding ulcers, PPI and antibiotic treatment	(298)
	Serological tests	82-91	53-70	Cheap, available, good for epidemiological studies	Low accuracy, do not differentiate between active or past infection, local cut off values are needed	(61)

Type of method	Diagnostic method	Sensitivity	Specificity	Advantages	Limitations	Reference
Invasive methods	Histology	90	93	Provides information about disease severity	Subjective assessment results in score variation, sampling error due to patchy growth	(299)
	Culture	> 90	100	High specificity, isolates can be used for antimicrobial susceptibility	Low sensitivity if improper transport medium is used and in bleeding ulcers, slow growth	(300)
	Polymerase chain reaction	95	97	High sensitivity, fast, no special transportation is needed	False negative due to DNA detection of dead bacteria	(301)
	Rapid urea test (RUT)	94	99	Rapid, inexpensive, widely available, highly specific	False negative due to antibiotics or PPI use	(299)

Table 1.2 Prevalence of *H. pylori* in developed countries

Country	Prevalence (%)	Type of Diagnostic Test	Number/ Population	Reference
Canada	23.1 38 7.1	Serology serology UBT-Rapid urease-stool antigen	1306/adults 316/adults 204/children	(75) (80) (81)
USA	32.5	Serology	7465/adults	(82)
Germany	13.3	UBT	195/mixed	(85)
Netherland	24	UBT	2200/mixed	(85)
Israel	44.1	UBT	5941/mixed	(85)
Italy	29.9	UBT	3855/mixed	(85)
Switzerland	26.6	UBT	522/mixed	(85)
Czech Republic	23.5 4.8	UBT	1826/adults 420/children	(84)
Russia	13	Serology	370/children	(86)

Table 1.3 *H. pylori* prevalence in developing and recently industrialized countries

Country	Prevalence (%)	Type of Diagnostic Test	Number/ Population	Reference
Iran	86.8	Histology	303/adults	(302)
	52.2	UBT	113/children	(303)
Turkey	63	Stool antigen test	200/adults	(304)
	68	UBT	1680/children	(305)
Egypt	91.7	Serology	605/mixed	(306)
	72.4	UBT	286/children	(307)
Saudi Arabia	54.9	Histology	488/adults	(308)
	27.4	UBT	314/children	(309)
India	59	Histology, RUT	147/adults	(310)
Mexico	66	Serology	156/adults	(311)
Bangladesh	92	Serology	181/adults	(312)
	50	Stool antigen test	238/children	(87)
China	63.4	UBT	5417/adults	(77)
Japan	51.3	Serology, stool antigen test	11470/adults	(89)
	3.7	Stool antigen test	108/children	(88)
South Korea	59.6	Serology	8020/adults	(90)
Taiwan	54.7	UBT	106/adults	(91)

Table 1.4 Functions of proteins encoded by *cag*PAI (313,314)

Protein	Function
CagA	• cell scattering • cytoskeletal rearrangements • cell elongation • cell proliferation, motility and inflammation
CagE	• Important for delivery of *H. pylori* proteins into the host • induces IL-8 secretion
CagL	• bridges type IV secretion system to integrins on target cells • role in cell signalling to activate kinases required for CagA phosphorylation • activates IL-8 independent of CagA translocation
CagY	• binds to host receptors (integrin β1)
CagI	• binds to host receptors (integrin β1)
CagZ	• essential for CagA translocation but not IL-8 induction
CagM	• role in CagA translocation • activation pf NF-κβ
Cagδ	• essential for CagA secretion and induction of IL-8
CagP	• role in *H. pylori* adherence to gastric epithelial cells
CagF	• interacts with CagA and facilitate translocation • acts as a chaperone protein for CagA in early stages of translocation
CagG	• role in cagA translocation and IL-8 induction • role in adherence to gastric epithelial cells

Table 1.5 Amino acid sequence of different EPIYA motifs

EPIYA motif	Amino acid sequence
EPIYA-A	EPIYA(Q/K)VNKKK(T/A)GQ
EPIYA-B	EPIY(A/T)QVAKKV
EPIYA-C	EPIYATIDDLGGPEPL
EPIYA-D	EPIYATIDFDEANQAG

Table1.6 Functions and clinical significance of *H. pylori* adhesins

Adhesin	Function and clinical significance	Reference
Blood group binding adhesin (BabA)	• mediates *H. pylori* binding to Lewis b antigen • might enhance CagA translocation and induction of inflammation	(315)
Sialic acid binding adhesin (SabA & SabB)	• binds to sialylated glycans sLe^x and sLe^a • maintains binding to gastric epithelial cells in chronic gastritis and gastric carcinoma • induction of phagocytosis	(316)
Adherence associated lipoprotein (AlpA & AlpB)	• role in adhesion to gastric epithelial cells • influences host cell signaling and cytokine production	(317)
Outer inflammatory proteinA (OipA)	• stimulates IL-8 production in presence of *cag*PAI • significantly associated with duodenal ulcers, gastric cancer, high *H. pylori* density and severe inflammation	(318)
H. pylori outer membrane protein (HopZ)	• Role in early phase colonization • Strong selection for HopZ 'on' status	(315)

Table 1.7 Resistance mechanisms and rates for antibiotics commonly used for *H. pylori* treatment

Antibiotic	Mechanisms of resistance	Resistance rate (%)	Country	Reference
Clarithromycin	• Point mutations within the bacterial 23S rRNA (A2142G, A2143G, A2142C, 2143C) • Efflux proteins	8 24 35.6 13.2 37.2 30 23 25 48.2 2.5	Canada Europe Span Ireland China Japan Iran Israel Turkey Brazil	(319) (320) (321) (322) (215) (323) (216) (324) (325) (326)
Metronidazole	• Mutations in *rdxA* gene which encodes for an oxygen insensitive NADPH nitro-reductase • Efflux proteins • Other genes may contribute to metronidazole resistance	20 25 31.5 64 63.9 19	Canada Europe Ireland Iran China Israel	(319) (322) (320) (216) (215) (324) (251)
Amoxicillin	• Multiple point mutations in the gene coding for penicillin binding proteins, PBP1A • Point mutations in genes coding for outer membrane porins	2.5 0.3 12.2	Iran China Israel	(216) (215) (218)
Tetracycline	• Point mutations in 16S rRNA gene (AGA965-967TTC) affect the binding of tetracycline to ribosome • Efflux proteins	0 1.2 2.4	Iran China Israel	(216) (215) (218)

Table 1.8 Vaccine trials for immunization against *H. pylori*

Antigen	Route of administration	Immunological response	Animal experiments	Adjuvant	Human experiments	Ref
Dextran-based glycoconjugate of LPS	intraperitoneal	humoral (IgG)	mice and rabbits	Titanus toxid and Diphtheria toxoid	-	(327)
Multi-epitope	intranasal or intramuscular	cell mediated	C57BL/6 mice	peptide spacer	-	(241)
NAP	intraperitoneal	humoral and cell mediated	mice	measles virus	-	(242)
UreA	intraperitoneal		BALB/c mice	Cholera toxin B	-	(243)
Urease	oral	cell mediated	-	*S. enterica*	Yes	(244)
VacA, CagA, NAP	intramuscular	humoral and cell mediated	-	aluminium hydroxide	Yes	(245)
urease B subunit	oral	humoral (IgG & IgA)	-	heat-labile enterotoxin B	Yes	(246)

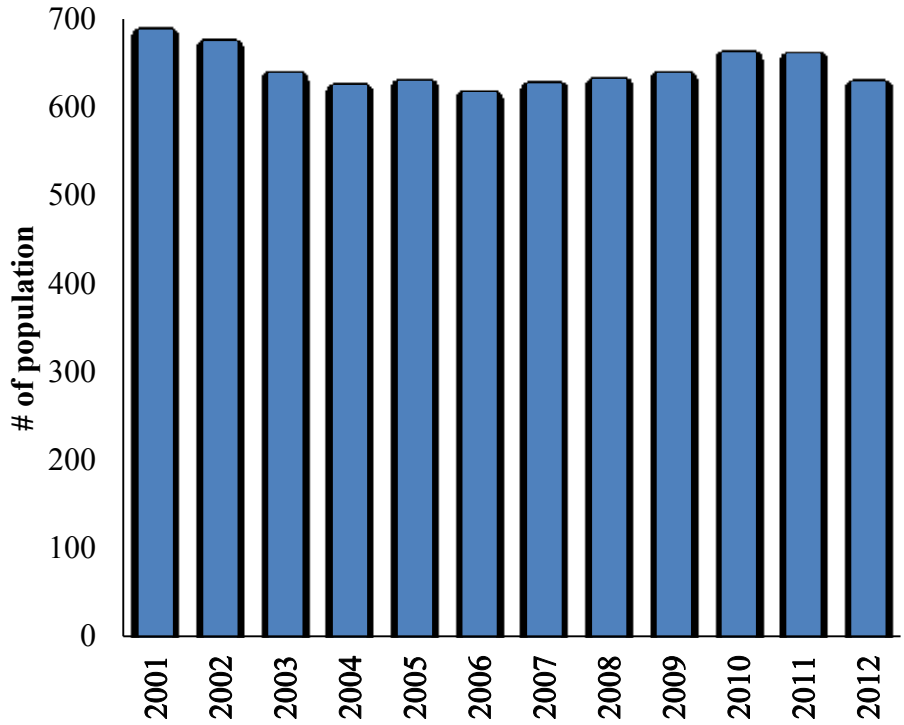

Figure 1.1 Aklavik population size (2001-2012)

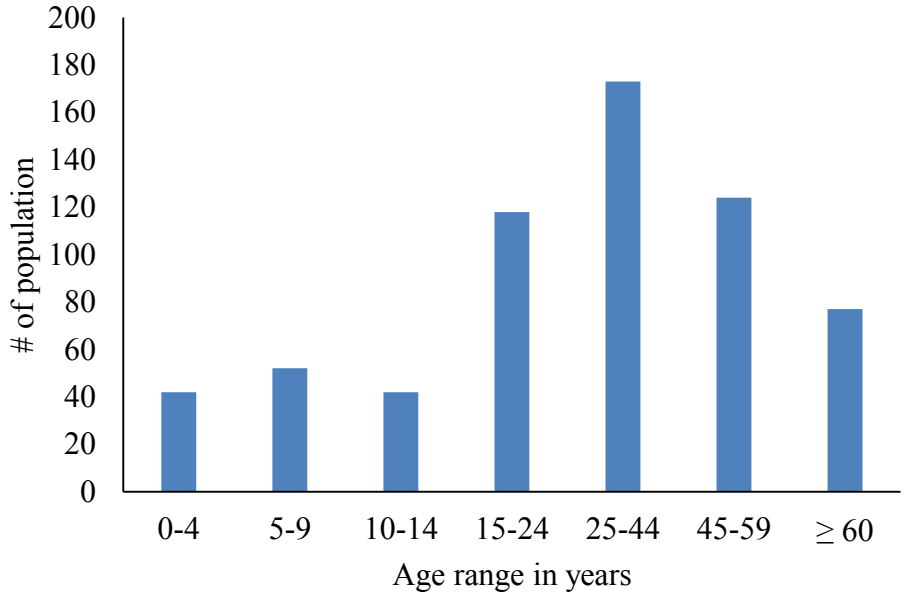

Figure 1.2 Aklavik population age range (2012)

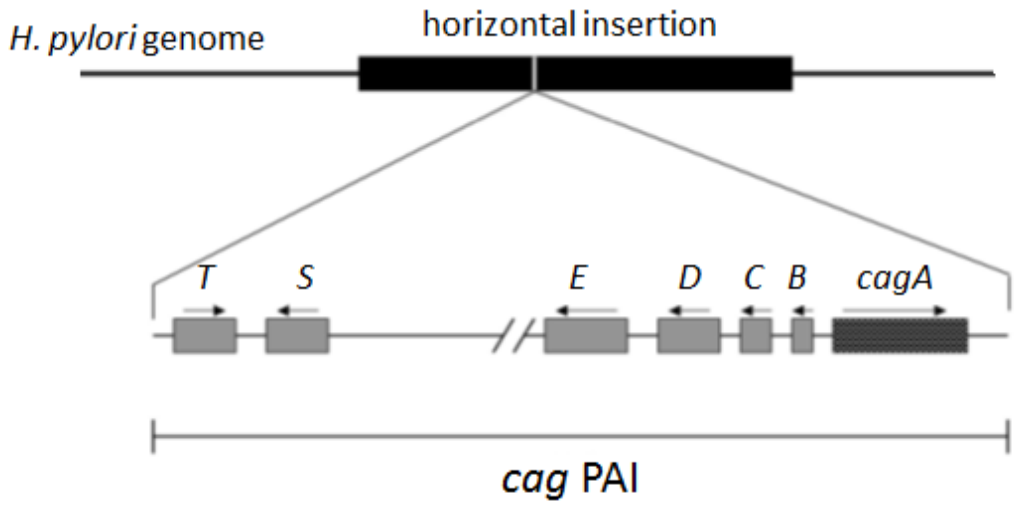

Figure 1.3 *H. pylori cag* pathogenicity island

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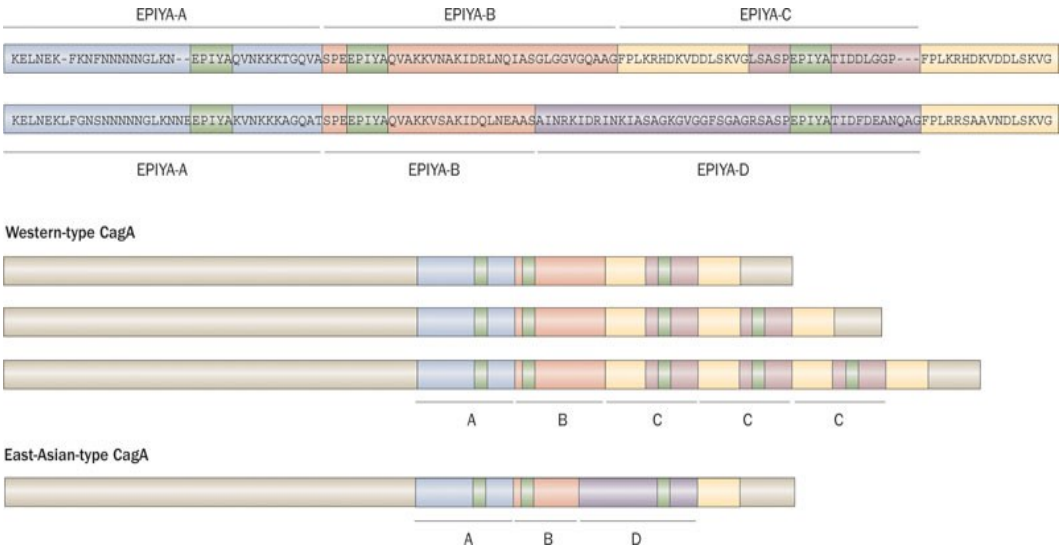

Figure 1.4 Sites of CagA phosphorylation

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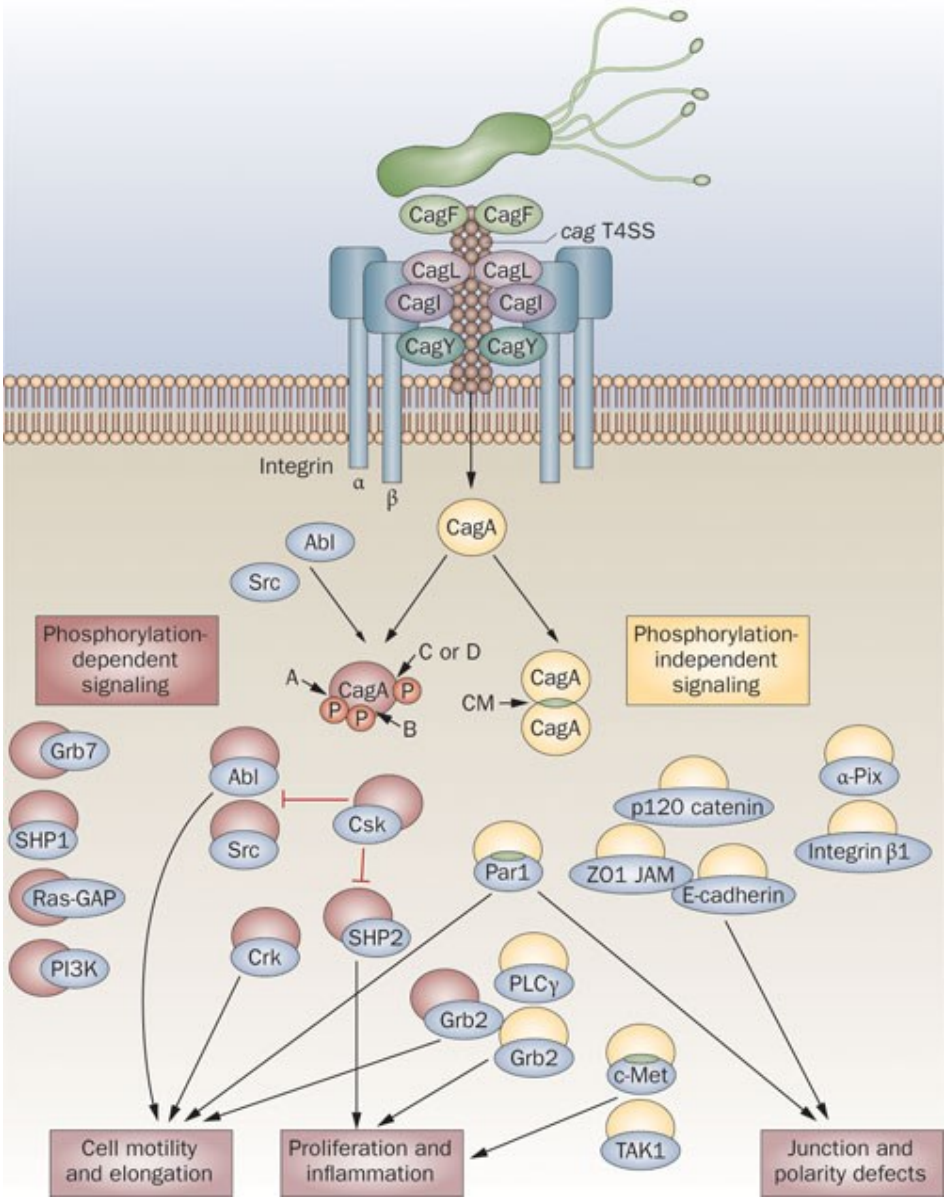

Figure 1.5 Actions of phosphorylated and non-phosphorylated CagA

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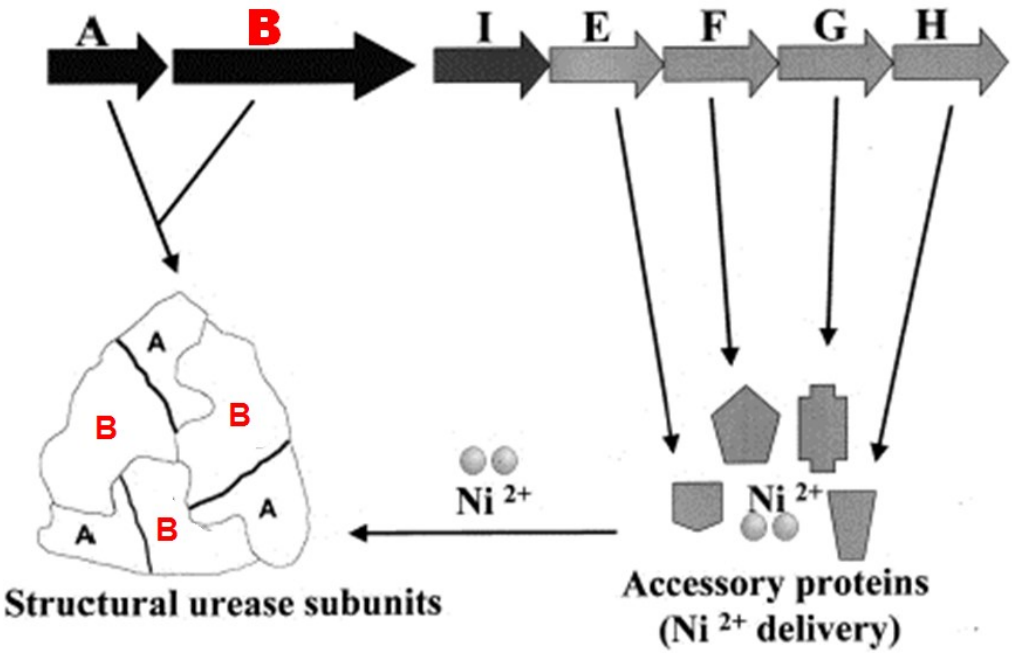

Figure 1.6 Structure of urease gene

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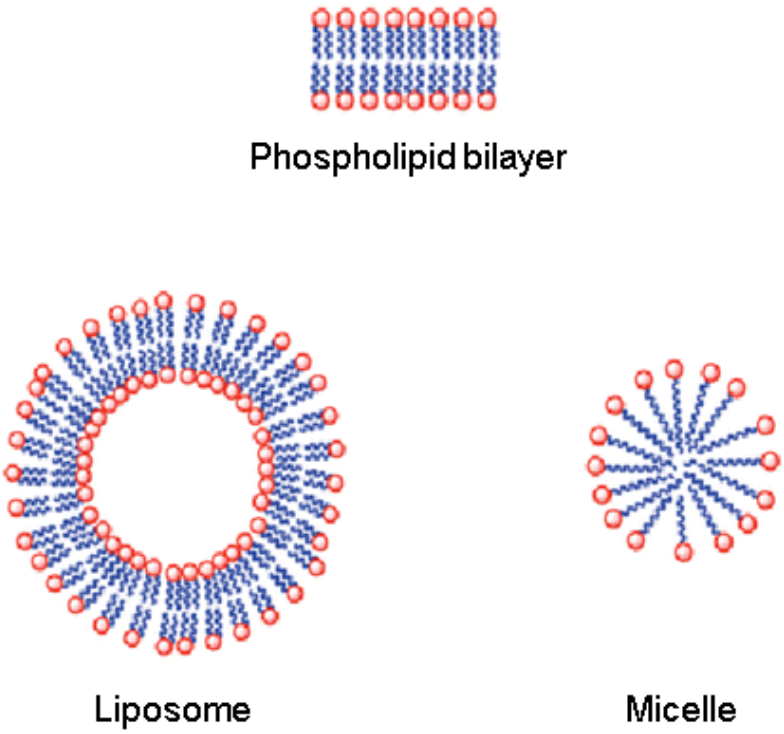

Figure 1.7 Structure of liposomes and micelles

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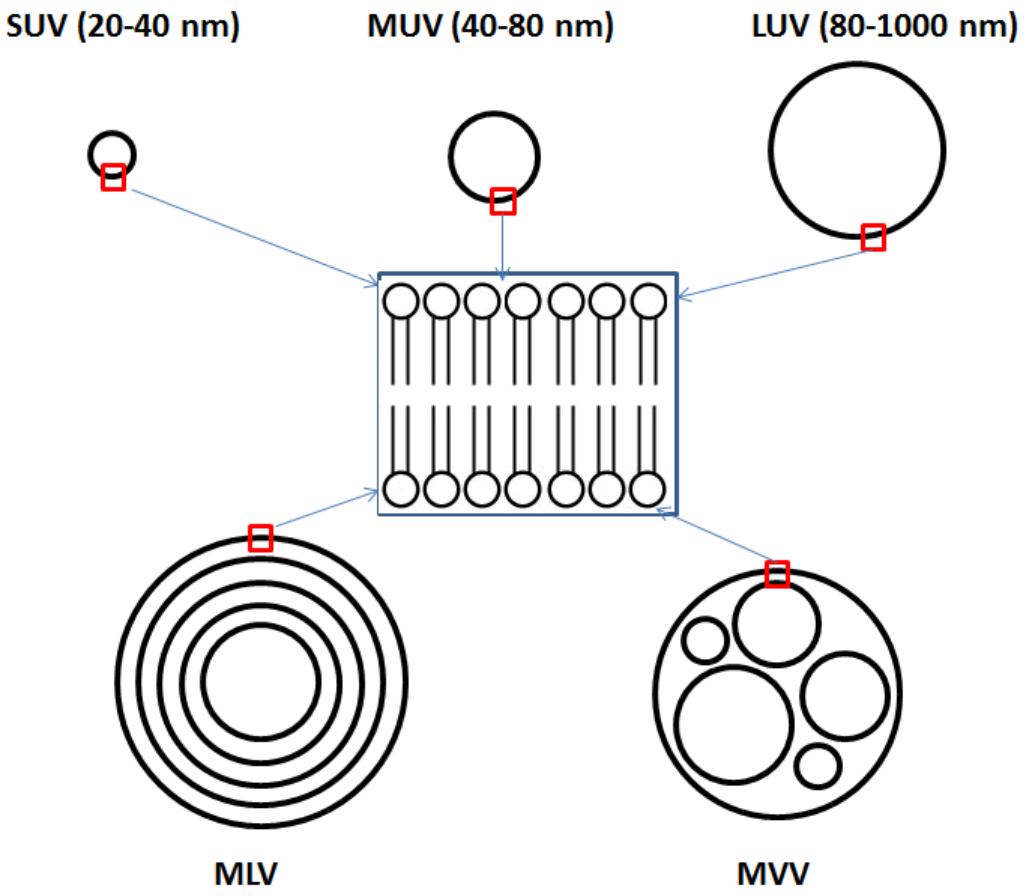

Figure 1.8 Types of liposome structures

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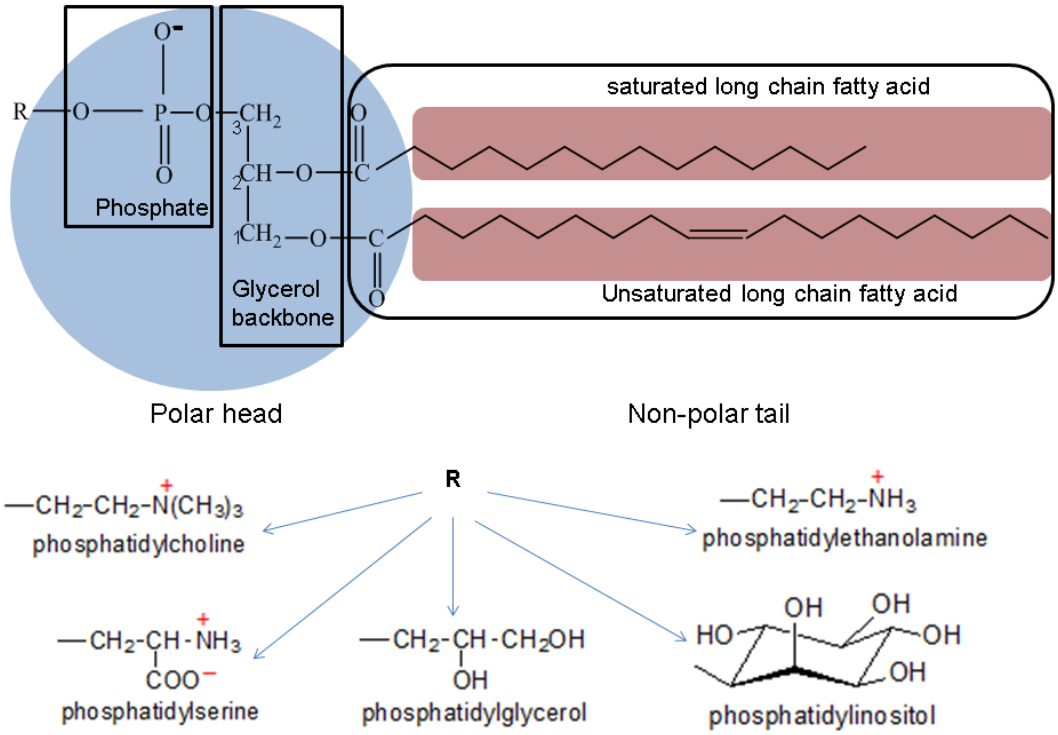
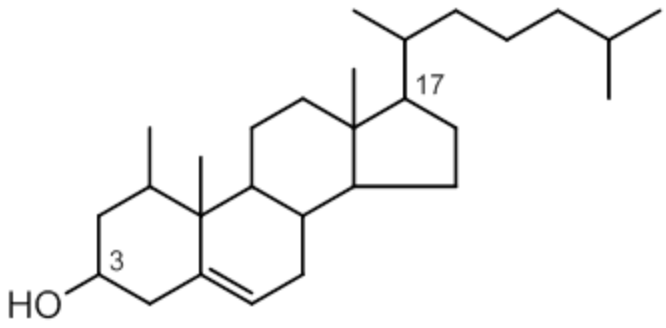
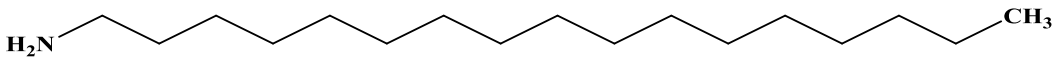


Figure 1.9 Structure of phospholipids



Cholesterol



Stearylamine

Figure 1.10 Structure of cholesterol and stearylamine

1.7 References

(1) Dunn BE, Cohen H, Blaser MJ. *Helicobacter pylori*. Clin Microbiol Rev 1997 Oct;10(4):720-741.

(2) Perez-Perez GI, Blaser MJ. *Campylobacter* and *Helicobacter*. In: Baron S, editor. Medical Microbiology. 4th ed. Galveston (TX): The University of Texas Medical Branch at Galveston; 1996.

(3) Kusters JG, van Vliet AH, Kuipers EJ. Pathogenesis of *Helicobacter pylori* infection. Clin Microbiol Rev 2006 Jul;19(3):449-490.

(4) Yamaoka Y. Pathogenesis of *Helicobacter pylori*-Related Gastroduodenal Diseases from Molecular Epidemiological Studies. Gastroenterol Res Pract 2012;2012:371503.

(5) O'Rourke J, Bode G. Morphology and Ultrastructure. In: Mobley HLT, Mendz GL, Hazell SL, editors. *Helicobacter pylori*: Physiology and Genetics Washington (DC): ASM Press; 2001.

(6) Benaissa M, Babin P, Quellard N, Pezennec L, Cenatiempo Y, Fauchere JL. Changes in *Helicobacter pylori* ultrastructure and antigens during conversion from the bacillary to the coccoid form. Infect Immun 1996 Jun;64(6):2331-2335.

(7) Steer HW. Ultrastructure of cell migration throught the gastric epithelium and its relationship to bacteria. J Clin Pathol 1975 Aug;28(8):639-646.

(8) Steer HW, Colin-Jones DG. Mucosal changes in gastric ulceration and their response to carbenoxolone sodium. Gut 1975 Aug;16(8):590-597.

(9) Ahmed N. 23 years of the discovery of *Helicobacter pylori*: is the debate over? Ann Clin Microbiol Antimicrob 2005 Oct 31;4:17.

(10) Marshall BJ, Armstrong JA, McGechie DB, Glancy RJ. Attempt to fulfil Koch's postulates for pyloric *Campylobacter*. Med J Aust 1985 Apr 15;142(8):436-439.

(11) Marshall BJ, Warren JR. Unidentified curved bacilli in the stomach of patients with gastritis and peptic ulceration. Lancet 1984 Jun 16;1(8390):1311-1315.

(12) Warren JR, Marshall BJ. Unidentified curved bacilli on gastric epithelium in active chronic gastritis. Lancet 1983 Jun 4;1(8336):1273-1275.

(13) Marshall BJ. One Hundred Years of Discovery and Rediscovery of *Helicobacter pylori* and Its Association with Peptic Ulcer Disease. In: Mobley

HLT, Mendz GL, Hazell SL, editors. *Helicobacter pylori*: Physiology and Genetics Washington (DC): ASM Press; 2001.

(14) Konturek JW. Discovery by Jaworski of *Helicobacter pylori* and its pathogenetic role in peptic ulcer, gastritis and gastric cancer. J Physiol Pharmacol 2003 Dec;54 Suppl 3:23-41.

(15) Graham DY, Opekun AR, Osato MS, El-Zimaity HM, Lee CK, Yamaoka Y, et al. Challenge model for *Helicobacter pylori* infection in human volunteers. Gut 2004 Sep;53(9):1235-1243.

(16) Morris A, Nicholson G. Ingestion of *Campylobacter pyloridis* causes gastritis and raised fasting gastric pH. Am J Gastroenterol 1987 Mar;82(3):192-199.

(17) McColl KE, el-Omar E, Gillen D. Interactions between *H. pylori* infection, gastric acid secretion and anti-secretory therapy. Br Med Bull 1998;54(1):121-138.

(18) Kusters JG, van Vliet AH, Kuipers EJ. Pathogenesis of *Helicobacter pylori* infection. Clin Microbiol Rev 2006 Jul;19(3):449-490.

(19) Kuipers EJ, Uyterlinde AM, Pena AS, Hazenberg HJ, Bloemena E, Lindeman J, et al. Increase of *Helicobacter pylori*-associated corpus gastritis during acid suppressive therapy: implications for long-term safety. Am J Gastroenterol 1995 Sep;90(9):1401-1406.

(20) Del Valle J. Peptic Ulcer Disease. In: Longo D, Fauci A, Kasper D, Hauser S, Jameson, JL and Loscalzo, J, editors. Harrison's Principles of Internal Medicine. 18th ed. USA: The McGraw-Hill Companies, Inc.; 2012.

(21) Van Zanten SJ, Dixon MF, Lee A. The gastric transitional zones: neglected links between gastroduodenal pathology and *Helicobacter* ecology. Gastroenterology 1999 May;116(5):1217-1229.

(22) Graham DY. History of *Helicobacter pylori*, duodenal ulcer, gastric ulcer and gastric cancer. World J Gastroenterol 2014 May 14;20(18):5191-5204.

(23) Kuipers EJ, Thijs JC, Festen HP. The prevalence of *Helicobacter pylori* in peptic ulcer disease. Aliment Pharmacol Ther 1995;9 Suppl 2:59-69.

(24) Kuipers EJ, Uyterlinde AM, Pena AS, Roosendaal R, Pals G, Nelis GF, et al. Long-term sequelae of *Helicobacter pylori* gastritis. Lancet 1995 Jun 17;345(8964):1525-1528.

(25) Capelle LG, de Vries AC, Haringsma J, Ter Borg F, de Vries RA, Bruno MJ, et al. The staging of gastritis with the OLGA system by using intestinal metaplasia

as an accurate alternative for atrophic gastritis. Gastrointest Endosc 2010 Jun;71(7):1150-1158.

(26) Filipe MI, Munoz N, Matko I, Kato I, Pompe-Kirn V, Jutersek A, et al. Intestinal metaplasia types and the risk of gastric cancer: a cohort study in Slovenia. Int J Cancer 1994 May 1;57(3):324-329.

(27) Miyaji H, Azuma T, Ito S, Abe Y, Gejyo F, Hashimoto N, et al. *Helicobacter pylori* infection occurs via close contact with infected individuals in early childhood. J Gastroenterol Hepatol 2000 Mar;15(3):257-262.

(28) Percival SL, Thomas JG. Transmission of *Helicobacter pylori* and the role of water and biofilms. J Water Health 2009 Sep;7(3):469-477.

(29) Luzza F, Mancuso M, Imeneo M, Contaldo A, Giancotti L, Pensabene L, et al. Evidence favouring the gastro-oral route in the transmission of *Helicobacter pylori* infection in children. Eur J Gastroenterol Hepatol 2000 Jun;12(6):623-627.

(30) Brown LM. *Helicobacter pylori*: epidemiology and routes of transmission. Epidemiol Rev 2000;22(2):283-297.

(31) Zou QH, Li RQ. *Helicobacter pylori* in the oral cavity and gastric mucosa: a meta-analysis. J Oral Pathol Med 2011 Apr;40(4):317-324.

(32) Burgers R, Schneider-Brachert W, Reischl U, Behr A, Hiller KA, Lehn N, et al. *Helicobacter pylori* in human oral cavity and stomach. Eur J Oral Sci 2008 Aug;116(4):297-304.

(33) Goodman KJ, Correa P, Tengana Aux HJ, Ramirez H, DeLany JP, Guerrero Pepinosa O, et al. *Helicobacter pylori* infection in the Colombian Andes: a population-based study of transmission pathways. Am J Epidemiol 1996 Aug 1;144(3):290-299.

(34) Vale FF, Vitor JM. Transmission pathway of *Helicobacter pylori*: does food play a role in rural and urban areas? Int J Food Microbiol 2010 Mar 31;138(1-2):1-12.

(35) Thomas JE, Gibson GR, Darboe MK, Dale A, Weaver LT. Isolation of *Helicobacter pylori* from human faeces. Lancet 1992 Nov 14;340(8829):1194-1195.

(36) Mapstone NP, Lynch DA, Lewis FA, Axon AT, Tompkins DS, Dixon MF, et al. PCR identification of *Helicobacter pylori* in faeces from gastritis patients. Lancet 1993 Feb 13;341(8842):447.

(37) Dore MP, Osato MS, Malaty HM, Graham DY. Characterization of a culture method to recover *Helicobacter pylori* from the feces of infected patients. Helicobacter 2000 Sep;5(3):165-168.

(38) Falsafi T, Valizadeh N, Najafi M, Ehsani A, Khani A, Landarani Z, et al. Culture of *Helicobacter pylori* from stool samples in children. Can J Microbiol 2007 Mar;53(3):411-416.

(39) Dore MP, Sepulveda AR, El-Zimaity H, Yamaoka Y, Osato MS, Mototsugu K, et al. Isolation of *Helicobacter pylori* from sheep-implications for transmission to humans. Am J Gastroenterol 2001 May;96(5):1396-1401.

(40) Quaglia NC, Dambrosio A, Normanno G, Parisi A, Patrono R, Ranieri G, et al. High occurrence of *Helicobacter pylori* in raw goat, sheep and cow milk inferred by glmM gene: a risk of food-borne infection? Int J Food Microbiol 2008 May 10;124(1):43-47.

(41) Safaei HG, Rahimi E, Zandi A, Rashidipour A. *Helicobacter pylori* as a zoonotic infection: the detection of *H. pylori* antigens in the milk and faeces of cows. J Res Med Sci 2011 Feb;16(2):184-187.

(42) Orozco A, Ogura T, Hirosawa T, Garduno R, Kubo I. In hydrolyzed cow's milk *Helicobacter pylori* becomes nonculturable and the growth of *Salmonella typhi* and *Escherichia coli* is inhibited. J Food Sci 2007 Oct;72(8):M306-9.

(43) Bunn JE, MacKay WG, Thomas JE, Reid DC, Weaver LT. Detection of *Helicobacter pylori* DNA in drinking water biofilms: implications for transmission in early life. Lett Appl Microbiol 2002;34(6):450-454.

(44) Horiuchi T, Ohkusa T, Watanabe M, Kobayashi D, Miwa H, Eishi Y. *Helicobacter pylori* DNA in drinking water in Japan. Microbiol Immunol 2001;45(7):515-519.

(45) Hulten K, Han SW, Enroth H, Klein PD, Opekun AR, Gilman RH, et al. *Helicobacter pylori* in the drinking water in Peru. Gastroenterology 1996 Apr;110(4):1031-1035.

(46) Linke S, Lenz J, Gemein S, Exner M, Gebel J. Detection of *Helicobacter pylori* in biofilms by real-time PCR. Int J Hyg Environ Health 2010 Jun;213(3):176-182.

(47) Adams BL, Bates TC, Oliver JD. Survival of *Helicobacter pylori* in a natural freshwater environment. Appl Environ Microbiol 2003 Dec;69(12):7462-7466.

(48) Fan XG, Chua A, Li TG, Zeng QS. Survival of *Helicobacter pylori* in milk and tap water. J Gastroenterol Hepatol 1998 Nov;13(11):1096-1098.

(49) Vincent P. Transmission and acquisition of *Helicobacter pylori* infection: evidences and hypothesis. Biomed Pharmacother 1995;49(1):11-18.

(50) Lu Y, Redlinger TE, Avitia R, Galindo A, Goodman K. Isolation and genotyping of *Helicobacter pylori* from untreated municipal wastewater. Appl Environ Microbiol 2002 Mar;68(3):1436-1439.

(51) Al-Sulami AA, Al-Edani TA, Al-Abdula AA. Culture Method and PCR for the Detection of *Helicobacter pylori* in Drinking Water in Basrah Governorate Iraq. Gastroenterol Res Pract 2012;2012:245167.

(52) Garza-Gonzalez E, Perez-Perez GI, Maldonado-Garza HJ, Bosques-Padilla FJ. A review of *Helicobacter pylori* diagnosis, treatment, and methods to detect eradication. World J Gastroenterol 2014 Feb 14;20(6):1438-1449.

(53) Morris A, Ali MR, Brown P, Lane M, Patton K. *Campylobacter pylori* infection in biopsy specimens of gastric antrum: laboratory diagnosis and estimation of sampling error. J Clin Pathol 1989 Jul;42(7):727-732.

(54) Bermejo F, Boixeda D, Gisbert JP, Defarges V, Sanz JM, Redondo C, et al. Rapid urease test utility for *Helicobacter pylori* infection diagnosis in gastric ulcer disease. Hepatogastroenterology 2002 Mar-Apr;49(44):572-575.

(55) Megraud F, Lehours P. *Helicobacter pylori* detection and antimicrobial susceptibility testing. Clin Microbiol Rev 2007 Apr;20(2):280-322.

(56) Chomvarin C, Kulsantiwong P, Chantarasuk Y, Chantrakooptungool S, Kanjanahareutai S. Comparison of media and antibiotic supplements for isolation of *Helicobacter pylori* from gastric biopsies. Southeast Asian J Trop Med Public Health 2006 Nov;37(6):1163-1169.

(57) Yin Y, He LH, Zhang JZ. Successful isolation of *Helicobacter pylori* after prolonged incubation from a patient with failed eradication therapy. World J Gastroenterol 2009 Mar 28;15(12):1528-1529.

(58) Andersen, P. and Wadström T. Basic Bacteriology and Culture. In: Mobley HLT, Mendz GL, Hazell SL, editor. *Helicobacter pylori*: Physiology and Genetics. Washington (DC): ASM; 2001.

(59) Rimbara E, Sasatsu M, Graham DY. PCR detection of *Helicobacter pylori* in clinical samples. Methods Mol Biol 2013;943:279-287.

(60) World gastroenterology organisation global guideline: *Helicobacter pylori* in developing countries. J Dig Dis 2011 Oct;12(5):319-326.

(61) Harris P, Perez-Perez G, Zylberberg A, Rollan A, Serrano C, Riera F, et al. Relevance of adjusted cut-off values in commercial serological immunoassays for *Helicobacter pylori* infection in children. Dig Dis Sci 2005 Nov;50(11):2103-2109.

(62) She RC, Wilson AR, Litwin CM. Evaluation of *Helicobacter pylori* Immunoglobulin G (IgG), IgA, and IgM serologic testing compared to stool antigen testing. Clin Vaccine Immunol 2009 Aug;16(8):1253-1255.

(63) Asfeldt AM, Lochen ML, Straume B, Steigen SE, Florholmen J, Goll R, et al. Accuracy of a monoclonal antibody-based stool antigen test in the diagnosis of *Helicobacter pylori* infection. Scand J Gastroenterol 2004 Nov;39(11):1073-1077.

(64) Iranikhah A, Ghadir MR, Sarkeshikian S, Saneian H, Heiari A, Mahvari M. Stool antigen tests for the detection of *Helicobacter pylori* in children. Iran J Pediatr 2013 Apr;23(2):138-142.

(65) Zhou X, Su J, Xu G, Zhang G. Accuracy of stool antigen test for the diagnosis of *Helicobacter pylori* infection in children: A meta-analysis. Clin Res Hepatol Gastroenterol 2014 Mar 10.

(66) Chehter EZ, Bacci MR, Fonseca FL, Goncalves JA, Buchalla G, Shiraichi SA, et al. Diagnosis of the infection by the *Helicobacter pylori* through stool examination: Method standardization in adults. Clin Biochem 2013 Oct;46(15):1622-1624.

(67) Torres J, Perez-Perez G, Goodman KJ, Atherton JC, Gold BD, Harris PR, et al. A comprehensive review of the natural history of *Helicobacter pylori* infection in children. Arch Med Res 2000 Sep-Oct;31(5):431-469.

(68) Clemens J, Albert MJ, Rao M, Huda S, Qadri F, Van Loon FP, et al. Sociodemographic, hygienic and nutritional correlates of *Helicobacter pylori* infection of young Bangladeshi children. Pediatr Infect Dis J 1996 Dec;15(12):1113-1118.

(69) Sarker SA, Mahalanabis D, Hildebrand P, Rahaman MM, Bardhan PK, Fuchs G, et al. *Helicobacter pylori*: prevalence, transmission, and serum pepsinogen II concentrations in children of a poor periurban community in Bangladesh. Clin Infect Dis 1997 Nov;25(5):990-995.

(70) Torres J, Leal-Herrera Y, Perez-Perez G, Gomez A, Camorlinga-Ponce M, Cedillo-Rivera R, et al. A community-based seroepidemiologic study of *Helicobacter pylori* infection in Mexico. J Infect Dis 1998 Oct;178(4):1089-1094.

(71) Ozaydin N, Turkyilmaz SA, Cali S. Prevalence and risk factors of *Helicobacter pylori* in Turkey: a nationally-representative, cross-sectional,

screening with the (1)(3)C-Urea breath test. BMC Public Health 2013 Dec 21;13:1215-2458-13-1215.

(72) Shi R, Xu S, Zhang H, Ding Y, Sun G, Huang X, et al. Prevalence and risk factors for *Helicobacter pylori* infection in Chinese populations. Helicobacter 2008 Apr;13(2):157-165.

(73) Malaty HM, Paykov V, Bykova O, Ross A, Graham DP, Anneger JF, et al. *Helicobacter pylori* and socioeconomic factors in Russia. Helicobacter 1996 Jun;1(2):82-87.

(74) Rothenbacher D, Winkler M, Gonser T, Adler G, Brenner H. Role of infected parents in transmission of *Helicobacter pylori* to their children. Pediatr Infect Dis J 2002 Jul;21(7):674-679.

(75) Naja F, Kreiger N, Sullivan T. *Helicobacter pylori* infection in Ontario: prevalence and risk factors. Can J Gastroenterol 2007 Aug;21(8):501-506.

(76) Hu D, Shao J, Wang L, Zheng H, Xu Y, Song G, et al. Prevalence and risk factors of *Helicobacter pylori* infection in Chinese maritime workers. Ann Hum Biol 2013 Nov-Dec;40(6):472-476.

(77) Zhu Y, Zhou X, Wu J, Su J, Zhang G. Risk Factors and Prevalence of *Helicobacter pylori* Infection in Persistent High Incidence Area of Gastric Carcinoma in Yangzhong City. Gastroenterol Res Pract 2014;2014:481365.

(78) Mhaskar RS, Ricardo I, Azliyati A, Laxminarayan R, Amol B, Santosh W, et al. Assessment of risk factors of *Helicobacter pylori* infection and peptic ulcer disease. J Glob Infect Dis 2013 Apr;5(2):60-67.

(79) Epidemiology of, and risk factors for, *Helicobacter pylori* infection among 3194 asymptomatic subjects in 17 populations. The EUROGAST Study Group. Gut 1993 Dec;34(12):1672-1676.

(80) Veldhuyzen van Zanten SJ, Pollak PT, Best LM, Bezanson GS, Marrie T. Increasing prevalence of *Helicobacter pylori* infection with age: continuous risk of infection in adults rather than cohort effect. J Infect Dis 1994 Feb;169(2):434-437.

(81) Segal I, Otley A, Issenman R, Armstrong D, Espinosa V, Cawdron R, et al. Low prevalence of *Helicobacter pylori* infection in Canadian children: a cross-sectional analysis. Can J Gastroenterol 2008 May;22(5):485-489.

(82) Everhart JE, Kruszon-Moran D, Perez-Perez GI, Tralka TS, McQuillan G. Seroprevalence and ethnic differences in *Helicobacter pylori* infection among adults in the United States. J Infect Dis 2000 Apr;181(4):1359-1363.

(83) Jackman RP, Schlichting C, Carr W, Dubois A. Prevalence of *Helicobacter pylori* in United States Navy submarine crews. Epidemiol Infect 2006 Jun;134(3):460-464.

(84) Bures J, Kopacova M, Koupil I, Seifert B, Skodova Fendrichova M, Spirkova J, et al. Significant decrease in prevalence of *Helicobacter pylori* in the Czech Republic. World J Gastroenterol 2012 Aug 28;18(32):4412-4418.

(85) Schmilovitz-Weiss H, Sehayek-Shabat V, Eliakim R, Skapa E, Avni Y, Shirin H. Applicability of a short/rapid 13C-urea breath test for *Helicobacter pylori*: retrospective multicenter chart review study. BMC Gastroenterol 2012 Jan 19;12:8-230X-12-8.

(86) Tkachenko MA, Zhannat NZ, Erman LV, Blashenkova EL, Isachenko SV, Isachenko OB, et al. Dramatic changes in the prevalence of *Helicobacter pylori* infection during childhood: a 10-year follow-up study in Russia. J Pediatr Gastroenterol Nutr 2007 Oct;45(4):428-432.

(87) Bhuiyan TR, Qadri F, Saha A, Svennerholm AM. Infection by *Helicobacter pylori* in Bangladeshi children from birth to two years: relation to blood group, nutritional status, and seasonality. Pediatr Infect Dis J 2009 Feb;28(2):79-85.

(88) Okuda M, Miyashiro E, Booka M, Tsuji T, Nakazawa T. *Helicobacter pylori* colonization in the first 3 years of life in Japanese children. Helicobacter 2007 Aug;12(4):324-327.

(89) Ueda J, Gosho M, Inui Y, Matsuda T, Sakakibara M, Mabe K, et al. Prevalence of *Helicobacter pylori* infection by birth year and geographic area in Japan. Helicobacter 2014 Apr;19(2):105-110.

(90) Yim JY, Kim N, Choi SH, Kim YS, Cho KR, Kim SS, et al. Seroprevalence of *Helicobacter pylori* in South Korea. Helicobacter 2007 Aug;12(4):333-340.

(91) Chi H, Bair MJ, Wu MS, Chiu NC, Hsiao YC, Chang KY. Prevalence of *Helicobacter pylori* infection in high-school students on Lanyu Island, Taiwan: risk factor analysis and effect on growth. J Formos Med Assoc 2009 Dec;108(12):929-936.

(92) World Health Organization. 2013;
Available at: www.who.int/topics/health_services_indigenous/en/.
Accessed 10/3, 2013.

(93) Bartlett JG, Madariaga-Vignudo L, O'Neil JD, Kuhnlein HV: Identifying indigenous peoples for health research in a global context: a review of perspectives and challenges. Int J Circumpolar Health 2007, 66(4)**:**287-307.

(94) Voyageur, C.J. and Calliou, B. (2000) Various shades of red: Diversity within Canada's Indigenous community London Journal of Canadian Studies, 16, 109-124.

(95) Turner A, Crompton S, and Langlois S. Statistics Canada. 2013; . Accessed 10/3, 2013.

(96) Bernstein CN, McKeown I, Embil JM, Blanchard JF, Dawood M, Kabani A, et al. Seroprevalence of *Helicobacter pylori*, incidence of gastric cancer, and peptic ulcer-associated hospitalizations in a Canadian Indian population. Dig Dis Sci 1999 Apr;44(4):668-674.

(97) McKeown I, Orr P, Macdonald S, Kabani A, Brown R, Coghlan G, et al. *Helicobacter pylori* in the Canadian arctic: seroprevalence and detection in community water samples. Am J Gastroenterol 1999 Jul;94(7):1823-1829.

(98) Cheung J, Goodman K, Munday R, Heavner K, Huntington J, Morse J, et al. *Helicobacter pylori* infection in Canada's arctic: searching for the solutions. Can J Gastroenterol 2008 Nov;22(11):912-916.

(99) Sethi A, Chaudhuri M, Kelly L, Hopman W. Prevalence of *Helicobacter pylori* in a First Nations population in northwestern Ontario. Can Fam Physician 2013 Apr;59(4):e182-7.

(100) Goodman KJ, Jacobson K, Veldhuyzen van Zanten S. *Helicobacter pylori* infection in Canadian and related Arctic Aboriginal populations. Can J Gastroenterol 2008 Mar;22(3):289-295.

(101) Northwest Territories Bureau of Statistics. 2014;
Available at: http://www.statsnwt.ca/community-data/infrastructure/aklavik.html. Accessed 10/6, 2014.

(102) Statistics Canada. Life expectancy, at birth at 65 years by privinces and territories. 2012; Available at: http://www.statcan.gc.ca/tables-tableaux/sum-som/l01/cst01/health72a-eng.htm. Accessed 10/16, 2014.

(103) Department of Health and Social Services, Government of the Northwest Territories. The NWT Health Status Report 2005. 2005.

(104) Salokangas, R. and Parlee, B. The influence of family history on learning opportunities of Inuvialuit youth. Erudit 2009;33:191.

(105) Cheung J, Goodman KJ, Girgis S, Bailey R, Morse J, Fedorak RN, et al. Disease manifestations of *Helicobacter pylori* infection in Arctic Canada: using

epidemiology to address community concerns. BMJ Open 2014 Jan 8;4(1):e003689-2013-003689.

(106) Department of Health and Social Services, Government of the Northwest Territories Cancer in the Northwest Territories, 1990-2000. A descriptive report. 2003.

(107) Parkinson AJ, Gold BD, Bulkow L, Wainwright RB, Swaminathan B, Khanna B, et al. High prevalence of *Helicobacter pylori* in the Alaska native population and association with low serum ferritin levels in young adults. Clin Diagn Lab Immunol 2000 Nov;7(6):885-888.

(108) Colquhoun A, Geary J, Goodman KJ. Challenges in conducting community-driven research created by differing ways of talking and thinking about science: a researcher's perspective. Int J Circumpolar Health 2013 Aug 5;72:10.3402/ijch.v72i0.21232. eCollection 2013.

(109) Valle J, Kekki M, Sipponen P, Ihamaki T, Siurala M. Long-term course and consequences of *Helicobacter pylori* gastritis. Results of a 32-year follow-up study. Scand J Gastroenterol 1996 Jun;31(6):546-550.

(110) Kuipers EJ. *Helicobacter pylori* and the risk and management of associated diseases: gastritis, ulcer disease, atrophic gastritis and gastric cancer. Aliment Pharmacol Ther 1997 Apr;11 Suppl 1:71-88.

(111) Peek RM,Jr, Crabtree JE. *Helicobacter* infection and gastric neoplasia. J Pathol 2006 Jan;208(2):233-248.

(112) Sgouros SN, Bergele C. Clinical outcome of patients with *Helicobacter pylori* infection: the bug, the host, or the environment? Postgrad Med J 2006 May;82(967):338-342.

(113) El-Omar EM, Carrington M, Chow WH, McColl KE, Bream JH, Young HA, et al. Interleukin-1 polymorphisms associated with increased risk of gastric cancer. Nature 2000 Mar 23;404(6776):398-402.

(114) Hatakeyama M. The role of *Helicobacter pylori* CagA in gastric carcinogenesis. Int J Hematol 2006 Nov;84(4):301-308.

(115) Wada A, Yamasaki E, Hirayama T. *Helicobacter pylori* vacuolating cytotoxin, VacA, is responsible for gastric ulceration. J Biochem 2004 Dec;136(6):741-746.

(116) Gerhard M, Lehn N, Neumayer N, Boren T, Rad R, Schepp W, et al. Clinical relevance of the *Helicobacter pylori* gene for blood-group antigen-binding adhesin. Proc Natl Acad Sci U S A 1999 Oct 26;96(22):12778-12783.

(117) Machida-Montani A, Sasazuki S, Inoue M, Natsukawa S, Shaura K, Koizumi Y, et al. Association of *Helicobacter pylori* infection and environmental factors in non-cardia gastric cancer in Japan. Gastric Cancer 2004;7(1):46-53.

(118) Shiota S, Suzuki R, Yamaoka Y. The significance of virulence factors in *Helicobacter pylori*. J Dig Dis 2013 Jul;14(7):341-349.

(119) Censini S, Lange C, Xiang Z, Crabtree JE, Ghiara P, Borodovsky M, et al. cag, a pathogenicity island of *Helicobacter pylori*, encodes type I-specific and disease-associated virulence factors. Proc Natl Acad Sci U S A 1996 Dec 10;93(25):14648-14653.

(120) Xiang Z, Censini S, Bayeli PF, Telford JL, Figura N, Rappuoli R, et al. Analysis of expression of CagA and VacA virulence factors in 43 strains of *Helicobacter pylori* reveals that clinical isolates can be divided into two major types and that CagA is not necessary for expression of the vacuolating cytotoxin. Infect Immun 1995 Jan;63(1):94-98.

(121) Weel JF, van der Hulst RW, Gerrits Y, Roorda P, Feller M, Dankert J, et al. The interrelationship between cytotoxin-associated gene A, vacuolating cytotoxin, and *Helicobacter pylori*-related diseases. J Infect Dis 1996 May;173(5):1171-1175.

(122) Stein M, Bagnoli F, Halenbeck R, Rappuoli R, Fantl WJ, Covacci A. c-Src/Lyn kinases activate *Helicobacter pylori* CagA through tyrosine phosphorylation of the EPIYA motifs. Mol Microbiol 2002 Feb;43(4):971-980.

(123) Stein M, Rappuoli R, Covacci A. Tyrosine phosphorylation of the *Helicobacter pylori* CagA antigen after cag-driven host cell translocation. Proc Natl Acad Sci U S A 2000 Feb 1;97(3):1263-1268.

(124) Higashi H, Yokoyama K, Fujii Y, Ren S, Yuasa H, Saadat I, et al. EPIYA motif is a membrane-targeting signal of *Helicobacter pylori* virulence factor CagA in mammalian cells. J Biol Chem 2005 Jun 17;280(24):23130-23137.

(125) Mueller D, Tegtmeyer N, Brandt S, Yamaoka Y, De Poire E, Sgouras D, et al. c-Src and c-Abl kinases control hierarchic phosphorylation and function of the CagA effector protein in Western and East Asian *Helicobacter pylori* strains. J Clin Invest 2012 Apr 2;122(4):1553-1566.

(126) Jones KR, Joo YM, Jang S, Yoo YJ, Lee HS, Chung IS, et al. Polymorphism in the CagA EPIYA motif impacts development of gastric cancer. J Clin Microbiol 2009 Apr;47(4):959-968.

(127) Azuma T. *Helicobacter pylori* CagA protein variation associated with gastric cancer in Asia. J Gastroenterol 2004;39(2):97-103.

(128) Segal ED, Cha J, Lo J, Falkow S, Tompkins LS. Altered states: involvement of phosphorylated CagA in the induction of host cellular growth changes by *Helicobacter pylori*. Proc Natl Acad Sci U S A 1999 Dec 7;96(25):14559-14564.

(129) Zhou X. SHP-2 tyrosine phosphatase-the target of CagA. Trends Microbiol 2002 Apr;10(4):164.

(130) Suzuki M, Mimuro H, Kiga K, Fukumatsu M, Ishijima N, Morikawa H, et al. *Helicobacter pylori* CagA phosphorylation-independent function in epithelial proliferation and inflammation. Cell Host Microbe 2009 Jan 22;5(1):23-34.

(131) Hatakeyama M. Oncogenic mechanisms of the *Helicobacter pylori* CagA protein. Nat Rev Cancer 2004 Sep;4(9):688-694.

(132) Hatakeyama M. Deregulation of SHP-2 tyrosine phosphatase by the *Helicobacter pylori* virulence factor CagA. Keio J Med 2002 Dec;51 Suppl 2:26-32.

(133) Bentires-Alj M, Paez JG, David FS, Keilhack H, Halmos B, Naoki K, et al. Activating mutations of the noonan syndrome-associated SHP2/*PTPN11* gene in human solid tumors and adult acute myelogenous leukemia. Cancer Res 2004 Dec 15;64(24):8816-8820.

(134) Clyne M, Dolan B, Reeves EP. Bacterial factors that mediate colonization of the stomach and virulence of *Helicobacter pylori*. FEMS Microbiol Lett 2007 Mar;268(2):135-143.

(135) Judd LM, Alderman BM, Howlett M, Shulkes A, Dow C, Moverley J, et al. Gastric cancer development in mice lacking the SHP2 binding site on the IL-6 family co-receptor gp130. Gastroenterology 2004 Jan;126(1):196-207.

(136) Huang JQ, Zheng GF, Sumanac K, Irvine EJ, Hunt RH. Meta-analysis of the relationship between *cagA* seropositivity and gastric cancer. Gastroenterology 2003 Dec;125(6):1636-1644.

(137) Miernyk K, Morris J, Bruden D, McMahon B, Hurlburt D, Sacco F, et al. Characterization of *Helicobacter pylori cagA* and *vacA* genotypes among Alaskans and their correlation with clinical disease. J Clin Microbiol 2011 Sep;49(9):3114-3121.

(138) Radosz-Komoniewska H, Bek T, Jozwiak J, Martirosian G. Pathogenicity of *Helicobacter pylori* infection. Clin Microbiol Infect 2005 Aug;11(8):602-610.

(139) Palframan SL, Kwok T, Gabriel K. Vacuolating cytotoxin A (VacA), a key toxin for *Helicobacter pylori* pathogenesis. Front Cell Infect Microbiol 2012;2:92.

(140) Ogura K, Maeda S, Nakao M, Watanabe T, Tada M, Kyutoku T, et al. Virulence factors of *Helicobacter pylori* responsible for gastric diseases in Mongolian gerbil. J Exp Med 2000 Dec 4;192(11):1601-1610.

(141) Jafari F, Shokrzadeh L, Dabiri H, Baghaei K, Yamaoka Y, Zojaji H, et al. *vacA* genotypes of *Helicobacter pylori* in relation to *cagA* status and clinical outcomes in Iranian populations. Jpn J Infect Dis 2008 Jul;61(4):290-293.

(142) Atherton JC, Cover TL, Twells RJ, Morales MR, Hawkey CJ, Blaser MJ. Simple and accurate PCR-based system for typing vacuolating cytotoxin alleles of *Helicobacter pylori*. J Clin Microbiol 1999 Sep;37(9):2979-2982.

(143) Atherton JC, Cao P, Peek RM,Jr, Tummuru MK, Blaser MJ, Cover TL. Mosaicism in vacuolating cytotoxin alleles of *Helicobacter pylori*. Association of specific *vacA* types with cytotoxin production and peptic ulceration. J Biol Chem 1995 Jul 28;270(30):17771-17777.

(144) Pagliaccia C, de Bernard M, Lupetti P, Ji X, Burroni D, Cover TL, et al. The m2 form of the *Helicobacter pylori* cytotoxin has cell type-specific vacuolating activity. Proc Natl Acad Sci U S A 1998 Aug 18;95(17):10212-10217.

(145) McClain MS, Cao P, Iwamoto H, Vinion-Dubiel AD, Szabo G, Shao Z, et al. A 12-amino-acid segment, present in type s2 but not type s1 *Helicobacter pylori* VacA proteins, abolishes cytotoxin activity and alters membrane channel formation. J Bacteriol 2001 Nov;183(22):6499-6508.

(146) Rhead JL, Letley DP, Mohammadi M, Hussein N, Mohagheghi MA, Eshagh Hosseini M, et al. A new *Helicobacter pylori* vacuolating cytotoxin determinant, the intermediate region, is associated with gastric cancer. Gastroenterology 2007 Sep;133(3):926-936.

(147) Chung C, Olivares A, Torres E, Yilmaz O, Cohen H, Perez-Perez G. Diversity of VacA intermediate region among *Helicobacter pylori* strains from several regions of the world. J Clin Microbiol 2010 Mar;48(3):690-696.

(148) Ogiwara H, Graham DY, Yamaoka Y. *vacA* i-region subtyping. Gastroenterology 2008 Apr;134(4):1267; author reply 1268.

(149) Zhang ZW, Dorrell N, Wren BW, Farthingt MJ. *Helicobacter pylori* adherence to gastric epithelial cells: a role for non-adhesin virulence genes. J Med Microbiol 2002 Jun;51(6):495-502.

(150) Boren T, Falk P, Roth KA, Larson G, Normark S. Attachment of *Helicobacter pylori* to human gastric epithelium mediated by blood group antigens. Science 1993 Dec 17;262(5141):1892-1895.

(151) Rad R, Gerhard M, Lang R, Schoniger M, Rosch T, Schepp W, et al. The *Helicobacter pylori* blood group antigen-binding adhesin facilitates bacterial colonization and augments a nonspecific immune response. J Immunol 2002 Mar 15;168(6):3033-3041.

(152) Wang J, Blanchard TG, Ernst PB. Host Inflammatory Response to Infection. In: Mobley HLT, Mendz GL, Hazell SL, editors. *Helicobacter pylori*: Physiology and Genetics Washington (DC): ASM Press; 2001.

(153) Ilver D, Arnqvist A, Ogren J, Frick IM, Kersulyte D, Incecik ET, et al. *Helicobacter pylori* adhesin binding fucosylated histo-blood group antigens revealed by retagging. Science 1998 Jan 16;279(5349):373-377.

(154) Oriol R, Mollicone R, Cailleau A, Balanzino L, Breton C. Divergent evolution of fucosyltransferase genes from vertebrates, invertebrates, and bacteria. Glycobiology 1999 Apr;9(4):323-334.

(155) Ma B, Simala-Grant JL, Taylor DE. Fucosylation in prokaryotes and eukaryotes. Glycobiology 2006 Dec;16(12):158R-184R.

(156) Appelmelk BJ, Simoons-Smit I, Negrini R, Moran AP, Aspinall GO, Forte JG, et al. Potential role of molecular mimicry between *Helicobacter pylori* lipopolysaccharide and host Lewis blood group antigens in autoimmunity. Infect Immun 1996 Jun;64(6):2031-2040.

(157) Pohl MA, Romero-Gallo J, Guruge JL, Tse DB, Gordon JI, Blaser MJ. Host-dependent Lewis (Le) antigen expression in *Helicobacter pylori* cells recovered from Leb-transgenic mice. J Exp Med 2009 Dec 21;206(13):3061-3072.

(158) Aspholm-Hurtig M, Dailide G, Lahmann M, Kalia A, Ilver D, Roche N, et al. Functional adaptation of BabA, the *H. pylori* ABO blood group antigen binding adhesin. Science 2004 Jul 23;305(5683):519-522.

(159) Benktander J, Angstrom J, Breimer ME, Teneberg S. Redefinition of the carbohydrate binding specificity of *Helicobacter pylori* BabA adhesin. J Biol Chem 2012 Sep 14;287(38):31712-31724.

(160) Alm RA, Bina J, Andrews BM, Doig P, Hancock RE, Trust TJ. Comparative genomics of *Helicobacter pylori*: analysis of the outer membrane protein families. Infect Immun 2000 Jul;68(7):4155-4168.

(161) Torres LE, Melian K, Moreno A, Alonso J, Sabatier CA, Hernandez M, et al. Prevalence of *vacA*, *cagA* and *babA2* genes in Cuban *Helicobacter pylori* isolates. World J Gastroenterol 2009 Jan 14;15(2):204-210.

(162) Mattar R, dos Santos AF, Eisig JN, Rodrigues TN, Silva FM, Lupinacci RM, et al. No correlation of *babA2* with *vacA* and *cagA* genotypes of *Helicobacter pylori* and grading of gastritis from peptic ulcer disease patients in Brazil. Helicobacter 2005 Dec;10(6):601-608.

(163) Mahdavi J, Sonden B, Hurtig M, Olfat FO, Forsberg L, Roche N, et al. *Helicobacter pylori* SabA adhesin in persistent infection and chronic inflammation. Science 2002 Jul 26;297(5581):573-578.

(164) Peck B, Ortkamp M, Diehl KD, Hundt E, Knapp B. Conservation, localization and expression of HopZ, a protein involved in adhesion of *Helicobacter pylori*. Nucleic Acids Res 1999 Aug 15;27(16):3325-3333.

(165) Odenbreit S, Till M, Hofreuter D, Faller G, Haas R. Genetic and functional characterization of the alpAB gene locus essential for the adhesion of *Helicobacter pylori* to human gastric tissue. Mol Microbiol 1999 Mar;31(5):1537-1548.

(166) Bauerfeind P, Garner R, Dunn BE, Mobley HL. Synthesis and activity of *Helicobacter pylori* urease and catalase at low pH. Gut 1997 Jan;40(1):25-30.

(167) Hu LT, Foxall PA, Russell R, Mobley HL. Purification of recombinant *Helicobacter pylori* urease apoenzyme encoded by *ureA* and *ureB*. Infect Immun 1992 Jul;60(7):2657-2666.

(168) Mobley HLT. Urease. In: Mobley HLT, Mendz GL, Hazell SL, editors. *Helicobacter pylori*: Physiology and Genetics Washington (DC): ASM Press; 2001.

(169) Tsujii M, Kawano S, Tsuji S, Fusamoto H, Kamada T, Sato N. Mechanism of gastric mucosal damage induced by ammonia. Gastroenterology 1992 Jun;102(6):1881-1888.

(170) Kuwahara H, Miyamoto Y, Akaike T, Kubota T, Sawa T, Okamoto S, et al. *Helicobacter pylori* urease suppresses bactericidal activity of peroxynitrite via carbon dioxide production. Infect Immun 2000 Aug;68(8):4378-4383.

(171) Mobley HL. The role of *Helicobacter pylori* urease in the pathogenesis of gastritis and peptic ulceration. Aliment Pharmacol Ther 1996 Apr;10 Suppl 1:57-64.

(172) Beswick EJ, Pinchuk IV, Minch K, Suarez G, Sierra JC, Yamaoka Y, et al. The *Helicobacter pylori* urease B subunit binds to CD74 on gastric epithelial cells and induces NF-kappaB activation and interleukin-8 production. Infect Immun 2006 Feb;74(2):1148-1155.

(173) Tsuda M, Karita M, Morshed MG, Okita K, Nakazawa T. A urease-negative mutant of *Helicobacter pylori* constructed by allelic exchange mutagenesis lacks

the ability to colonize the nude mouse stomach. Infect Immun 1994 Aug;62(8):3586-3589.

(174) Eaton KA, Brooks CL, Morgan DR, Krakowka S. Essential role of urease in pathogenesis of gastritis induced by *Helicobacter pylori* in gnotobiotic piglets. Infect Immun 1991 Jul;59(7):2470-2475.

(175) Umamaheshwari RB, Jain NK. Receptor-mediated targeting of lipobeads bearing acetohydroxamic acid for eradication of *Helicobacter pylori*. J Control Release 2004 Sep 14;99(1):27-40.

(176) Rajinikanth PS, Mishra B. Preparation and in vitro characterization of gellan based floating beads of acetohydroxamic acid for eradication of *H. pylori*. Acta Pharm 2007 Dec;57(4):413-427.

(177) Fischer W, Hofreuter D, Haas R. Natural Transformation, Recombination, and Repair. In: Mobley HLT, Mendz GL, Hazell SL, editors. *Helicobacter pylori*: Physiology and Genetics Washington (DC): ASM Press; 2001.

(178) Tsuda M, Karita M, Nakazawa T. Genetic transformation in *Helicobacter pylori*. Microbiol Immunol 1993;37(1):85-89.

(179) Smeets LC, Kusters JG. Natural transformation in *Helicobacter pylori*: DNA transport in an unexpected way. Trends Microbiol 2002 Apr;10(4):159-62; discussion 162.

(180) Rohrer S, Holsten L, Weiss E, Benghezal M, Fischer W, Haas R. Multiple pathways of plasmid DNA transfer in *Helicobacter pylori*. PLoS One 2012;7(9):e45623.

(181) Hofreuter D, Odenbreit S, Henke G, Haas R. Natural competence for DNA transformation in *Helicobacter pylori*: identification and genetic characterization of the comB locus. Mol Microbiol 1998 Jun;28(5):1027-1038.

(182) Terradot L, Bayliss R, Oomen C, Leonard GA, Baron C, Waksman G. Structures of two core subunits of the bacterial type IV secretion system, VirB8 from Brucella suis and ComB10 from *Helicobacter pylori*. Proc Natl Acad Sci U S A 2005 Mar 22;102(12):4596-4601.

(183) Fernandez-Gonzalez E, Backert S. DNA transfer in the gastric pathogen *Helicobacter pylori*. J Gastroenterol 2014 Apr;49(4):594-604.

(184) Kuipers EJ, Israel DA, Kusters JG, Blaser MJ. Evidence for a conjugation-like mechanism of DNA transfer in *Helicobacter pylori*. J Bacteriol 1998 Jun;180(11):2901-2905.

(185) Backert S, Kwok T, Konig W. Conjugative plasmid DNA transfer in *Helicobacter pylori* mediated by chromosomally encoded relaxase and TraG-like proteins. Microbiology 2005 Nov;151(Pt 11):3493-3503.

(186) Miller JF, Dower WJ, Tompkins LS. High-voltage electroporation of bacteria: genetic transformation of *Campylobacter jejuni* with plasmid DNA. Proc Natl Acad Sci U S A 1988 Feb;85(3):856-860.

(187) Segal ED, Tompkins LS. Transformation of *Helicobacter pylori* by electroporation. BioTechniques 1993 Feb;14(2):225-226.

(188) de la Cruz F, Frost LS, Meyer RJ, Zechner EL. Conjugative DNA metabolism in Gram-negative bacteria. FEMS Microbiol Rev 2010 Jan;34(1):18-40.

(189) Wang Y, Roos KP, Taylor DE. Transformation of *Helicobacter pylori* by chromosomal metronidazole resistance and by a plasmid with a selectable chloramphenicol resistance marker. J Gen Microbiol 1993 Oct;139(10):2485-2493.

(190) Kawata Y, Yano S, Kojima H. *Escherichia coli* can be transformed by a liposome-mediated lipofection method. Biosci Biotechnol Biochem 2003 May;67(5):1179-1181.

(191) Vaucheret H, Fagard M. Transcriptional gene silencing in plants: targets, inducers and regulators. Trends Genet 2001 Jan;17(1):29-35.

(192) Han J, Kim D, Morris KV. Promoter-associated RNA is required for RNA-directed transcriptional gene silencing in human cells. Proc Natl Acad Sci U S A 2007 Jul 24;104(30):12422-12427.

(193) Matzke M, Matzke AJ, Kooter JM. RNA: guiding gene silencing. Science 2001 Aug 10;293(5532):1080-1083.

(194) van Vliet AH, Wren BW. New levels of sophistication in the transcriptional landscape of bacteria. Genome Biol 2009;10(8):233.

(195) Brantl S. Regulatory mechanisms employed by *cis*-encoded antisense RNAs. Curr Opin Microbiol 2007 Apr;10(2):102-109.

(196) Brantl S. Antisense-RNA regulation and RNA interference. Biochim Biophys Acta 2002 May 3;1575(1-3):15-25.

(197) Weaver KE. Emerging plasmid-encoded antisense RNA regulated systems. Curr Opin Microbiol 2007 Apr;10(2):110-116.

(198) Wen Y, Feng J, Scott DR, Marcus EA, Sachs G. A *cis*-encoded antisense small RNA regulated by the HP0165-HP0166 two-component system controls expression of ureB in *Helicobacter pylori*. J Bacteriol 2011 Jan;193(1):40-51.

(199) Delihas N. Regulation of gene expression by *trans*-encoded antisense RNAs. Mol Microbiol 1995 Feb;15(3):411-414.

(200) Papenfort K, Vogel J. Regulatory RNA in bacterial pathogens. Cell Host Microbe 2010 Jul 22;8(1):116-127.

(201) Brantl S, Bruckner R. Small regulatory RNAs from low-GC Gram-positive bacteria. RNA Biol 2014 May;11(5):443-456.

(202) Jeon B, Zhang Q. Sensitization of *Campylobacter jejuni* to fluoroquinolone and macrolide antibiotics by antisense inhibition of the CmeABC multidrug efflux transporter. J Antimicrob Chemother 2009 May;63(5):946-948.

(203) Ouattara B, Simard RE, Holley RA, Piette GJ, Begin A. Antibacterial activity of selected fatty acids and essential oils against six meat spoilage organisms. Int J Food Microbiol 1997 Jul 22;37(2-3):155-162.

(204) Patil SD, Sharma R, Srivastava S, Navani NK, Pathania R. Downregulation of *yidC* in *Escherichia coli* by Antisense RNA Expression Results in Sensitization to Antibacterial Essential Oils Eugenol and Carvacrol. PLoS One 2013;8(3):e57370.

(205) Wang H, Meng J, Jia M, Ma X, He G, Yu J, et al. *oprM* as a new target for reversion of multidrug resistance in *Pseudomonas aeruginosa* by antisense phosphorothioate oligodeoxynucleotides. FEMS Immunol Med Microbiol 2010 Dec;60(3):275-282.

(206) Yanagihara K, Tashiro M, Fukuda Y, Ohno H, Higashiyama Y, Miyazaki Y, et al. Effects of short interfering RNA against methicillin-resistant *Staphylococcus aureus* coagulase in vitro and in vivo. J Antimicrob Chemother 2006 Jan;57(1):122-126.

(207) Croxen MA, Ernst PB, Hoffman PS. Antisense RNA modulation of alkyl hydroperoxide reductase levels in *Helicobacter pylori* correlates with organic peroxide toxicity but not infectivity. J Bacteriol 2007 May;189(9):3359-3368.

(208) Meyer JM, Silliman NP, Wang W, Siepman NY, Sugg JE, Morris D, et al. Risk factors for *Helicobacter pylori* resistance in the United States: the surveillance of *H. pylori* antimicrobial resistance partnership (SHARP) study, 1993-1999. Ann Intern Med 2002 Jan 1;136(1):13-24.

(209) Cameron EA, Bell GD, Baldwin L, Powell KU, Williams SG. Long-term study of re-infection following successful eradication of *Helicobacter pylori* infection. Aliment Pharmacol Ther 2006 May 1;23(9):1355-1358.

(210) Al-Eidan FA, McElnay JC, Scott MG, McConnell JB. Management of *Helicobacter pylori* eradication--the influence of structured counselling and follow-up. Br J Clin Pharmacol 2002 Feb;53(2):163-171.

(211) Graham DY, Fischbach L. *Helicobacter pylori* treatment in the era of increasing antibiotic resistance. Gut 2010 Aug;59(8):1143-1153.

(212) Laheij RJ, Rossum LG, Jansen JB, Straatman H, Verbeek AL. Evaluation of treatment regimens to cure *Helicobacter pylori* infection-a meta-analysis. Aliment Pharmacol Ther 1999 Jul;13(7):857-864.

(213) Kadayifci A, Buyukhatipoglu H, Cemil Savas M, Simsek I. Eradication of *Helicobacter pylori* with triple therapy: an epidemiologic analysis of trends in Turkey over 10 years. Clin Ther 2006 Nov;28(11):1960-1966.

(214) Morse AL, Goodman KJ, Munday R, Chang HJ, Morse JW, Keelan M, et al. A randomized controlled trial comparing sequential with triple therapy for *Helicobacter pylori* in an Aboriginal community in the Canadian North. Can J Gastroenterol 2013 Dec;27(12):701-706.

(215) Gao W, Cheng H, Hu F, Li J, Wang L, Yang G, et al. The evolution of *Helicobacter pylori* antibiotics resistance over 10 years in Beijing, China. Helicobacter 2010 Oct;15(5):460-466.

(216) Tomatari, F. Haghi, A. Mohabbati Mobarez, M. Amini, D. Hosseini, and A. Talebi Bezmin Abadi. Resistance to Metronidazole and Clarithromycin in Dyspeptic Patients in Iran. Iranian Red Crescent Medical Journal 2010;12.

(217) Dore MP, Graham DY, Sepulveda AR. Different penicillin-binding protein profiles in amoxicillin-resistant *Helicobacter pylori*. Helicobacter 1999 Sep;4(3):154-161.

(218) Peretz A, Paritsky M, Nasser O, Brodsky D, Glyatman T, Segal S, et al. Resistance of *Helicobacter pylori* to tetracycline, amoxicillin, clarithromycin and metronidazole in Israeli children and adults. J Antibiot (Tokyo) 2014 Apr 30.

(219) Berrutti M, Pellicano R, Astegiano M, Smedile A, Saracco G, Morgando A, et al. *Helicobacter pylori* eradication: metronidazole or tinidazole? Data from Turin, Italy. Minerva Gastroenterol Dietol 2008 Dec;54(4):355-358.

(220) Allmon A, Stevermer JJ. Sequential therapy boosts *H pylori* eradication rates. J Fam Pract 2008 Oct;57(10):651-654.

(221) Vaira D, Zullo A, Hassan C, Fiorini G, Vakil N. Sequential Therapy for *Helicobacter pylori* Eradication: The Time is Now! Therap Adv Gastroenterol 2009 Nov;2(6):317-322.

(222) Chung JW, Jung YK, Kim YJ, Kwon KA, Kim JH, Lee JJ, et al. Ten-day sequential versus triple therapy for *Helicobacter pylori* eradication: a prospective, open-label, randomized trial. J Gastroenterol Hepatol 2012 Nov;27(11):1675-1680.

(223) Zhou L, Zhang J, Chen M, Hou X, Li Z, Song Z, et al. A comparative study of sequential therapy and standard triple therapy for *Helicobacter pylori* infection: a randomized multicenter trial. Am J Gastroenterol 2014 Apr;109(4):535-541.

(224) Kim JS, Ji JS, Choi H, Kim JH. Sequential therapy or triple therapy for *Helicobacter pylori* infection in Asians: systematic review and meta-analysis. Clin Res Hepatol Gastroenterol 2014 Feb;38(1):118-125.

(225) Nasa M, Choksey A, Phadke A, Sawant P. Sequential therapy versus standard triple-drug therapy for *Helicobacter pylori* eradication: a randomized study. Indian J Gastroenterol 2013 Nov;32(6):392-396.

(226) Lahaie R, Farley A, Dallaire C, Archambault A, Fallone CA, Ponich T, et al. Bismuth-based quadruple therapy with bismuth subcitrate, metronidazole, tetracycline and omeprazole in the eradication of *Helicobacter pylori*. Can J Gastroenterol 2001 Sep;15(9):581-585.

(227) Malfertheiner P, Megraud F, O'Morain CA, Atherton J, Axon AT, Bazzoli F, et al. Management of *Helicobacter pylori* infection-the Maastricht IV/ Florence Consensus Report. Gut 2012 May;61(5):646-664.

(228) Malfertheiner P, Bazzoli F, Delchier JC, Celinski K, Giguere M, Riviere M, et al. *Helicobacter pylori* eradication with a capsule containing bismuth subcitrate potassium, metronidazole, and tetracycline given with omeprazole versus clarithromycin-based triple therapy: a randomised, open-label, non-inferiority, phase 3 trial. Lancet 2011 Mar 12;377(9769):905-913.

(229) Venerito M, Krieger T, Ecker T, Leandro G, Malfertheiner P. Meta-analysis of bismuth quadruple therapy versus clarithromycin triple therapy for empiric primary treatment of *Helicobacter pylori* infection. Digestion 2013;88(1):33-45.

(230) Corthesy-Theulaz I, Porta N, Glauser M, Saraga E, Vaney AC, Haas R, et al. Oral immunization with *Helicobacter pylori* urease B subunit as a treatment against Helicobacter infection in mice. Gastroenterology 1995 Jul;109(1):115-121.

(231) Michetti P, Corthesy-Theulaz I, Davin C, Haas R, Vaney AC, Heitz M, et al. Immunization of BALB/c mice against Helicobacter felis infection with *Helicobacter pylori* urease. Gastroenterology 1994 Oct;107(4):1002-1011.

(232) Zhang S, Moise L, Moss SF. *H. pylori* vaccines: why we still don't have any. Hum Vaccin 2011 Nov;7(11):1153-1157.

(233) Bogstedt AK, Nava S, Wadstrom T, Hammarstrom L. *Helicobacter pylori* infections in IgA deficiency: lack of role for the secretory immune system. Clin Exp Immunol 1996 Aug;105(2):202-204.

(234) Akhiani AA, Stensson A, Schon K, Lycke NY. IgA antibodies impair resistance against *Helicobacter pylori* infection: studies on immune evasion in IL-10-deficient mice. J Immunol 2005 Jun 15;174(12):8144-8153.

(235) Koch M, Meyer TF, Moss SF. Inflammation, immunity, vaccines for *Helicobacter pylori* infection. Helicobacter 2013 Sep;18 Suppl 1:18-23.

(236) Salama NR, Hartung ML, Muller A. Life in the human stomach: persistence strategies of the bacterial pathogen *Helicobacter pylori*. Nat Rev Microbiol 2013 Jun;11(6):385-399.

(237) Mori J, Vranac T, Smrekar B, Cernilec M, Serbec VC, Horvat S, et al. Chimeric flagellin as the self-adjuvanting antigen for the activation of immune response against *Helicobacter pylori*. Vaccine 2012 Aug 31;30(40):5856-5863.

(238) Nilsson CL, Larsson T, Gustafsson E, Karlsson KA, Davidsson P. Identification of protein vaccine candidates from *Helicobacter pylori* using a preparative two-dimensional electrophoretic procedure and mass spectrometry. Anal Chem 2000 May 1;72(9):2148-2153.

(239) Rossi G, Ruggiero P, Peppoloni S, Pancotto L, Fortuna D, Lauretti L, et al. Therapeutic vaccination against *Helicobacter pylori* in the beagle dog experimental model: safety, immunogenicity, and efficacy. Infect Immun 2004 Jun;72(6):3252-3259.

(240) Chen J, Lin L, Li N, She F. Enhancement of *Helicobacter pylori* outer inflammatory protein DNA vaccine efficacy by co-delivery of interleukin-2 and B subunit heat-labile toxin gene encoded plasmids. Microbiol Immunol 2012 Feb;56(2):85-92.

(241) Moss SF, Moise L, Lee DS, Kim W, Zhang S, Lee J, et al. HelicoVax: epitope-based therapeutic *Helicobacter pylori* vaccination in a mouse model. Vaccine 2011 Mar 3;29(11):2085-2091.

(242) Iankov ID, Haralambieva IH, Galanis E. Immunogenicity of attenuated measles virus engineered to express *Helicobacter pylori* neutrophil-activating protein. Vaccine 2011 Feb 11;29(8):1710-1720.

(243) Guo L, Li X, Tang F, He Y, Xing Y, Deng X, et al. Immunological features and the ability of inhibitory effects on enzymatic activity of an epitope vaccine composed of cholera toxin B subunit and B cell epitope from *Helicobacter pylori* urease A subunit. Appl Microbiol Biotechnol 2012 Mar;93(5):1937-1945.

(244) Aebischer T, Bumann D, Epple HJ, Metzger W, Schneider T, Cherepnev G, et al. Correlation of T cell response and bacterial clearance in human volunteers challenged with *Helicobacter pylori* revealed by randomised controlled vaccination with Ty21a-based *Salmonella* vaccines. Gut 2008 Aug;57(8):1065-1072.

(245) Malfertheiner P, Schultze V, Rosenkranz B, Kaufmann SH, Ulrichs T, Novicki D, et al. Safety and immunogenicity of an intramuscular *Helicobacter pylori* vaccine in noninfected volunteers: a phase I study. Gastroenterology 2008 Sep;135(3):787-795.

(246) Zeng M, Mao XH, Li JX, Tong WD, Wang B, Zhang YJ, et al. Efficacy, safety, and immunogenicity of an oral recombinant *Helicobacter pylori* vaccine in children in China: a randomised, double-blind, placebo-controlled, phase 3 trial. Lancet 2015 Oct 10;386(10002):1457-1464.

(247) Jain P, Jain S, Prasad KN, Jain SK, Vyas SP. Polyelectrolyte coated multilayered liposomes (nanocapsules) for the treatment of *Helicobacter pylori* infection. Mol Pharm 2009 Mar-Apr;6(2):593-603.

(248) Singh DY, Prasad NK. Double liposomes mediated dual drug targeting for treatment of *Helicobacter pylori* infections. Pharmazie 2011 May;66(5):368-373.

(249) Jain AK, Jain SK. Development and characterization of nanolipobeads-based dual drug delivery system for *H. pylori* targeting. J Drug Target 2013 Jul;21(6):593-603.

(250) Bardonnet PL, Faivre V, Boullanger P, Piffaretti JC, Falson F. Pre-formulation of liposomes against *Helicobacter pylori*: characterization and interaction with the bacteria. Eur J Pharm Biopharm 2008 Aug;69(3):908-922.

(251) Francesco VD, Zullo A, Hassan C, Giorgio F, Rosania R, Ierardi E. Mechanisms of *Helicobacter pylori* antibiotic resistance: An updated appraisal. World J Gastrointest Pathophysiol 2011 Jun 15;2(3):35-41.

(252) Obonyo M, Zhang L, Thamphiwatana S, Pornpattananangkul D, Fu V, Zhang L. Antibacterial activities of liposomal linolenic acids against antibiotic-resistant *Helicobacter pylori*. Mol Pharm 2012 Sep 4;9(9):2677-2685.

(253) Bangham AD, Standish MM, Watkins JC. Diffusion of univalent ions across the lamellae of swollen phospholipids. J Mol Biol 1965 Aug;13(1):238-252.

(254) Mufamadi MS, Pillay V, Choonara YE, Du Toit LC, Modi G, Naidoo D, et al. A review on composite liposomal technologies for specialized drug delivery. J Drug Deliv 2011;2011:939851.

(255) Shashi K., Satinder K., and Bharat P. A complete review on: Liposomes. International Research Journal of Pharmacy 2012;3(7):10-16.

(256) Storm, G. and Crommelin, D. J.A. Liposomes: quo vadis? research focus 1998;1(1):19.

(257) Allen TM, Hansen C. Pharmacokinetics of stealth versus conventional liposomes: effect of dose. Biochim Biophys Acta 1991 Sep 30;1068(2):133-141.

(258) Maruyama K. PEG-immunoliposome. Biosci Rep 2002 Apr;22(2):251-266.

(259) Caracciolo G, Amenitsch H. Cationic liposome/DNA complexes: from structure to interactions with cellular membranes. Eur Biophys J 2012 Oct;41(10):815-829.

(260) Wang D, Jing NH, Lin QS. Stearylamine liposome as a new efficient reagent for DNA transfection of eukaryotic cells. Biochem Biophys Res Commun 1996 Sep 13;226(2):450-455.

(261) Kent C. Eukaryotic phospholipid biosynthesis. Annu Rev Biochem 1995;64:315-343.

(262) Dufourc EJ. Sterols and membrane dynamics. J Chem Biol 2008 Nov;1(1-4):63-77.

(263) Coderch L, Fonollosa J, De Pera M, Estelrich J, De La Maza A, Parra JL. Influence of cholesterol on liposome fluidity by EPR. Relationship with percutaneous absorption. J Control Release 2000 Jul 31;68(1):85-95.

(264) Pomorski T, Hrafnsdottir S, Devaux PF, van Meer G. Lipid distribution and transport across cellular membranes. Semin Cell Dev Biol 2001 Apr;12(2):139-148.

(265) Cevc G editor. Phospholipids Handbook. New York, USA: Marcel Dekker Inc.; 1993.

(266) Simons K, Sampaio JL. Membrane organization and lipid rafts. Cold Spring Harb Perspect Biol 2011 Oct 1;3(10):a004697.

(267) van Meer G, de Kroon AI. Lipid map of the mammalian cell. J Cell Sci 2011 Jan 1;124(Pt 1):5-8.

(268) O'Toole PW, Clyne M. Cell Envelope. In: Mobley HLT, Mendz GL, Hazell SL, editors. *Helicobacter pylori*: Physiology and Genetics Washington (DC): ASM Press; 2001.

(269) Haque M, Hirai Y, Yokota K, Mori N, Jahan I, Ito H, et al. Lipid profile of Helicobacter spp.: presence of cholesteryl glucoside as a characteristic feature. J Bacteriol 1996 Apr;178(7):2065-2070.

(270) Dua J.S., Rana A. C. and Bhandari A. K. Liposome: methods of preparation and applications. International Journal of Pharmaceutical Studies and Research 2012;3(2).

(271) Lasch J., Weissig V. and Brandle M. Preparation of liposomes. In: Torchilin V. P., Weissig V., editor. Liposomes. second ed. UK: Oxford University Press; 2003. p. 3.

(272) Traikia M, Warschawski DE, Recouvreur M, Cartaud J, Devaux PF. Formation of unilamellar vesicles by repetitive freeze-thaw cycles: characterization by electron microscopy and 31P-nuclear magnetic resonance. Eur Biophys J 2000;29(3):184-195.

(273) Olson F, Hunt CA, Szoka FC, Vail WJ, Papahadjopoulos D. Preparation of liposomes of defined size distribution by extrusion through polycarbonate membranes. Biochim Biophys Acta 1979 Oct 19;557(1):9-23.

(274) Barenholz, Y. and Lasic, D.D editor. Handbook of Nonmedical Applications of Liposomes from design to microreactors. United States of America: CRC Press Inc.; 1996.

(275) Akbarzadeh A, Rezaei-Sadabady R, Davaran S, Joo SW, Zarghami N, Hanifehpour Y, et al. Liposome: classification, preparation, and applications. Nanoscale Res Lett 2013 Feb 22;8(1):102-276X-8-102.

(276) Bhai S. A., Yadav V.,Mamatha Y. and Prasanth V.V. Liposomes: an overview. Journal of pharmaceutical and scientific innovation 2012;1(1).

(277) Nii T, Ishii F. Encapsulation efficiency of water-soluble and insoluble drugs in liposomes prepared by the microencapsulation vesicle method. Int J Pharm 2005 Jul 14;298(1):198-205.

(278) Kelly C, Jefferies C, Cryan SA. Targeted liposomal drug delivery to monocytes and macrophages. J Drug Deliv 2011;2011:727241.

(279) Qiu J, Wei XH, Geng F, Liu R, Zhang JW, Xu YH. Multivesicular liposome formulations for the sustained delivery of interferon alpha-2b. Acta Pharmacol Sin 2005 Nov;26(11):1395-1401.

(280) Layek B, Mukherjee B. Tamoxifen citrate encapsulated sustained release liposomes: preparation and evaluation of physicochemical properties. Sci Pharm 2010;78(3):507-515.

(281) Drulis-Kawa Z, Dorotkiewicz-Jach A. Liposomes as delivery systems for antibiotics. Int J Pharm 2010 Mar 15;387(1-2):187-198.

(282) Nicolau C, Le Pape A, Soriano P, Fargette F, Muh JP, Juhel MF. In vivo transfer and expression of the liposome encapsulated rat insulin gene. Prog Clin Biol Res 1982;102 pt A:321-330.

(283) Karmali PP, Chaudhuri A. Cationic liposomes as non-viral carriers of gene medicines: resolved issues, open questions, and future promises. Med Res Rev 2007 Sep;27(5):696-722.

(284) Campanha MT, Mamizuka EM, Carmona-Ribeiro AM. Interactions between cationic liposomes and bacteria: the physical-chemistry of the bactericidal action. J Lipid Res 1999 Aug;40(8):1495-1500.

(285) Vieira DB, Carmona-Ribeiro AM. Cationic lipids and surfactants as antifungal agents: mode of action. J Antimicrob Chemother 2006 Oct;58(4):760-767.

(286) Banerjee A, Roychoudhury J, Ali N. Stearylamine-bearing cationic liposomes kill Leishmania parasites through surface exposed negatively charged phosphatidylserine. J Antimicrob Chemother 2008 Jan;61(1):103-110.

(287) Kanazawa A, Ikeda T, Endo T. A novel approach to mode of action of cationic biocides: morphological effect on antibacterial activity. J Appl Bacteriol 1995 Jan;78(1):55-60.

(288) Desbois AP, Smith VJ. Antibacterial free fatty acids: activities, mechanisms of action and biotechnological potential. Appl Microbiol Biotechnol 2010 Feb;85(6):1629-1642.

(289) Marshall BJ, Barrett LJ, Prakash C, McCallum RW, Guerrant RL. Urea protects *Helicobacter (Campylobacter) pylori* from the bactericidal effect of acid. Gastroenterology 1990 Sep;99(3):697-702.

(290) Kaijser B, Falen EMarshall BJ, Barrett LJ, Prakash C, et al. Protection of *Campylobacter pyloridis* (CP) but not *Campylobacter jejuni* (CJ) against acid susceptibility by urea. In: Kaijser B, Falen E, eds. *Campylobacter* IV. Proceedings of the 4th International Workshop on *Campylobacter* Infections. Goteborg: University of Goteborg, 1988:402–3.

(291) Tsuda M, Karita M, Morshed MG, Okita K, Nakazawa T. A urease-negative mutant of *Helicobacter pylori* constructed by allelic exchange mutagenesis lacks the ability to colonize the nude mouse stomach. Infect Immun 1994 Aug;62(8):3586-3589.

(292) Eaton KA, Brooks CL, Morgan DR, Krakowka S. Essential role of urease in pathogenesis of gastritis induced by *Helicobacter pylori* in gnotobiotic piglets. Infect Immun 1991 Jul;59(7):2470-2475.

(293) Kosikowska P, Berlicki L. Urease inhibitors as potential drugs for gastric and urinary tract infections: a patent review. Expert Opin Ther Pat 2011 Apr 4.

(294) Wang D, Jing NH, Lin QS. Stearylamine liposome as a new efficient reagent for DNA transfection of eukaryotic cells. Biochem Biophys Res Commun 1996 Sep 13;226(2):450-455.

(295) Bardonnet PL, Faivre V, Boullanger P, Piffaretti JC, Falson F. Pre-formulation of liposomes against *Helicobacter pylori*: characterization and interaction with the bacteria. Eur J Pharm Biopharm 2008 Aug;69(3):908-922.

(296) Ladewig K, Niebert M, Xu ZP, Gray PP, Lu GQ. Efficient siRNA delivery to mammalian cells using layered double hydroxide nanoparticles. Biomaterials 2010 Mar;31(7):1821-1829.

(297) Leal YA, Flores LL, Fuentes-Panana EM, Cedillo-Rivera R, Torres J. 13C-urea breath test for the diagnosis of *Helicobacter pylori* infection in children: a systematic review and meta-analysis. Helicobacter 2011 Aug;16(4):327-337.

(298) Korkmaz H, Kesli R, Karabagli P, Terzi Y. Comparison of the diagnostic accuracy of five different stool antigen tests for the diagnosis of *Helicobacter pylori* infection. Helicobacter 2013 Oct;18(5):384-391.

(299) Calvet X, Sanchez-Delgado J, Montserrat A, Lario S, Ramirez-Lazaro MJ, Quesada M, et al. Accuracy of diagnostic tests for *Helicobacter pylori*: a reappraisal. Clin Infect Dis 2009 May 15;48(10):1385-1391.

(300) Hirschl AM, Makristathis A. Methods to detect *Helicobacter pylori*: from culture to molecular biology. Helicobacter 2007 Nov;12 Suppl 2:6-11.

(301) Ottiwet O, Chomvarin C, Chaicumpar K, Namwat W, Mairiang P. Nested polymerase chain reaction for detection of *Helicobacter pylori* in gastric biopsy specimens. Southeast Asian J Trop Med Public Health 2010 Nov;41(6):1423-1431.

(302) Shokrzadeh L, Baghaei K, Yamaoka Y, Shiota S, Mirsattari D, Porhoseingholi, A.and Reza Zali, M. Prevalence of *Helicobacter pylori* infection in dyspeptic patients in Iran. Gastroenterology insights 2012;4(1).

(303) Dehghani SM, Karamifar H, Raeesi T, Haghighat M. Growth parameters in children with dyspepsia symptoms and *Helicobacter pylori* infection. Indian Pediatr 2013 Mar;50(3):324-326.

(304) Yucel T, Aygin D, Sen S, Yucel O. The prevalence of *Helicobacter pylori* and related factors among university students in Turkey. Jpn J Infect Dis 2008 May;61(3):179-183.

(305) Seyda T, Derya C, Fusun A, Meliha K. The relationship of *Helicobacter pylori* positivity with age, sex, and ABO/Rhesus blood groups in patients with gastrointestinal complaints in Turkey. Helicobacter 2007 Jun;12(3):244-250.

(306) El Dine SS, Mubarak M, Salama R, El Raziky M, El Sherbiny, E. and others. Low seroprevalence of anti-CagA antibodies Inspite of high seroprevalence of anti-*H pylori* antibodies in rural Egyptian community. . Res J Med Med Sci. 2008;3:118.

(307) Mohammad MA, Hussein L, Coward A, Jackson SJ. Prevalence of *Helicobacter pylori* infection among Egyptian children: impact of social background and effect on growth. Public Health Nutr 2008 Mar;11(3):230-236.

(308) Ayoola AE, Ageely HM, Gadour MO, Pathak VP. Prevalence of *Helicobacter pylori* infection among patients with dyspepsia in South-Western Saudi Arabia. Saudi Med J 2004 Oct;25(10):1433-1438.

(309) Telmesani AM. *Helicobacter pylori*: prevalence and relationship with abdominal pain in school children in Makkah City, western Saudi Arabia. Saudi J Gastroenterol 2009 Apr;15(2):100-103.

(310) Singh V, Trikha B, Nain CK, Singh K, Vaiphei K. Epidemiology of *Helicobacter pylori* and peptic ulcer in India. J Gastroenterol Hepatol 2002 Jun;17(6):659-665.

(311) Alvarado-Esquivel C. Seroepidemiology of *Helicobacter pylori* Infection in Tepehuanos Aged 15 Years and Older in Durango, Mexico. J Pathog 2013;2013:243246.

(312) Ahmad MM, Rahman M, Rumi AK, Islam S, Huq F, Chowdhury MF, et al. Prevalence of *Helicobacter pylori* in asymptomatic population-a pilot serological study in Bangladesh. J Epidemiol 1997 Dec;7(4):251-254.

(313) Tegtmeyer N, Wessler S, Backert S. Role of the cag-pathogenicity island encoded type IV secretion system in *Helicobacter pylori* pathogenesis. FEBS J 2011 Apr;278(8):1190-1202.

(314) Cendron L, Zanotti G. Structural and functional aspects of unique type IV secretory components in the *Helicobacter pylori* cag-pathogenicity island. FEBS J 2011 Apr;278(8):1223-1231.

(315) Posselt G, Backert S, Wessler S. The functional interplay of *Helicobacter pylori* factors with gastric epithelial cells induces a multi-step process in pathogenesis. Cell Commun Signal 2013 Oct 7;11:77-811X-11-77.

(316) Yamaoka Y. Increasing evidence of the role of *Helicobacter pylori* SabA in the pathogenesis of gastroduodenal disease. J Infect Dev Ctries 2008 Jun 1;2(3):174-181.

(317) Senkovich OA, Yin J, Ekshyyan V, Conant C, Traylor J, Adegboyega P, et al. *Helicobacter pylori* AlpA and AlpB bind host laminin and influence gastric inflammation in gerbils. Infect Immun 2011 Aug;79(8):3106-3116.

(318) Backert S, Clyne M, Tegtmeyer N. Molecular mechanisms of gastric epithelial cell adhesion and injection of CagA by *Helicobacter pylori*. Cell Commun Signal 2011 Nov 1;9:28-811X-9-28.

(319) Bourke B, Ceponis P, Chiba N, Czinn S, Ferraro R, Fischbach L, et al. Canadian *Helicobacter* Study Group Consensus Conference: Update on the approach to *Helicobacter pylori* infection in children and adolescents-an evidence-based evaluation. Can J Gastroenterol 2005 Jul;19(7):399-408.

(320) Koletzko S, Richy F, Bontems P, Crone J, Kalach N, Monteiro ML, et al. Prospective multicentre study on antibiotic resistance of *Helicobacter pylori* strains obtained from children living in Europe. Gut 2006 Dec;55(12):1711-1716.

(321) Agudo S, Perez-Perez G, Alarcon T, Lopez-Brea M. High prevalence of clarithromycin-resistant *Helicobacter pylori* strains and risk factors associated with resistance in Madrid, Spain. J Clin Microbiol 2010 Oct;48(10):3703-3707.

(322) O'connor A, Taneike I, Nami A, Fitzgerald N, Murphy P, Ryan B, et al. *Helicobacter pylori* resistance to metronidazole and clarithromycin in Ireland. Eur J Gastroenterol Hepatol 2010 Sep;22(9):1123-1127.

(323) Horiki N, Omata F, Uemura M, Suzuki S, Ishii N, Iizuka Y, et al. Annual change of primary resistance to clarithromycin among *Helicobacter pylori* isolates from 1996 through 2008 in Japan. Helicobacter 2009 Oct;14(5):86-90.

(324) Zevit N, Levy I, Shmuely H, Samra Z, Yahav J. Antibiotic resistance of *Helicobacter pylori* in Israeli children. Scand J Gastroenterol 2010 May;45(5):550-555.

(325) Onder G, Aydin A, Akarca U, Tekin F, Ozutemiz O, Ilter T. High *Helicobacter pylori* resistance rate to clarithromycin in Turkey. J Clin Gastroenterol 2007 Sep;41(8):747-750.

(326) Suzuki RB, Lopes RA, da Camara Lopes GA, Hung Ho T, Speranca MA. Low *Helicobacter pylori* primary resistance to clarithromycin in gastric biopsy specimens from dyspeptic patients of a city in the interior of Sao Paulo, Brazil. BMC Gastroenterol 2013 Dec 4;13:164-230X-13-164.

(327) Altman E, Chandan V, Harrison B. The potential of dextran-based glycoconjugates for development of *Helicobacter pylori* vaccine. Glycoconj J 2014 Jan;31(1):13-24.

(328) Yamaoka Y. Mechanisms of disease: *Helicobacter pylori* virulence factors. Nat Rev Gastroenterol Hepatol 2010 Nov;7(11):629-641.

(329) Azagarsamy MA, Gomez-Escudero A, Yesilyurt V, Vachet RW, Thayumanavan S. Amphiphilic nanoassemblies for the detection of peptides and proteins using fluorescence and mass spectrometry. Analyst 2009 Apr;134(4):635-649.

(330) Daniels, R. (April 2001). Galenic principles of modern skin care products. Skin care forum Issue 25. http://www.skin-care-forum.basf.com. Accessed 21/02/2014.

Chapter 2

Genotypic Features of *Helicobacter pylori* Isolated from Residents of Aklavik, Northwest Territories and the Relation of Genotypic Features to Gastric Histopathology[1]

2.1 Introduction

Helicobacter pylori is a gram-negative spiral shaped bacterium that was first cultured by Marshall and Warren in 1982 (1,2). The prevalence of *H. pylori* infection was estimated by Brown (2000) to be about 40% in developed countries and 70% in developing countries (3). *H. pylori* infection is a risk factor for the development of gastric diseases such as gastritis, peptic ulcer, mucosa associated lymphoid tissue lymphoma and gastric adenocarcinoma (4).

Gastric cancer is a major health concern. It is the fifth most common type of cancer after lung, breast, colorectal and prostate cancers. A recent study estimated 260 000 cases of cardia gastric cancer (starts in the area of the stomach near the oesophageal-gastric junction) and 691 000 cases of non-cardia gastric cancer (starts in the distal regions of the stomach) worldwide in 2012 with the highest rates observed in Eastern/Southeastern Asia (5). Non-cardia gastric cancer, which accounts for 73% of total gastric cancer cases, is the subsite that is strongly associated with *H. pylori* infection (5).

Aklavik is a small community of about 600 people living in the Northwest Territories, Canada. Residents of Aklavik have long been concerned about the perceived increased incidence of gastric cancer in their community and its association with *H. pylori* infection diagnosed in many residents (6). Between 1992 and 2000, the age-adjusted rate for gastric cancer among Northwest Territories males was 1.9 times higher than among Canadian males (8). In addition, gastric cancer was the second most frequently diagnosed cancer in Inuit males and the third most frequently diagnosed cancer in Dene First Nations males in the Northwest

[1] The work presented here will be included in a future publication of the CAN*Help* Working Group that presents results of an analysis that includes an increased sample size achieved by combining data from several Arctic communities.

Territories, although it ranked 10th in males across Canada (8). Moreover, Aklavik is a home for number of immigrants from Alaska where high rates of gastric cancer have been previously reported (6,9). The Canadian North *Helicobacter pylori* (CAN*Help*) Working Group launched the Aklavik *H. pylori* Project to address these concerns. The community concern is justified by studies performed by the CAN*Help* Working Group. Of 333 project participants screened for *H. pylori* infection by urea breath test as part of the Aklavik *H. pylori* Project in January and February 2008, 58% were positive for *H. pylori* infection, indicating a high prevalence of *H. pylori* infection in the community. A recent cross-sectional study compared the frequency of gastric histopathology diagnoses among Aklavik research participants with those of University of Alberta Hospital patients assessed for the same conditions. Relative to University of Alberta Hospital patients, Aklavik residents had a much higher prevalence of *H. pylori* infection (66% versus 14% by histology). Moreover, *H pylori*-positive Aklavik residents had a much higher prevalence of severe gastric inflammation and gastric atrophy (7).

A study conducted during November 2011 through June 2012 to estimate the incidence and re-infection rates in Aklavik *H. pylori* Project participants who initially tested negative or received treatment to eliminate *H. pylori* infection during 2008 through 2010 yielded an estimated re-infection proportion of 4.7% (95% CI: 0.6–16.0%). Aboriginal participants had a combined re-infection/incidence rate of 2.4% (95% CI: 0.8–5.9% per year). Neither the 9 non-Aboriginal participants nor the 23 participants aged 55 years and above included in the follow-up study showed evidence of re-infection. The re-infection study indicates that the Aklavik *H. pylori* project has substantially reduced the prevalence of this infection in Aklavik since the project started in 2008 (10). The clinical outcomes of *H. pylori* infection are hypothesized to be dependent on several bacterial factors identified as having the potential to influence virulence, as well as specified environmental and host factors (11). *H. pylori* organisms produce several factors that facilitate their colonization and survival in the hostile gastric environment (12-14) and are hypothesized to determine virulence. The *cag* pathogenicity island (*cag*-PAI) is the most extensively studied virulence marker of *H. pylori*. The *cag*-PAI contains 27-31

genes (the number is variable in different strains) including the *cagE* and *cagA* genes (12). The *cagE* gene is one of 7 genes of the *cag*-PAI hypothesized to influence secretion of chemokines such as interleukin 8 from infected host epithelial cells (15,16). The *cagA* gene codes for an immunodominant protein known as CagA (cytotoxin associated protein) (17). Inside gastric cells, CagA is phosphorylated on tyrosine phosphorylation motifs containing the Glu-Pro-Ile-Tyr-Ala (EPIYA) amino acid sequence (12). The EPIYA motif is present in multiple copies in the protein and is referred to as the EPIYA repeat region. The EPIYA motif may be defined as a combination of EPIYA-A, -B -C, or -D, depending upon the amino acid sequence that follows the EPIYA (18-20). The CagA protein species nearly always contain EPIYA-A and EPIYA-B sites, followed by one to five repeats of EPIYA-C in Western-type *H. pylori* cultures (African and Indo-European ancestry) or EPIYA-D site in East Asian-type cultures (21). Evidence supports the hypothesis that the virulence of *H. pylori* increases with the number of EPIYA-C motifs (18,22). Phosphorylated CagA protein has the ability to disturb the cellular functions by interacting with a cellular protein, SHP-2, which plays an important role in mitogenic cellular transduction (20).

The *vacA* gene, which sometimes but not always induces vacuolation, is present in nearly all *H. pylori* strains (13). The massive production of autophagic vacuoles has been linked to cell death (13) due to destruction of parts of the cytoplasm including organelles caused by lysosomal activity within vacuoles (23). It may be that genetic polymorphism within *vacA* affects the ability of a particular strain to induce vacuolation (24). Two major polymorphic regions have been identified within *vacA*: the signal region (*s1* or *s2*) and the midregion (*m1* or *m2*) (25). Characterization of 59 cultures from the United States with respect to virulence based on in vitro cytotoxin activity and co-occuring clinical conditions in the human hosts, resulted in *vacA s1/m1* strains being classified as the most virulent, while those with *s1/m2* were classified as having intermediate virulence and those with *s2/m2* as the least virulent (26). Of note, *vacA s2* strains consistently failed to produce detectable vacuolatization *in vitro*; only strains with *vacA* s1 produced such activity (26). In Germany, Miehlke *et al.,* compared the genotypic features of

H. pylori isolated from 34 patients with gastric cancer to those from 35 subjects with asymptomatic gastritis and reported an increased frequency of *vacA s1m1* in patients with gastric cancer (27). Another study of 167 *H. pylori* cultures in Italy reported an apparent interaction between *cagA*, *babA2,* and *vacA s1m1*, given that in combination they were associated with higher intestinal metaplasia and inflammation scores (28). In addition to *vacA s* and *m* regions, another *vacA* polymorphic site, designated as the intermediate (*i*) region, was identified as having two sequence types (*i1* and *i2*) (29). In lab studies, Winter *et al,* reported a strong association between *vacA i1* and precancerous intestinal metaplasia in mice, although *H. pylori* producing the *s2/i2* form of *vacA* colonized mice more efficiently than *vacA*-negative mutants or mutants producing *vacA s1i1* (30). From a study of 73 Iranian patients, Rhead reported that those with the *i1* type had a higher frequency of gastric adenocarcinoma than those with the *i2* type (29). However, Ogiwara *et al.,* reported that *vacA s/i/m* genotypes were not good markers of clinical outcomes among 314 strains isolated from East Asian and Southeast Asian countries (31).

BabA (blood group antigen binding adhesin), encoded by the *babA2* gene, is thought to contribute to the ability of *H. pylori* to adhere to the gastric mucosa (32). BabA adherence is mediated through the fucosylated Lewis b (Le^b) blood-group antigen (33). Three different *bab* alleles have been identified: *babA1*, *babA2*, and *babB*. The *babA1*gene lacks the translational initiation codon while both *babA2* and *babB* encode homologus proteins which have polymorphic midregion sequences. BabA mediates Le^b binding, but, BabB does not (32,34). Since Le^b is not expressed in all humans, other adhesins may be involved such as SabA (sialic acid-binding adhesin) and SabB (35), HopQ (*H. pylori* outer membrane protein) (36) and AlpA and AlpB (adherence associated lipoprotein) (37).

The possibility that *H. pylori* genotypes are linked to clinical outcomes presents the potential for identifying virulent strains that can be prioritized for elimination, and in this way reduce overly general antibiotic use. Based on this rationale, for the present study I used data from the Aklavik *H. pylori* Project to describe the prevalence of *cagA, cagE, vacA* and *babA2* genes, and describe the

characteristics of these genes, in association with relevant histopathology outcomes. This study also describes the prevalence of histopathology outcomes, and estimates associations between genotypic features of *H. pylori* isolated from gastric biopsies and characteristics observed in histopathologic examination of gastric biopsies from the same individuals. The main goal of the work presented in this chapter was to characterize Aklavik *H. pylori* cultures for the main virulence genes to identify targets for novel treatment approaches.

2.2 Materials and Methods

In this section, I describe the laboratory procedures I carried out and those performed by other members of the research team. Unless otherwise stated, chemicals, disposables and glassware were purchased from Fisher Scientific (Ottawa, ON, Canada), antibiotics were purchased from Sigma-Aldrich (Oakville, ON, Canada), 0.22 µm filters were purchased from Merck KGaA, Darmstadt, Germany and enzymes were purchased from Invitrogen (Carlsbad, CA, USA).

2.2.1 *H. pylori* isolation from gastric biopsies

Gastric biopsies used in this research came from the Aklavik *H. pylori* Project, conducted by the Canadian North *Helicobacter pylori* (CAN*Help*) Working Group to address community concerns regarding cancer risks from *H. pylori* infection. For one component of the Aklavik *H. pylori* Project, gastroenterologists obtained gastric biopsies (1-2 for microbiology, 4-6 for pathology) from 194 participants through upper gastrointestinal endoscopy performed at the Aklavik Heath Centre in February 2008. All participants gave informed consent. Research protocols were approved by the Health Research Ethics Board (HREB) at the University of Alberta and the Aurora Research Institute, which issues research licenses for the Northwest Territories. Gastric biopsies were immediately placed in transport media containing, 3.7% brain heart infusion (BHI) (Oxoid LTD., Basingstoke, England) - 0.5% yeast extract (YE) (Difco, Sparks, USA) - 5% horse serum (HS) (Gibco, Scotland, UK) - 20% glycerol broth supplemented with antibiotic mixture containing vancomycin (8 µg/mL), amphotericin (8 µg/mL), cefsulodin (5 µg/mL), trimethoprim (5 µg/mL), β-

cyclodextrin (2 µg/mL), polymixin B (0.2149 µg/mL). Project field staff arranged for the transport of gastric biopsies from Aklavik to Dr. Monika Keelan's microbiology research lab at the University of Alberta and to the pathology lab at the University of Alberta Hospital in Edmonton. Gastric biopsies were evaluated according to the updated Sydney protocol (38) by a single tertiary-care centre gastrointestinal pathologist, Dr. Safwat Girgis, who was blinded to endoscopic findings. Gastric biopsy sections were stained with Hematoxylin & Eosin for regular histology and sections were also stained with Giemsa to detect *H. pylori* (7).

Laboratory staff isolated *H. pylori* from the gastric biopsies by first homogenizing each biopsy vial (three quick bursts at maximum speed) using the Omni international TH homogenizer (Kennesaw, GA, USA) to break down tissue samples and liberate *H. pylori*. For each homogenate, 100 uL aliquots were plated in duplicate onto 3.7% BHI / 0.5% YE / 5% HS / 1.5% agar (Oxoid LTD., Basingstoke, England) plates containing the same transport media antibiotic mixture and incubated them under microaerobic conditions (5% O_2, 5-10% CO_2, and balance N_2) at 37°C for up to 21 days.

Transport and culture media were prepared without horse serum or antibiotics and autoclaved at 20 psi and 121°C for 20-30 minutes. After cooling the media for an hour in a 56°C water bath, horse serum and filter-sterilized antibiotics were added to the media.

2.2.2 Identification of *H. pylori*

Laboratory staff initially identified *H. pylori* cultures using typical colony morphology (circular, convex and translucent), Gram stain (negative spiral rods) and positive urease, catalase, and oxidase tests. The *H. pylori* isolated from the gastric biopsies from one individual is referred to as a primary culture of one person's biopsies.

2.2.2.1 Urease test

Laboratory staff used a sterile swab to collect *H. pylori* colonies from plate culture with a sterile swab and immersed it into a microcentrifuge tube containing

urease test medium (0.1% peptone, 0.5% NaCl, 0.2% KH_2PO_4, 0.1% dextrose, 2% urea and 0.0012% phenol red, sterilized by filtration). *H. pylori* produces urease which breaks down urea into CO_2 and NH_3. The phenol red indicator changes from yellow to pink when the pH becomes alkaline (> 8.2) (39,40). The test was considered positive when *H. pylori* colonies turned pink due to ammonia liberation detected by the change in pH to an alkaline pH.

2.2.2.2 Catalase test

H. pylori colonies from plate culture were collected with a sterile swab and immersed into a microcentrifuge tube containing 3% H_2O_2. *H. pylori* produces catalase which breaks down H_2O_2 into H_2O and O_2. The test was considered positive when bubbles formed due to O_2 liberation (40).

2.2.2.3 Oxidase test

H. pylori colonies from plate culture were collected with a sterile swab and dipped into a microcentrifuge tube containing 1% N,N,N',N'-tetramethyl-p-phenylenediamine dihydrochloride (TMPD) (Becton and Dickinson company, USA). *H. pylori* produces cytochrome oxidase which oxidizes colourless TMPD to indophenols which are purple (41). The test was considered positive if the purple colour formed within 30 seconds.

2.2.3 Preservation of *H. pylori*

Primary *H. pylori* plate cultures were suspended in BHI/YE/20% glycerol broth, transferred to cryovials, rapidly frozen in liquid nitrogen, and stored at -80°C.

2.2.4 *H. pylori* genotyping

Genomic DNA was isolated from pure plate cultures and quantified with help from laboratory staff. We confirmed the biochemical identification of *H. pylori* by 16S rRNA gene PCR. We also detected virulence genes using PCR, and performed nucleotide sequence analysis when PCR failed to detect the gene.

2.2.4.1 Genomic bacterial DNA isolation

Crystals of frozen primary stock cultures were plated onto BHI/YE/HS agar containing vancomycin (15 μg/ml), amphotericin (15 μg/ml) for 3-5 days. The

bacteria were harvested with 500 µL of DNA extraction buffer (0.15 M NaCl, 0.1 M EDTA, pH 8). The cells were lysed using 20-40 µL of 20% SDS, gently mixed until the mixture became clear. Genomic DNA was extracted using phenol/chloroform (1:1) followed by centrifugation at 13,000 rpm for 10 minutes using Accuspin^{TM} Micro, Fisher Scientific (Ottawa, ON, Canada). The upper layer was transferred to a new microcentrifuge tube and precipitated with cold (-20°C) 100% ethanol (42). After overnight storage at -20°C, the tube was briefly centrifuged and the DNA pellet dissolved in 50 µL sterile deionized water. DNA concentration was determined prior to storage at -20°C.

2.2.4.2 DNA concentration

DNA concentration was determined by measuring the optical density at 260 nm (OD_{260}) using the NanoVue spectrophotometer (GE Healthcare, Buckinghamshire, UK). The concentration of double-stranded DNA (dsDNA) is based on the relationship that 1.0 optical density unit at OD_{260} is equal to 50 µg DNA/mL. Since inaccurate spectrophotometric DNA quantitation occurs with the presence of protein or chemicals (42), the OD_{260}/OD_{280} ratios were determined for an indication of DNA purity. Pure DNA has an OD_{260}/OD_{280} ratio of ~1.8. Ratios below 1.8 could be caused by protein contamination (aromatic amino acids absorb at 280 nm) or phenol contamination (phenol absorbs maximally at 270 nm). Contaminated DNA preparations were discarded and fresh cultures obtained for DNA extraction.

2.2.4.3 *H. pylori* gene detection by PCR

PCR was used to confirm the identification of *H. pylori* by 16S rRNA and to detect the presence or absence of virulence genes *cagA*, *cagE*, *vacA* and *babA2* using primers that were previously published in the literature (**Table 2.1**). I determined the number and type of EPIYA motifs of *cagA*$^{+}$ *H. pylori* cultures using two different methods. The first method was based on the size of the amplicon obtained by a single primer pair (43). However, sequence analysis of the amplicons was not consistent with the EPIYA type obtained with the first method. For this reason, I used a second PCR method that utilized a specific primer pair for each

EPIYA type and I verified the method by DNA sequence analysis (19). For all PCR reactions, 20 ng of DNA was added to each PCR mixture of 50 µL containing 0.1-0.2 µM primer, 1.5 mM $MgCl_2$, 1X PCR buffer (20 mM Tris-HCl, pH 8.4, 50 mM KCl), 0.2 mM dATP, dCTP, dGTP and dTTP, and 1 U Platinum® *Taq* DNA polymerase under PCR conditions specified in **Table 2.2**. I separated PCR products (amplicons) using agarose gel electrophoresis. PCR product aliquots of 10 µL were mixed with 2 µL of 6X loading dye (30% (v/v) glycerol, 0.25% (w/v) bromophenol blue and 0.25% (w/v) xylene cyanol) and then loaded into 1.5% agarose gels in TAE electrophoresis buffer (40 mM **T**ris-**A**cetate and 1 mM **E**DTA, pH 8.5). A reference DNA molecular weight (MW) size ladder (5 µL of 1 Kb plus DNA ladder, Invitrogen, Carlsbad, CA, USA) was loaded into a separate well. Electrophoresis was performed at 100 volts (E-C Apparatus Corporation electrophoresis power supply, USA) for 30-60 minutes. Gels were stained in 0.5 µg/mL ethidium bromide for 10-15 minutes, washed by soaking in water for 30 minutes to reduce background staining, and then visualized under ultraviolet light using a UV imager (Bio-RaD Gel DocTM EZ, Mississauga, Ontario) (44). Amplicon sizes were compared to bands of the reference DNA MW size ladder. Reference *H. pylori* strains served as positive controls for the different genotypes detected in this study: J99 *(cagA, cagE, vacA)*, 26695 *(babA2)*, G27 (EPIYA motifs) and SS1 *(babA2)*. The presence of a gene is indicated as *"positive"* or "+" immediately following the name of the gene (e.g. *cagA positive or cagA$^+$)*. The absence of a gene is indicated to be *"negative"* or "-" immediately following the gene name (e.g. *cagA negative* or *cagA$^-$)*. The detection of more than one genotype per primary culture indicates the presence of more than one *H. pylori* strain present. When more than one *vacA* genotype was detected for the same primary culture, I included these cultures in my analyses because individuals infected with more than one strain may be exposed to a putative virulent *vacA* genotype. PCR primers used for *cagA*, *cagE* and *babA2* identification did not detect more than one type of these genes. Nucleotide sequence analysis of these genes was not performed to confirm presence or absence of more than one genotype.

2.2.4.4 Nucleotide sequence analysis

cagA, vacA and *babA2* genes were amplified from *H. pylori* genomic DNA using Platinum® *Taq* DNA polymerase high fidelity according to the same PCR conditions specified in **Table 2.2** but at an extension temperature of 68°C. PCR amplicons were purified using the QIAquick® PCR purification kit (Qiagen, Maryland, USA). The kit contains a silica membrane assembly for DNA binding in high salt buffer at pH ≤ 7.5. The high salt buffer was added directly to the PCR sample (10:1), then the mixture was loaded onto the QIAquick spin column and centrifuged for 1 min at 6000 rpm. PCR products were eluted with water by centrifugation for 1 min at 17,900 x *g*. The purification procedure removes primers, nucleotides, enzymes and other impurities from DNA samples that may interfere with sequencing. The laboratory staff at the Applied Genomic centre, University of Alberta sequenced the purified products using the Applied Biosystems Big Dye® Terminator kit which uses fluorescent dye primer-based sequencing. DNA sequencing is done using four different fluorescent dyes, which are attached to the ddNTPs (dideoxy nucleoside triphosphates), thereby only one reaction tube per sample is required. Each ddNTP (ddATP, ddCTP, ddGTP, or ddTTP) is labelled with a different color of dye. DNA template, primer, buffer, four dNTPs, four fluorescently labeled ddNTPs, and Taq DNA Polymerase are added to one reaction tube. When dye-labeled ddNTPs are incorporated, the reaction is randomly terminated, creating DNA fragments of different lengths. All terminated fragments contain a dye at their 3' end and can be analyzed to the corresponding DNA sequence (45). I analyzed the obtained DNA sequences using DNAStar software (Madison, USA).

2.2.5 *babA2* expression

To investigate observed variations in *babA2* amplicon size, I extracted RNA and performed reverse transcription PCR (RT-PCR) of *babA2* mRNA to cDNA to determine whether differences in the *babA2* gene expression were detected by differences in the *babA2* gene region amplified by PCR.

2.2.5.1 RNA extraction

Total RNA was extracted for each of two Aklavik *H. pylori* primary cultures,, (A62, A68) and one reference *H. pylori* strain J99 (positive control for *babA2* expression) using TRIzol® reagent (Invitrogen, Carlsbad, CA, USA), which is a monophasic solution of phenol, guanidine isothiocyanate, and other proprietary components that facilitate the isolation of RNA from cells and tissues. A 24 h fresh culture of *H. pylori* on BHI/YE/HS agar plates was suspended in BHI/YE broth to achieve $OD_{600} = 0.1$. Cell suspension aliquots of 250 µL were centrifuged at 12,000 × g for 15 minutes to pellet the cells. TRIzol® Reagent (750 µL) was directly added to cell pellets without washing and incubated for 5 minutes at room temperature (RT) to permit complete dissociation of the nucleoprotein complex. Then, 200 µL of chloroform was added and the tubes shaken vigorously by hand for 15 seconds followed by incubation for 2–3 minutes at RT. The mixtures were centrifuged at 12,000 × g for 15 minutes at 4°C, then the colourless upper aqueous phase transferred to a new tube. RNA was precipitated by adding 0.5 mL of 100% isopropanol to the aqueous phase followed by centrifugation at 12,000 × g for 10 minutes at 4°C. RNA pellets were dissolved in 50 µL RNase free sterile water. RNA concentration and purity were assessed as previously described in section 2.3.1 except that RNA quantitation is based on the relationship that an OD_{260} equal to 1.0 corresponds to an RNA concentration of 40 µg/mL.

2.2.5.2 RT- PCR

RNA samples were incubated with DNase I to digest any remaining genomic DNA prior to performing RT-PCR using a high capacity cDNA reverse transcription kit (Applied Biosystems, USA). A concurrent reaction in the absence of reverse transcriptase was performed to confirm the absence of genomic DNA (negative control). All reactions were performed in a 20 µL total volume containing 2 µL 10X RT buffer, 0.8 µL 25X dNTP mix (100 mM), 2 µL 10X random primer, 1 µL MultiScribe™ reverse transcriptase, 4.2 µL nuclease free water and 10 µL RNA (20 µg/µL). The reaction conditions were: 25°C for 10 min, 37°C for 120 min

and 85°C for 5 min. *babA2* cDNA was detected as an indicator of *babA2* transcription and likely BabA protein expression.

2.2.6 Statistical analysis

I performed all the statistical analyses using IBM SPSS® version 20 (New York, USA). To describe the distribution of *H. pylori* genotypes isolated from residents of Aklavik, relative frequencies of genotype categories were tabulated. The prevalence of histopathology outcomes among research participants with recoverable primary *H. pylori* cultures was estimated by dividing the number with the outcome by the total number included in the analysis. The prevalence of histopathology outcomes were also estimated within genotype categories. If a primary culture from an individual's biopsy contained a single *vacA* genotype, this was interpreted as being consistent with only one strain type being present and the participant was classified in the analysis as having a single genotype. If a primary culture from an individual's biopsy contained more than one *vacA* genotype, this was interpreted as being consistent with more than one strain type being present and the participant was classified in the analysis as having multiple genotypes. If a participant with multiple genotypes had one identified as a putative virulent genotype, the participant was classified as having the virulent genotype based on exposure to the virulent genotype. Crude odds ratios (OR) and 95% confidence intervals (CI) were calculated to estimate associations between genotypes and prevalence odds of histopathology outcomes.

2.3 Results

2.3.1 *H. pylori* culture positive gastric biopsies

Of 194 gastric biopsies processed for *H. pylori* culture, 140 (72%) were positive for *H. pylori* by biochemical testing (urease positive, catalase positive, oxidase positive) and Gram stain. Anaerobic jar failure resulted in the loss of 12 cultures. Another 7 cultures could not be recovered after storage at -80°C, which left a total of 121 primary cultures available for genotyping studies.

2.3.2 Demographics of Aklavik *H. pylori* Project participants

Cultures included in this study were isolated from 121 Aklavik *H. pylori* Project participants. Seventy two (60%) females and 49 (41%) males were included in this study. The majority of participants (95%) were Aboriginal people (Inuit, Gwich'in First Nations, or Metis). Participants' ages ranged from 11-80 years; 58 (48%) were 11 - 34 years old, 42 (35%) were 35 - 54 years old and 21 (17%) were older than 55 years.

2.3.3 Genotypic features of Aklavik *H. pylori*

The prevalence of *cagA, cagE*, *vacA* and *babA2* genes in 121 Aklavik *H. pylori* primary cultures are presented in **Table 2.3**. The *cagA* gene was detected in 36% (43/121) of *H. pylori* cultures. The *cagE* gene was detected in 31% (38/121) of cultures and all *cagE*-positive cultures were also *cagA*-positive. The *vacA* and *babA2* genes were present in nearly all *H. pylori* cultures, 96% (116/121) and 97% (117/121), respectively.

When subtyping the *vacA* gene by PCR, 24% (29/121) of cultures were untypeable for the *s* region (*vacA* $s_{unknown}$), because no amplicon was detected with the primer pairs used in this study (**Figure 2.1**). Sequence analysis of the *vacA* gene of these cultures confirmed the presence of the *s1* type in each of them (**Figure 2.2**). PCR successfully differentiated *vacA i1* and *i2* (**Figure 2.3**) and *vacA m1* and *m2* (**Figure 2.4**) for all the cultures tested. Eleven primary cultures (10 *cagA* positive, 1 *cagA* negative) had more than one *vacA* type. Since the *vacA* gene is present in a single copy in the *H. pylori* genome, the presence of more than one *vacA* type indicates the presence of more than one strain. For 10 cultures, amplicons were detected for *vacA i1* and *i2* when typed for *vacA i* and so those cultures were identified as having both types. Similarly, 9 cultures were identified as having both *m1* and *m2* types when more than one *vacA* genotype was present. Six different *vacA* types were detected; *s1/i1/m1, s1/i1/m2, s2/i1/m2, s1/i2/m2, s2/i2/m2*, and *s1/i2/m1*. Five of these *vacA* types occurred in genotypes that were positive for the *cagA* gene. In contrast, only 3 *vacA* types occurred in genotypes that were *cagA* negative. The *cagA* gene was observed in nearly all cultures with v*acA s1/i1/m1*

genotypes (26/27) while nearly all cultures with *vacA s1/i2/m2* (43/44) or *vacA s2/i2/m2* (28/29) strain types did not have the *cagA* gene.

The EPIYA-ABC type was detected in 91% (39/43) of *cagA*-positive *H. pylori*, whereas EPIYA-AB and EPIYA-ABCC types were detected in 2% (1/43) and 7% (3/43) of *cagA*-positive *H. pylori*, respectively. Sequence analysis of three cultures that could not be amplified using the EPIYA-C primers ($ABC_{unknown}$) revealed a difference in a single amino acid (glutamic acid instead of aspartic acid) in the C region (ABC*). This EPIYA-ABC* mutation was not reported previously in the literature (**Figures 2.5 and 2.6**).

Two different amplicon sizes were detected using *babA2* primers; an expected one at 271 bp and an unexpected one at 248 bp. Of Aklavik cultures, 71% (86/121), had the unexpected 248 bp amplicon size. Of note, variation in the *babA2* amplicon size was observed in reference strain G27 as well as in *H. pylori* cultured from an Alberta individual (**Figure 2.7**). Sequence analysis of *babA2* of two Aklavik *H. pylori* strains, identified as A62 (271 bp) and A68 (248 bp), showed a deletion of 13 and 33 bp respectively (**Figure 2.8**). A62 had the same *babA2* amplicon size as reference strain J99 but did not express the gene (as indicated by failure to detect *babA2* mRNA using reverse transcriptase PCR), while J99 did. A68 had the unexpected *babA2* amplicon size (248 bp) but expressed the gene (**Figure 2.9**).

2.3.4 Distribution of histopathology outcomes

A total of 121 research participants with histopathology data had *H. pylori* cultures available for this analysis. However, 119 participants were included in analyses of acute gastritis because data was missing for 2 participants. The distribution of gastritis severity, atrophy and intestinal metaplasia among 121 participants is presented in **Table 2.4**. Acute and chronic gastritis were detected in 81% (96/119) and 84% (102/121), respectively, of participants with recovered *H. pylori* cultures. However, *H. pylori* was negative by histopathology in 21 participants with positive results by culture; none of these 21 participants had acute gastritis and only two had chronic gastritis, which was mild in both cases. *H. pylori*

may have patchy distribution and so it is possible that its detection may be missed by histopathology. Atrophy was detected in 21% (26/121) of participants, while intestinal metaplasia was detected in 9% (11/121) of participants. Severe acute gastritis was observed in only 7% (8/119) of participants while moderate gastritis was seen in 27% (32/119) of participants. Almost half (47%, 56/119) of the participants had mild acute gastritis, and no gastritis was detected in 19% (23/119) of participants. The picture was quite different for chronic gastritis where more than three quarters (76%) of participants had moderate to severe chronic gastritis. Severe chronic gastritis was detected in 40% (49/121) while moderate chronic gastritis was observed in 36% (44/121) of participants. Nine participants (7%) had mild chronic gastritis and no signs of chronic gastritis were detected in 16% (19/121). Due to the small number of cases of severe acute gastritis, moderate and severe gastritis were combined for further analysis: 33% (40/121) of participants had moderate or severe acute gastritis, while 77% had moderate or severe chronic gastritis.

2.3.5 Prevalence of histopathology outcomes by genotype

The estimated associations of genotypes with the prevalence odds of acute gastritis, chronic gastritis, atrophy and intestinal metaplasia are presented in **Tables 2.9**, **2.9a and 2.10**. Outcomes were dichotomized based on their distribution across severity categories. The chronic gastritis outcome was dichotomized as more severe or less severe in two manners. Table 2.9 shows results for the dichotomization as moderate/severe versus absent/mild while table 2.9a shows results for dichotomization as severe versus absent/mild/moderate. For acute gastritis, participants with *cagA*-positive *H. pylori* had 2.2 (95% CI: 1.0 – 4.8) times the odds of moderate/severe acute gastritis relative to participants with *cagA*-negative *H. pylori*. For the *vacA* gene, participants with *vacA i1 H. pylori* had 2.0 (95% CI: 0.9 - 4.4) times the odds of moderate/severe acute gastritis relative to participants with *vacA i1*-negative *H. pylori*; participants with *vacA m1 H. pylori* had 1.7 (95% CI: 0.7 - 3.7) times the odds of moderate/severe acute gastritis relative to participants with *vacA m1*-negative *H. pylori*. Acute gastritis was minimally associated with *vacA s1* and *babA2*.

Chronic gastritis had weak-to-moderate inverse associations with *cagA-positive*, *vacA i1,* or *vacA m1,* but a weak positive association with *vacA s1* (odds ratio: 1.2, 95% CI: 0.5 – 3.0). When *H. pylori* participants with *babA2* 248 bp cultures were compared to participants with *H. pylori* cultures that had a different amplicon size of *babA2* or were *babA2-negative*, the odds ratio for chronic gastritis was 1.8, 95% CI: 0.7 – 4.3.

When severe chronic gastritis was compared to absent/mild/moderate acute gastritis, moderate-to-null associations were observed for all the investigated virulent genotypes with the exception of *babA2* 248 bp where an odds ratio of 1.8 (95% CI: 0.8 - 4.0) was estimated.

For the presence or absence of atrophy, weak-to-moderate positive associations were observed for all of the investigated putative virulent genotypes, with the strongest being 1.8 (95% CI: 0.7 – 4.3) for *cagA*. For the presence or absence of intestinal metaplasia, participants with *cagA*-positive or *vacA s1 H. pylori* had 2.4 (95% CI: 0.7 – 8.3) and 2.3 (95% CI: 0.5 – 11.1) times the odds of intestinal metaplasia, respectively relative to participants with *cagA*-negative or *vacA s1*-negative *H. pylori*. Weak associations with intestinal metaplasia were observed for the rest of the investigated putative virulence genotypes.

2.4 Discussion

H. pylori is a gastric pathogen that causes one of the most common bacterial infections. Based on a modest body of literature, it has been inferred that the prevalence of *H. pylori* infection has declined in the overall population of Canada (46), but it is still high in Canadian Aboriginal communities (47). The seroprevalence of *H pylori* in Canada was previously reported to vary between 21% and 95%, but for Aboriginal communities, a prevalence of 50% or more was reported (7,48).

In the present study, 121 Aklavik *H. pylori* cultures were typed for four genes (*cagA*, *cagE*, *vacA* and *babA2*) using PCR. As expected, the majority of cultures have the *vacA* and *babA2* genes but approximately one third have the genes *cagA* and *cagE* belonging to the *cag* pathogenicity island. High prevalence of acute

and chronic gastritis was observed (81% and 84% respectively) in participants, while atrophy and intestinal metaplasia were less frequently observed, in 21% and 9% of participants, respectively. Individuals with *H. pylori* cultures genotyped as positive for *cagA, vacA i1,* or *vacA m1* showed increased prevalence odds of moderate/severe acute gastritis compared to individuals with *H. pylori* cultures that were negative for these genotypes. Odds ratios greater than 2.0 with very wide CIs were estimated for intestinal metaplasia when individuals with *H. pylori* cultures genotyped as positive for *cagA* or *vacA i1* were compared to individuals with *H. pylori* cultures that were negative for these genotypes. Individuals with *H. pylori* cultures genotyped as positive for *babA2* 248 bp showed increased prevalence odds of acute and chronic gastritis compared to individuals with *H. pylori* cultures lacking this amplicon size. For gastric atrophy, individuals with *H. pylori* cultures genotyped as *cagA-positive* showed increased prevalence odds of gastric atrophy compared to individuals with *H. pylori* cultures that were *cagA-negative*. No clear evidence of a positive association between the putative virulent genotype and increased odds of more severe histopathology was observed for the rest of the tested genotypes.

The observed prevalence of the *cagA* gene in the Aklavik study was 36%, which is slightly higher than the observed prevalence of *cagA* in two Brazilian studies (49,50) but lower than the prevalence reported from studies conducted in other countries (**Table 2.10**). In the present study, *cagA* positivity was associated with higher prevalence odds of moderate-severe acute gastritis, presence of atrophy and presence of intestinal metaplasia. A summary odds ratio of similar magnitude 2.1 (95% CI, 1.5-3.0) was estimated for the association between *cagA* positivity and gastric cancer in a meta-analysis of 44 studies that included a combined total of 17,374 patients.(51).

The detection of at least one EPIYA-C motif in 98% of *cagA*-positive Aklavik cultures and the absence of EPIYA-D motif is consistent with a Western origin of these cultures. A recent study identified Aklavik *H. pylori* strains as predominantly of European and Amerindian origin (52). In the present study, it was not possible to investigate whether the number of EPIYA-C sequences is associated

with a higher prevalence of abnormal pathology because more than one EPIYA-C motif (two repeats) was detected in only three cultures. Each of the cultures with two EPIYA-C repeats came from a different participant; each of these three participants had severe chronic gastritis, but this is not enough evidence for drawing conclusions about the effect of multiple EPIYA-C sequences. Authors of a recent study of 84 *cagA*-positive *H. pylori* cultures from Colombia reported a lack of association between the number of EPIYA-C-repeats and abnormal cellular response (IL-8 production or cellular elongation) (53). Similar to the Aklavik study, the EPIYA-ABC motif was the most frequently detected type, observed in 61% (51/84) of the Colombian cultures.

Evidence of more than one *vacA* type in gastric biopsy culture from a single participant was found in 9% of cultures (11/121). Similar proportions of cultures with more than one *vacA* type have been reported by others (54,55). Gatti *et al.,* reported that 12% of 89 gastric biopsies taken from Brazilian adult patients had more than one *vacA* type (55). Another study in Brazil reported that 17% of 165 gastric biopsies had more than one *H. pylori* strain upon typing for *vacA* (54). The nearly universal prevalence of the *vacA* gene detected in the Aklavik cultures was similar to estimates from studies conducted in other countries (56-59). Although the observed prevalence of *cagA* and *babA2* in *H. pylori* isolated from different regions varies, *vacA* prevalence is consistently high. In the present study, participants with cultures of the *vacA i1* subtype had approximately twice the prevalence odds of acute gastritis compared to participants with cultures of the *vacA i2* subtype. Winter *et al,* reported a strong association between *vacA i1* and precancerous intestinal metaplasia in mice (30). A meta-analysis that included adult populations infected with *H. pylori* isolated in four Southeast Asian countries (Vietnam, Thailand, Singapore, and Malaysia), estimated an association between *vacA m1,* compared to *vacA m2,* and increased prevalence odds of peptic ulcer disease (OR: 1.5, 95% CI: 1.0-2.1), but did not observe such an association when comparing *vacA s1* or *i1 with s2* or *i2,* respectively (60). A recent meta-analysis of 42 studies reported summary ORs indicating increased odds of duodenal ulcer when comparing *vacA s1* to *s2* (OR = 3.0, 95 % CI = 2.3–3.8), *m1* to *m2* (OR = 1.5, 95 %

CI = 1.1–2.0) and *s1m1* to *s2m2* (OR = 1.9, 95 % CI = 1.5–2.4) (61). In the Aklavik study, the estimated odds ratio for the presence of intestinal metaplasia was 2.3 (95% CI, 0.5-11.1) when *vacA s1* was compared to *vacA s2*. Only three Aklavik *H. pylori* cultures were obtained from participants with a diagnosed gastric ulcer, and therefore estimating the association between *vacA* type and peptic ulcer was not an objective of this study. Two participants with gastric ulcer had *H. pylori* cultures with only *vacA s1i2m2,* while the third participant had *vacA s1i1m2/s2i2m2,* suggesting the presence of more than one strain of *H. pylori* in the biopsy.

In the present study, the *babA2* gene was detected in nearly all Aklavik cultures (97%), so the association between the presence of the *babA2* gene and severe histopathology could not be estimated. However, participants with the unexpected amplicon size of *babA2* (248 bp) had 1.8 (95% CI: 0.8 – 4.0) times the odds of severe chronic gastritis relative to participants without the 248 bp amplicon size, raising the question of whether the expression of the *babA2* gene differed depending on the amplicon size. In the present study, the expression of the *babA2* gene was studied in two Aklavik cultures that represent each of the two amplicon sizes, with the expected amplicon size lacking *babA2* expression and the unexpected amplicon size expressing *babA2*. This observation provides some support for the hypothesis that the *babA2* amplicon size influences *babA2* expression, though this needs be examined in more cultures to generate enough evidence for drawing conclusions. However, cultures that lack the expressed form of *babA2* may have other adhesins that facilitate their adhesion and contribute to their virulence, which may obscure the effect of the *babA2* gene on histopathology outcomes. A recent meta-analysis of 38 studies from around the world including a total of 1,535 patients (63), showed that the prevalence of *babA2* and its association with clinical outcomes varied across studies (**Table 2.11**). Estimates of associations between the presence of the *babA2* gene and increased prevalence of gastric inflammation, peptic ulcer, gastric cancer or intestinal metaplasia were inconsistent across studies. A summary odds ratio of 2.1 (95% CI, 1.5-2.8) was estimated for

the association between *babA2* positivity and the prevalence odds of peptic ulcer disease (63).

The data used for this analysis come from a community-based research project that screened more than 50% of Aklavik residents for *H. pylori* infection by urea breath test. Of note, 39% of all research participants and 26% of participants with recovered *H. pylori* cultures were asymptomatic. Studying both symptomatic and asymptomatic participants allows for better understanding of the role of virulence factors as predictors of specific clinical outcomes. Most published studies on the association between *H. pylori* genotype and clinical outcomes have been restricted to patients seeking clinical attention because they suffer from symptoms of dyspepsia. Exclusion of asymptomatic participants in research on virulence factors may distort the estimates of the effects of virulence factors on specific clinical outcomes.

Although a large proportion of the residents of the small community of Aklavik participated in the community-based project, this analysis was limited by the small numbers in specific genotype and histopathology categories. There are other limitations to consider when interpreting these results. It is not possible to determine whether the infecting *H. pylori* organisms were replaced by others with distinct genotypes over time or whether the genotype of persistent *H. pylori* organisms changed over time within each individual. A participant may have been infected with a *cagA*-positive *H. pylori* strain and the bacteria may have lost *cagA* over the course of the infection after inducing damage to the gastric epithelium. Additionally, the present study estimated only crude odds ratios which did not control for potential confounding variables. More detailed analyses that identify potential confounders and control for confounding will be presented in future publications of the CAN*Help* Working Group. This future work will also estimate effects of a greater number of genotype profiles, using larger samples sizes achieved by the inclusion of data from other communities in the Yukon and Northwest Territories. Increasing the sample size and examining additional genotype profiles using multivariable analysis will yield more informative results

on the relationship between hypothesized *H. pylori* virulence factors and histopathology outcomes.

In conclusion, there is a high prevalence of *H. pylori* infection and selected gastric histopathology outcomes among participants in the Aklavik *H. pylori* Project. Acute and chronic gastritis were detected in more than 80% of research participants with *H. pylori* cultured from gastric biopsies. Investigation of potential precancerous lesions revealed that approximately one in five participants had atrophy while one in ten had intestinal metaplasia. Estimation of the association of three major virulence factors with gastric histopathology revealed that individuals with acute gastritis, atrophy or intestinal metaplasia had a higher frequency of infection with *cagA*-positive, *vacA i1* and/or *vacA m1*-positive *H. pylori* while individuals with chronic gastritis, atrophy or intestinal metaplasia had a higher frequency of infection with *vacA s1* or *babA2* 248 bp strains. The present study contributes to other work done by the CAN*Help* Working Group to address the concerns raised by community members regarding the perceived risk of gastric cancer.

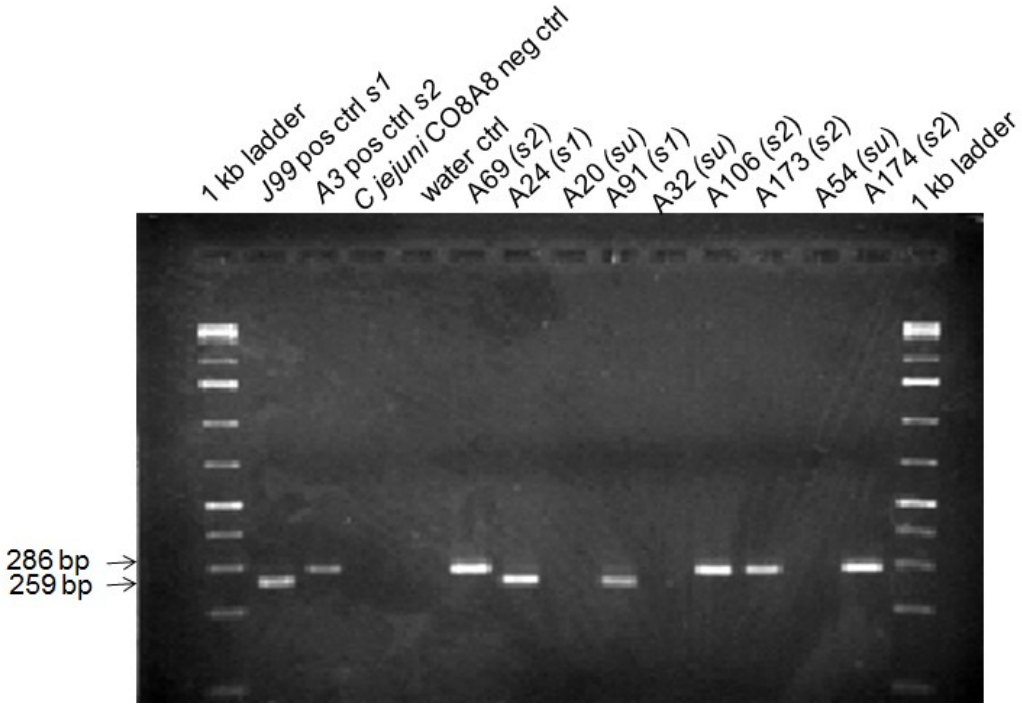


Figure 2.1 Typing of *H. pylori vacA* gene for *s*-region

A single primer pair was used to type the *vacA* gene for the *s*-region as described in section 2.2.4.3. The *vacA s1* type amplicon size is 259 bp, while the *vacA s2* type amplicon size is 286 bp. The *vacA su* (*s unknown*) type represents a non-typable *vacA* that could not be amplified with the primer pairs used in this study.

H. pylori J99 served as a positive control for the *vacA s1* type, while *H. pylori* A3 served as a positive control for the *vacA s2* type. *C. jejuni* CO848 served as a non-*H. pylori* DNA negative control. Water served as a no DNA negative control. "A" samples represent *H. pylori* isolated from residents of Aklavik. A 1 Kb plus DNA ladder served as a reference to estimate amplicon size.

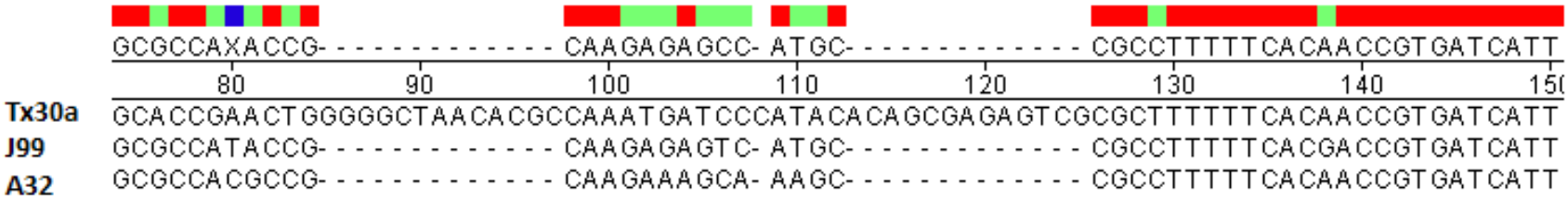


Figure 2.2 Sequence analysis of Aklavik *H. pylori* A32 *vacA su*

Sequence analysis (as described in section 2.2.4.4) of A32 *vacA su* (*s* type unknown by PCR) compared with *H. pylori* reference strains J99 *vacA s1* (NCBI Reference Sequence: NC_000921.1) and Tx30a *vacA s2* (ATCC 51932, GenBank U29401)

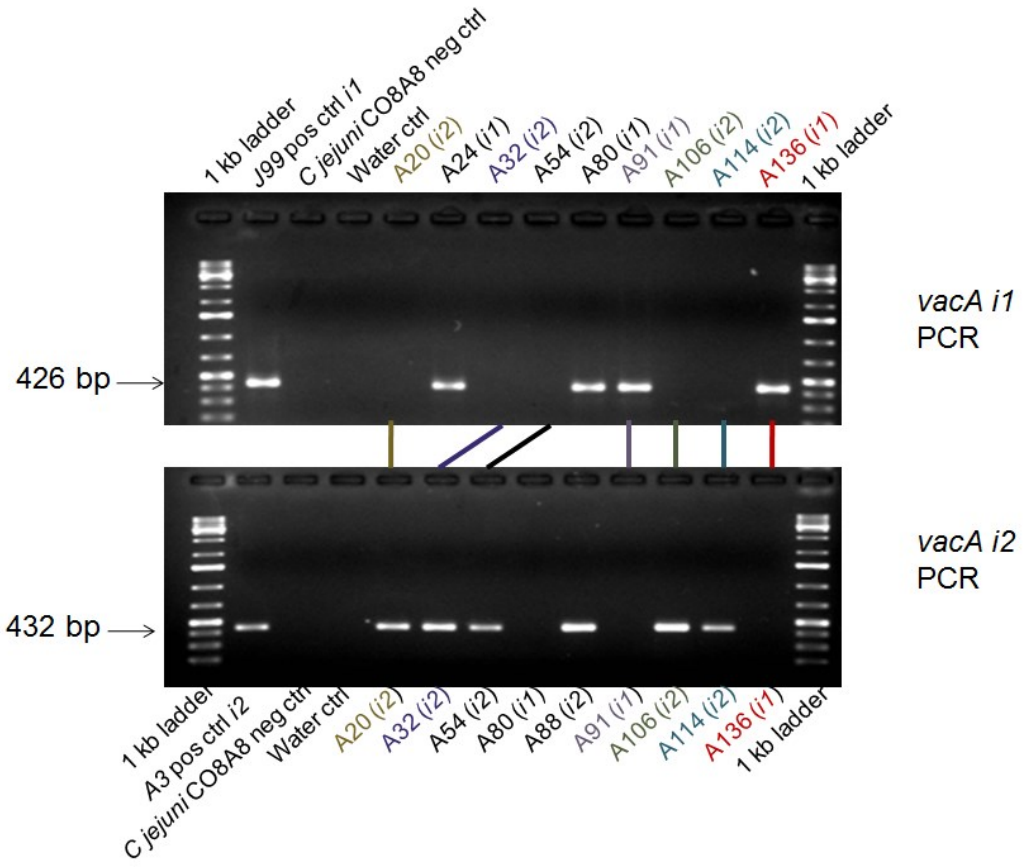


Figure 2.3 Typing of *vacA* gene for *i*-region

Two different primer pairs were used to type the *vacA* gene for the *i*-region as described in section 2.2.4.3. The amplicon size obtained by *vacA i1* PCR was 426 bp while the amplicon size obtained by *vacA i2* PCR was 432 bp. Mixed *H. pylori* cultures had amplicons for both *vacA i1*and *vacA i2* PCR.

H. pylori J99 served as a positive control for the *vacA i1* type, while *H. pylori* A3 served as a positive control for the *vacA i2* type. *C. jejuni* CO848 served as a non-*H. pylori* DNA negative control. Water served as a no DNA negative control. "A" samples represent *H. pylori* isolated from residents of Aklavik. A 1 Kb plus DNA ladder served as a reference to estimate amplicon size.

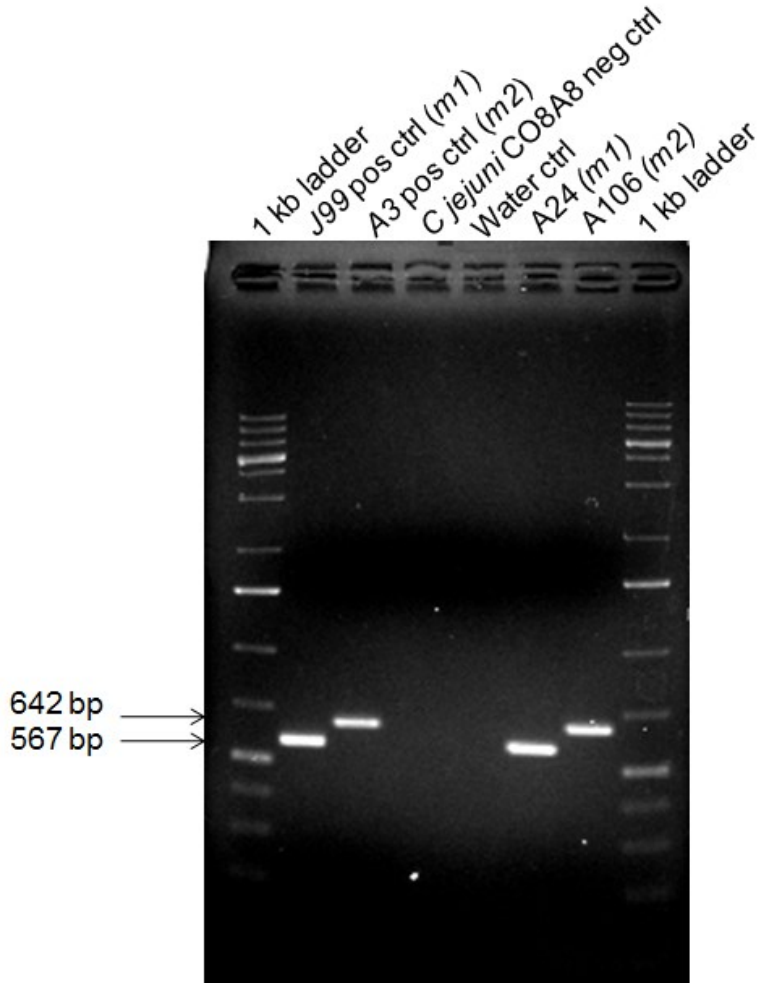


Figure 2.4 Typing of *vacA* gene for *m*-region

A single primer pair was used to type the *vacA* gene for the *m*-region as described in section 2.2.4.3. The *vacA m1* type amplicon size is 567 bp while the *vacA m2* type amplicon size is 642 bp. Mixed *H. pylori* cultures had amplicons for both *vacA m1* and *vacA m2*.

H. pylori J99 served as a positive control for the *vacA m1* type, while *H. pylori* A3 served as a positive control for the *vacA m2* type. *C. jejuni* CO848 served as a non-*H. pylori* DNA negative control. Water served as a no DNA negative control. "A" samples represent *H. pylori* isolated from residents of Aklavik. A 1 Kb plus DNA ladder served as a reference to estimate amplicon size.

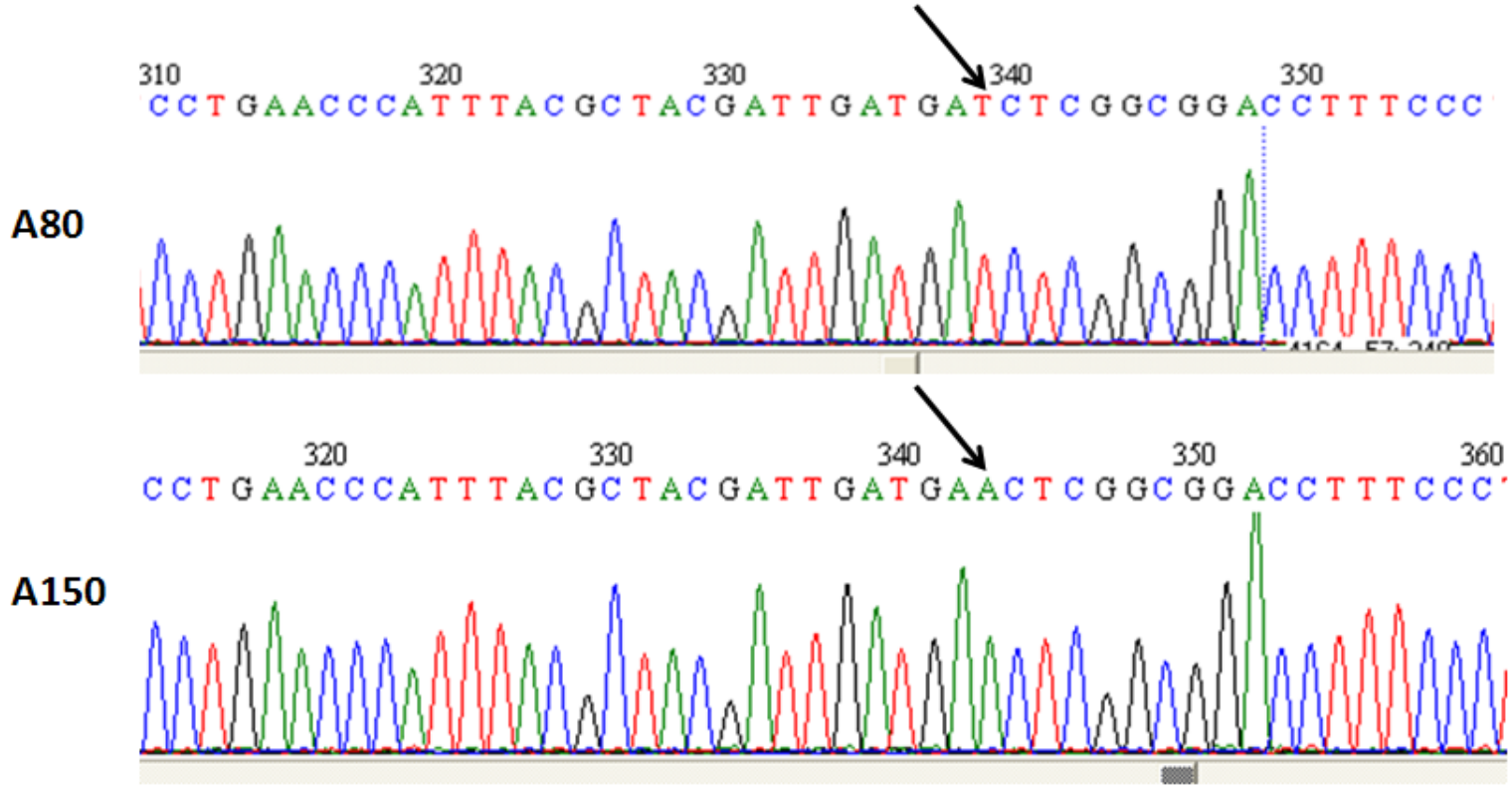


Figure 2.5 Sequence analysis of EPIYA regions of two Aklavik cultures

Sequence analysis (as described in section 2.2.4.4) of A150 (EPIYA-ABC$_{unknown}$ by PCR) revealed a difference in a single nucleotide (A) instead of (T) when compared with another Aklavik isolate (A80) that had EPIYA-ABC type.

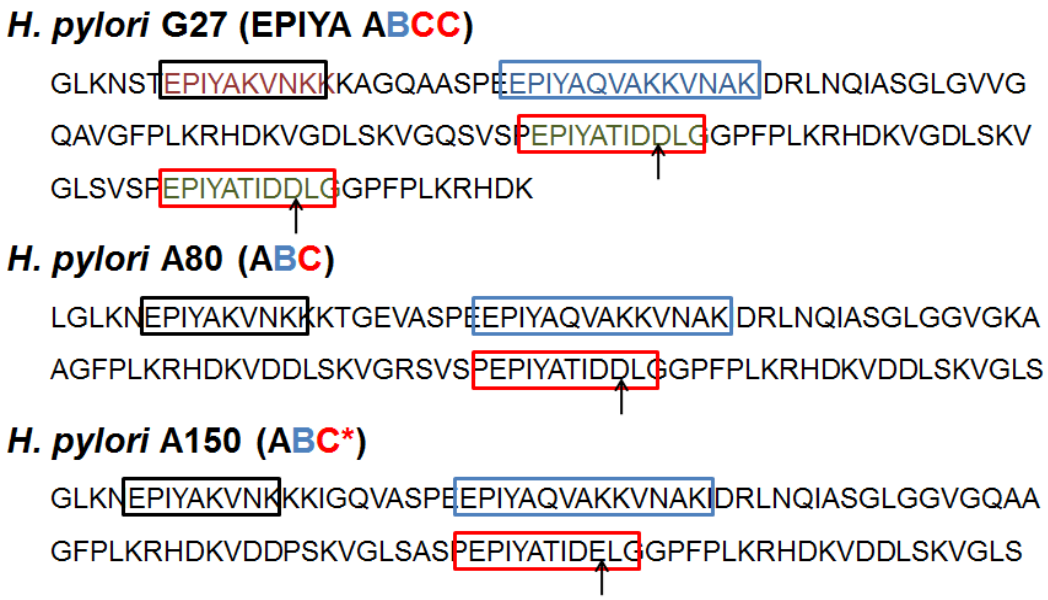


Figure 2.6 Amino acid sequences of EPIYA regions of reference *H. pylori* isolate G27 and two Aklavik cultures

Amino acid sequence analysis of A150 that could not be amplified using the EPIYA-C primers ($ABC_{unknown}$) revealed a difference in a single amino acid (glutamic acid instead of aspartic acid) in the C region (ABC*).

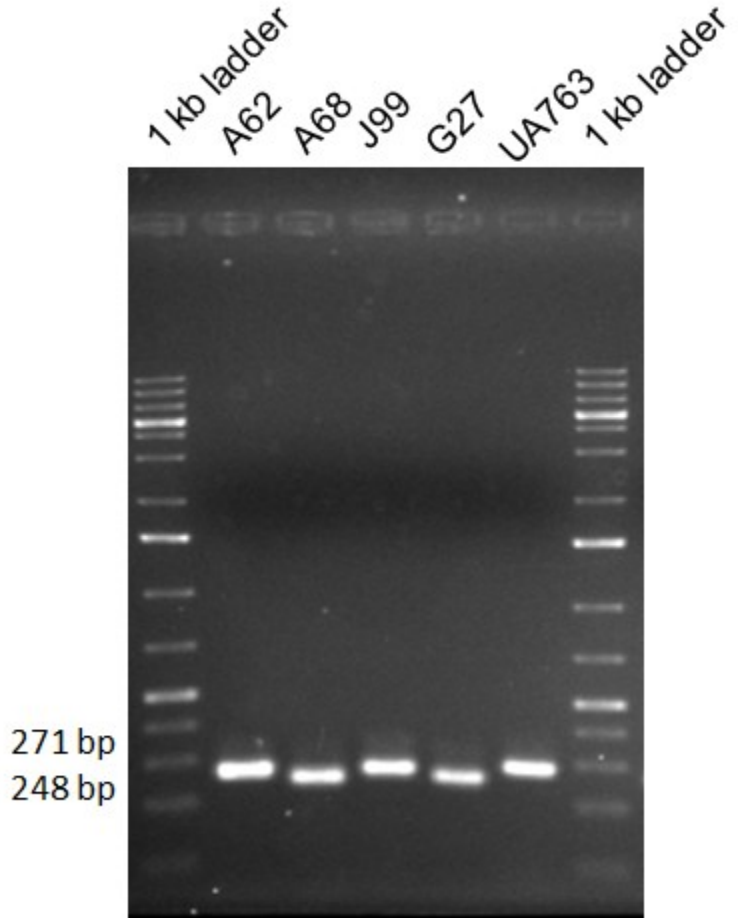
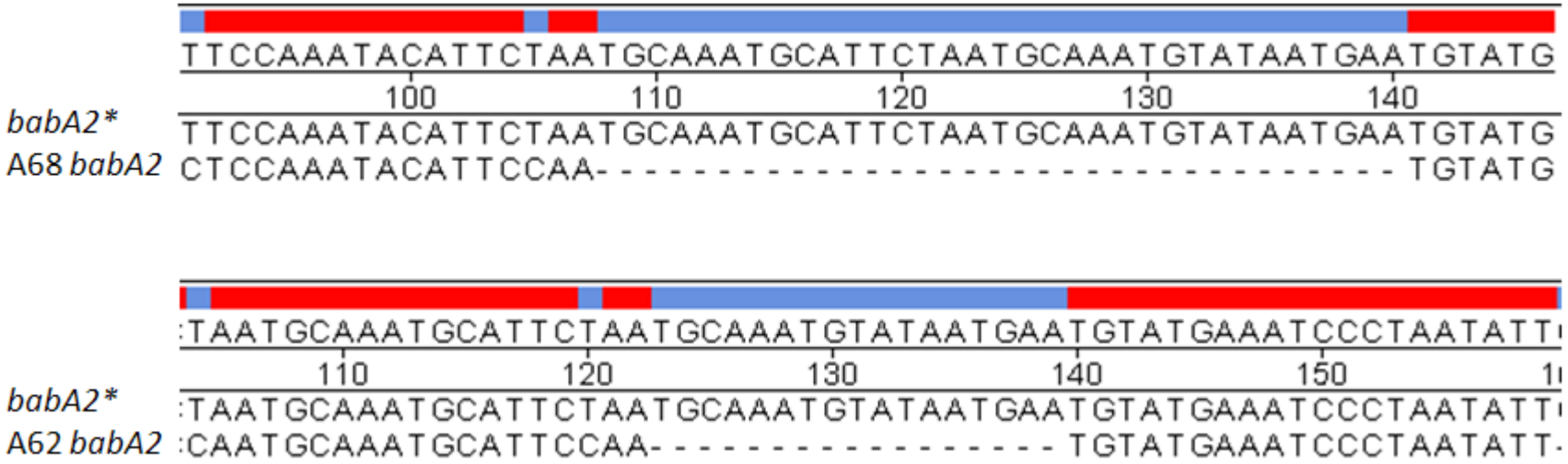


Figure 2.7 *babA2* gene amplification for *H. pylori* reference strains, Alberta and Aklavik cultures

The *babA2* gene was detected by PCR for reference strain G27, Alberta isolate UA 763, and Aklavik cultures as described in section 2.2.4.3. A 1 Kb plus DNA ladder served as a reference to estimate amplicon size.



***** *babA2* of *H. pylori* strain CCUG17875

Figure 2.8 Sequence analysis of *babA2* of two Aklavik *H. pylori* cultures

Sequence analysis (as described in section 2.2.4.4) of *babA2* of A62 and A68 indicate a deletion (represented by ------) of 13 bp and 33 bp, respectively. The sequences aligned with the *babA2* sequence published in GenBank (AF033654.1)

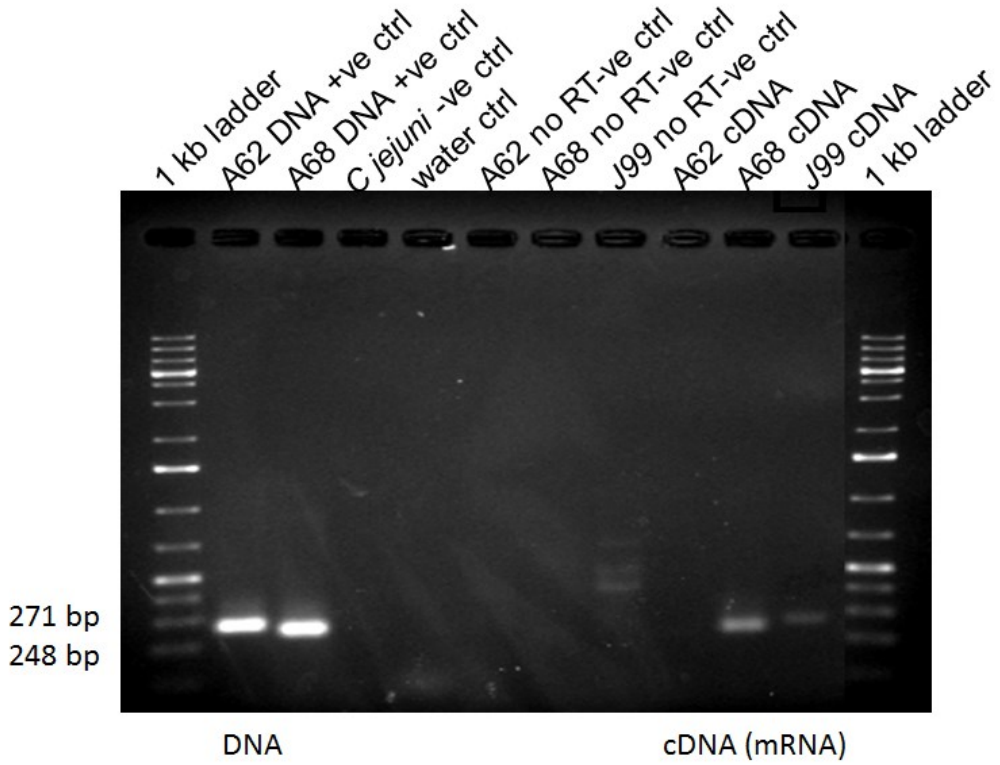


Figure 2.9 *babA2* expression of J99 (reference strain) and two Aklavik cultures (A62 and A68)

Expression of the *babA* gene was performed as described in section 2.2.5. Genomic DNA from Aklavik cultures A62 (271 bp) and A68 (248 bp) served as positive controls for the presence of the *babA2* gene. *C. jejuni* CO848 served as a non-*H. pylori* DNA negative control. Water served as a no DNA negative control. The "no-RT" negative controls did not contain reverse transcriptase and served as controls for the absence of genomic DNA. cDNA represents *babA2* mRNA transcripts assessed for reference strain *H. pylori* J99 (271 bp), A62 and A68. A 1 Kb plus DNA ladder served as a reference to estimate amplicon size.

Table 2.1 PCR primer sets used for Aklavik *H. pylori* genotyping

Gene	Primer	Sequence	Amplicon size (bp)	Source
16S rRNA	*HPF*	5'-GCGACCTGCTGGAACATTAC-3'	138	(64)
	HPR	5'-CGTTAGCTGCATTACTGGAGA-3'		
cagA	*cagP1*	5'-CCATTTTAAGCAACTCCATAAACC-3'	1027	(65)
	cagB1	5'-CTGCAAAAGATTGTTTGGCAGA-3'		
cagE	*cagE(f)*	5'-TTGAAAACTTCAAGGATAGGATAGAGC-3'	508	(66)
	cagE(r)	5'-GCCTAGCGTAATATCACCATTACCC-3'		
EPIYA	*cagA25305*	5'-GTTAARAATRGTAAYGG-3'	470-670	(43)
	cagA3000AS	5'-TTTAGCTTCTGATACCGC-3'		
EPIYA-A	*cagA28F*	5'-TTCTCAAAGGAGCAATTGGC-3'	264	(19)
	cagA-P1C	5'-GTCCTGTTTTCTTTTTATAACTTTAG-3'		
EPIYA-B	*cagA28F*	5'-TTCTCAAAGGAGCAATTGGC-3'	309	
	cagAP2CG	5'-TTTAGCAACTTGAGGGTAAATGGG-3'		
EPIYA-C	*cagA28F*	5'-TTCTCAAAGGAGCAATTGGC-3'	465-498	
	cagA-P3E	5'-ATCAATCGTAGCGTAAATGGG-3'		
vacA s	*VA1-F*	5'-ATGGAAATACAACAAACACAC-3'	259 (s1)	(26)
	VA1-R	5'-CTGCTTGAATGCGCCAAAC-3'	286 (s2)	
vacA i1	*VacF1(+)*	5'-GTTGGGATTGGGGGAATGCCG-3'		(29)
	C1R (-)	5'-TTAATTTAACGCTGTTTGAAG-3'	426 (i1)	
vacA i2	*C2R (-)*	5'-GATCAACGCTCTGATTTGA-3'	432 (i2)	
vacA m	*VAG-F*	5'-CAATCTGTCCAATCAAGCGAG-3'	567 (m1)	(25)
	VAG-R	5'-GCGTCAAAATAATTCCAAGG-3'	642 (m2)	
babA2	*babA-F*	5'-CCA AAC GAA ACA AAA AGC GT-3'	271	(67)
	babA-R	5'-GCT TGT GTA AAA GCC GTC GT-3'		
babA2	*babA2(+)*	5′-AATCCAAAAAGGAGAAAAAGTATGAAA-3′	850	(14)
	babA2(-)	5′-TGTTAGTGATTTCGGTGTAGGACA-3′		

Table 2.2 PCR reaction conditions used for Aklavik *H. pylori* genotyping

Gene	Denaturation	Annealing	Extension	N of cycles	Source
	Temperature °C (Time)				
16S rRNA	95 (30 sec)	60 (1 min)	72 (1 min)	35	(64)
cagA	94 (30 sec)	50 (45 sec)	72 (45 sec)	40	(65)
cagE	94 (45 sec)	53 (30 sec)	72 (90 sec)	35	(66)
EPIYA	94 (30 sec)	50 (45 sec)	72 (45 sec)	35	(43)
EPIYA	94 (1 min)	45 (1 min)	72 (1 min)	35	(19)
vacA s	94 (1 min)	52 (1 min)	72 (1 min)	35	(26)
vacA i	95 (30 sec)	52 (1 min)	72 (1 min)	35	(29)
vacA m	94 (1 min)	57 (1 min)	72 (1 min)	35	(25)
babA2	94 (1 min)	45 (1 min)	72 (1 min)	30	(67)
babA2	94 (1 min)	45 (1 min)	72 (1 min)	30	(14)

Table 2.3 Genotype distribution of 121 Aklavik *H. pylori* cultures

Genotype	Aklavik *H. pylori* cultures	
	n	% of 121
cagA+	43	36
cagE+	38	31
babA2+	117	97
vacA+	116	96
vacA type		
s1/i1/m1	27	22
s1/i2/m2	44	36
s2/i2/m2	29	24
s1/i1/m2	1	1
s2/i1/m2	3	2
s1/i2/m1	1	1
s1/i1/m1 + s1/i1/m2	1	1
s2/i1/m2 + s2/i2/m2	2	2
s1/i1/m1 + s1/i2/m1 + s1/i1/m2 + s1/i2/m2	8	7
Presence of *vacA* alleles hypothesized to be more virulent, with or without other alleles		
s1	82	68
*i1**	32	26
*m1**	28	23

*10 participants with *i1* also had an *i2* strain; 9 participants with *m1* also had an *m2* strain

Table 2.4 Distribution of gastritis severity, atrophy and intestinal metaplasia among research participants

Histopathology	Distribution	
	n (total)	%
Acute gastritis	96 (119)	81
Absent	23	19
Mild	56	47
Moderate	32	27
Severe	8	7
Chronic gastritis	102 (121)	84
Absent	19	16
Mild	9	7
Moderate	44	36
Severe	49	40
Gastric atrophy	26 (121)	21
Absent	95	79
Mild	23	19
Moderate	2	2
Severe	1	1
Intestinal metaplasia	11 (121)	9
Absent	110	91
Mild	7	6
Moderate	3	2
Severe	1	1

Table 2.5 Acute gastritis severity by *H. pylori* genotype

Genotype			Acute Gastritis Severity			
			Severe	Moderate	Mild	Absent
		Total n	%	%	%	%
		119	7	27	47	19
cagA	absent	77	9	18	53	19
	present	42	2	43	36	19
vacAs	*s2*	33	6	24	45	24
	s1	81	6	28	48	17
vacAi	*i2 only*	73	8	19	55	18
	i2 +/- others	82	7	21	52	20
	i1 only	32	3	44	34	19
	i1 +/- others	41	2	41	34	22
vacAm	*m2 only*	78	8	22	53	18
	m2 +/- others	86	7	23	51	19
	m1 only	28	4	39	36	21
	m1 +/- others	36	3	39	36	22
vacA	absent	5	20	20	40	20
	s1i1m1only	27	4	37	37	22
	s1i1m1+/- others	35	3	37	37	23
	s1i1 only	29	3	38	38	21
	s1i1+/- others	36	3	39	36	22
	s1m1only	28	4	39	36	21
	s1m1+/- others	36	3	39	36	22
	i1m1 only	27	4	37	37	22
	i1m1+/- others	35	3	37	37	23
	s2i2m2	28	7	18	50	25
	s2i2m2+/- others	30	7	17	50	27
	s2i2	28	7	18	50	25
	s2i2+/- others	30	7	17	50	27
	s2m2	33	6	24	45	24
	s2m2+/- others	33	6	24	45	24
	i2m2	72	8	18	56	18
	i2m2+/- others	81	7	20	53	20
babA2	absent	4	0	25	75	0
	271	31	0	39	32	29
	248	83	10	23	51	17
	present*	1	0	0	100	0

* identified positive by another primer pair

Table 2.6 Chronic gastritis severity by *H. pylori* genotype

Genotype			Chronic Gastritis Severity			
			Severe	Moderate	Mild	Absent
	Total	n	%	%	%	%
		121	40	36	7	16
cagA	absent	78	38	40	5	17
	present	43	44	30	12	14
vacAs	*s2*	34	41	32	9	18
	s1	82	40	38	7	15
vacAi	*i2 only*	74	38	42	4	16
	i2 +/- others	84	37	40	6	17
	i1 only	32	50	25	13	13
	i1 +/- others	42	45	26	14	14
vacAm	*m2 only*	79	39	41	5	15
	m2 +/- others	88	40	39	6	16
	m1 only	28	43	29	14	14
	m1 +/- others	37	43	27	14	16
vacA	absent	5	40	40	0	20
	s1i1m1only	27	48	26	11	15
	s1i1m1+/- others	36	47	25	11	17
	s1i1 only	29	52	24	10	14
	s1i1+/- others	37	47	24	11	16
	s1m1only	28	46	29	11	14
	s1m1+/- others	37	46	27	11	16
	i1m1 only	27	48	26	11	15
	i1m1+/- others	36	47	25	11	17
	s2i2m2	29	41	31	7	21
	s2i2m2+/- others	31	39	32	10	19
	s2i2	29	41	31	7	21
	s2i2+/- others	31	39	32	10	19
	s2m2	34	44	34	9	19
	s2m2+/- others	34	44	34	9	19
	i2m2	73	38	41	4	16
	i2m2+/- others	83	37	40	6	17
babA2	absent	4	25	75	0	0
	271	31	35	29	16	19
	248	85	44	36	5	15
	present*	1	0	100	0	0

* identified positive by another primer pair

Table 2.7 Gastric atrophy severity by *H. pylori* genotype

Genotype			Gastric Atrophy Severity			
			Severe	Moderate	Mild	Absent
	Total	n	%	%	%	%
		121	1	2	19	79
cagA	absent	78	1	1	15	82
	present	43	0	2	26	72
vacA s	*s2*	34	3	0	18	79
	s1	82	0	2	21	77
vacA i	*i2 only*	74	1	1	19	78
	i2 +/- others	84	1	2	18	79
	i1 only	32	0	0	25	75
	i1 +/- others	42	0	2	21	76
vacA m	*m2 only*	79	1	1	18	80
	m2 +/- others	88	1	2	17	80
	m1 only	28	0	0	29	71
	m1 +/- others	37	0	3	24	73
vacA	absent	5	0	0	0	100
	s1i1m1only	27	0	0	26	74
	s1i1m1+/- others	36	0	3	22	75
	s1i1 only	29	0	0	24	76
	s1i1+/- others	37	0	3	22	76
	s1m1only	28	0	0	29	71
	s1m1+/- others	37	0	3	24	73
	i1m1 only	27	0	0	26	74
	i1m1+/- others	36	0	3	22	75
	s2i2m2	29	3	0	17	79
	s2i2m2+/- others	31	3	0	16	81
	s2i2	29	3	0	17	79
	s2i2+/- others	31	3	0	16	81
	s2m2	34	3	0	18	79
	s2m2+/- others	34	3	0	18	79
	i2m2	73	1	1	18	79
	i2m2+/- others	83	1	2	17	80
babA2	absent	4	0	0	0	100
	271	31	0	0	23	77
	248	85	1	2	19	78
	present*	1	0	0	0	100

* identified positive by another primer pair

Table 2.8 Intestinal metaplasia severity by *H. pylori* genotype

Genotype			Intestinal Metaplasia Severity			
			Severe	Moderate	Mild	Absent
		Total	n	%	%	%
			121	1	2	6	91
cagA	absent		78	1	3	3	94
	present		43	0	2	12	86
vacA s	*s2*		34	3	0	3	94
	s1		82	0	4	7	89
vacA i	*i2 only*		74	1	3	5	91
	i2 +/- others		84	1	4	5	90
	i1 only		32	0	0	9	91
	i1 +/- others		42	0	2	7	90
vacA m	*m2 only*		79	1	3	5	91
	m2 +/- others		88	1	3	5	91
	m1 only		28	0	0	11	89
	m1 +/- others		37	0	3	8	89
vacA	absent		5	0	0	0	100
	s1i1m1only		27	0	0	19	81
	s1i1m1+/- others		36	0	3	14	83
	s1i1 only		29	0	0	17	83
	s1i1+/- others		37	0	3	14	84
	s1m1only		28	0	0	18	82
	s1m1+/- others		37	0	3	14	84
	i1m1 only		27	0	0	19	81
	i1m1+/- others		36	0	3	14	83
	s2i2m2		29	3	0	3	93
	s2i2m2+/- others		31	3	0	3	94
	s2i2		29	3	0	3	93
	s2i2+/- others		31	3	0	3	94
	s2m2		34	3	0	3	94
	s2m2+/- others		34	3	0	3	94
	i2m2		73	1	3	5	90
	i2m2+/- others		83	1	4	5	90
babA2	absent		4	0	0	0	100
	271		31	0	0	6	94
	248		85	1	4	5	91
	present*		1	0	0	100	0

* identified positive by another primer pair

Table 2.9 Estimated effects of genotype on the prevalence odds of acute gastritis and chronic gastritis

Genotype		Total n	Acute Gastritis Severity: moderate/severe n	Acute Gastritis Severity: absent/mild n	OR	95% CI
cagA	absent	77	21	56	1.0	
	present	42	19	23	2.2	1.0 - 4.8
vacA s	*s1* absent	38	12	26	1.0	
	s1 present	81	28	53	1.1	0.5 - 2.6
vacAi	*i1* absent	78	22	56	1.0	
	i1 present	41	18	23	2.0	0.9 - 4.4
vacAm	*m1* absent	83	25	58	1.0	
	m1 present	36	15	21	1.7	0.7 - 3.7
babA2	248 absent	36	13	23	1.0	
	248 present	83	27	56	0.9	0.4 - 1.9
			Chronic Gastritis Severity			
cagA	absent	78	61	17	1.0	
	present	43	32	11	0.8	0.3 - 1.9
vacA s	*s1* absent	39	29	10	1.0	
	s1 present	82	64	18	1.2	0.5 - 3.0
vacAi	*i1* absent	79	63	16	1.0	
	i1 present	42	30	12	0.6	0.3 - 1.5
vacAm	*m1* absent	84	67	17	1.0	
	m1 present	37	26	11	0.6	0.3 - 1.5
babA2	248 absent	36	25	11	1.0	
	248 present	85	68	17	1.8	0.7 - 4.3

Table 2.9a Estimated effects of genotype on the prevalence odds of chronic gastritis using an alternate categorization

Genotype		Total n	Chronic Gastritis Severity		OR	95% CI
			severe	moderate/mild/absent		
			n	n		
cagA	absent	78	30	48	1.0	
	present	43	19	24	1.3	0.6 - 2.7
vacA s	*s1* absent	39	16	23	1.0	
	s1 present	82	33	49	1.0	0.4 - 2.1
vacAi	*i1* absent	79	30	49	1.0	
	i1 present	42	19	23	1.3	0.6 - 2.9
vacAm	*m1* absent	84	33	51	1.0	
	m1 present	37	16	21	1.2	0.5 - 2.6
babA2	248 absent	36	11	25	1.0	
	248 present	85	37	48	1.8	0.8 - 4.0

Table 2.10 Estimated effects of genotype on the prevalence odds of gastric atrophy and intestinal metaplasia

Genotype		Total n	Gastric Atrophy Severity: Present n	Absent n	OR	95% CI
cagA	absent	78	14	64	1.0	
	present	43	12	31	1.8	0.7-4.3
vacA s	*s1* absent	39	7	32	1.0	
	s1 present	82	19	63	1.4	0.5-3.6
vacAi	*i1* absent	79	16	63	1.0	
	i1 present	42	10	32	1.2	0.5-3.0
vacAm	*m1* absent	84	16	68	1.0	
	m1 present	37	10	27	1.6	0.6-3.9
babA2	248 absent	36	7	29	1.0	
	248 present	85	19	66	1.2	0.5-3.1
Intestinal Metaplasia Severity						
cagA	absent	78	5	73	1.0	
	present	43	6	37	2.4	0.7-8.3
vacA s	*s1* absent	39	2	37	1.0	
	s1 present	82	9	73	2.3	0.5-11.1
vacAi	*i1* absent	79	7	72	1.0	
	i1 present	42	4	38	1.1	0.3-3.9
vacAm	*m1* absent	84	7	77	1.0	
	m1 present	37	4	33	1.3	0.4-4.9
babA2	248 absent	36	3	33	1.0	
	248 present	85	8	77	1.1	0.3-4.6

Table 2.11 Prevalence of *cagA*-positivity among people with *H. pylori* infection in studies conducted in different countries

Location of study	Prevalence of *cagA*-positive *H. pylori* (%)	n/total	Reported positive association of clinical outcomes with *cagA*-positivity	Reference
Alaska	85	242/286	None	(68)
Brazil	30	16/54	None	(49)
Colombia	66	114/174	None	(69)
Russia	79	135/170	Gastric cancer and peptic ulcer	(70)
Iran	69	79/115	None	(71)
Iraq	73	112/154	Peptic ulcer	(72)
China	61	86/141	Gastric cancer	(73)
Japan	100	169/169	None	(69)
Gambia	61	74/121	Gastric disease	(74)
South Africa	95	56/59	None	(75)
Poland	61	79/130	Duodenal ulcer	(76)
Slovenia	61	101/165	Acute and chronic inflammation	(77)
Italy	72	139/193	Gastric cancer and peptic ulcer	(78)
Spain	959	103/176	None	(79)
Netherlands	67	63/94	Peptic ulcer	(80)

Table 2.12 Prevalence of *babA2*-positivity among people with *H. pylori* infection in studies conducted in different countries

Location of study	Prevalence of *babA2*- positive *H. pylori*		Reported positive association of clinical outcomes with *babA2*-positivity	Reference
	(%)	n/total		
Brazil	46	96/208	Duodenal ulcer & gastric cancer	(81)
Mexico	22	31/143	None	(82)
Cuba	82	107/130	Duodenal ulcer	(59)
Iran	41	65/160	Gastric cancer	(83)
Iraq	45	69/154	Peptic ulcer disease	(72)
China	80	83/104	Lymphocytic infiltration & atrophy	(84)
Japan	85	152/179	None	(85)
Thailand	92	103/112	None	(86)
Poland	23	30/130	Duodenal ulcer	(76)
Slovenia	48	78/163	Gastric inflammation	(87)
Italy	36	60/167	Intestinal metaplasia	(28)
Germany	72	82/114	Duodenal ulcer & adenocarcinoma	(14)

2.5 References

(1) Marshall BJ, Warren JR. Unidentified curved bacilli in the stomach of patients with gastritis and peptic ulceration. Lancet 1984 Jun 16;1(8390):1311-1315.

(2) Warren JR, Marshall BJ. Unidentified curved bacilli on gastric epithelium in active chronic gastritis. Lancet 1983 Jun 4;1(8336):1273-1275.

(3) Brown LM. *Helicobacter pylori*: epidemiology and routes of transmission. Epidemiol Rev 2000;22(2):283-297.

(4) Suerbaum S, Michetti P. *Helicobacter pylori* infection. N Engl J Med 2002 Oct 10;347(15):1175-1186.

(5) Colquhoun A, Arnold M, Ferlay J, Goodman KJ, Forman D, Soerjomataram I. Global patterns of cardia and non-cardia gastric cancer incidence in 2012. Gut 2015 Mar 6.

(6) Cheung J, Goodman K, Munday R, Heavner K, Huntington J, Morse J, et al. *Helicobacter pylori* infection in Canada's arctic: searching for the solutions. Can J Gastroenterol 2008 Nov;22(11):912-916.

(7) Cheung J, Goodman KJ, Girgis S, Bailey R, Morse J, Fedorak RN, et al. Disease manifestations of *Helicobacter pylori* infection in Arctic Canada: using epidemiology to address community concerns. BMJ Open 2014 Jan 8;4(1):e003689-2013-003689.

(8) Department of Health and Social Services, Government of the Northwest Territories Cancer in the Northwest Territories, 1990-2000. A descriptive report. 2003.

(9) Parkinson AJ, Gold BD, Bulkow L, Wainwright RB, Swaminathan B, Khanna B, et al. High prevalence of *Helicobacter pylori* in the Alaska native population and association with low serum ferritin levels in young adults. Clin Diagn Lab Immunol 2000 Nov;7(6):885-888.

(10) Carraher S, Chang HJ, Munday R, Goodman KJ, CAN*Help* Working Group. *Helicobacter pylori* incidence and re-infection in the Aklavik *H. pylori* Project. Int J Circumpolar Health 2013 Aug 5;72:10.3402/ijch.v72i0.21594. eCollection 2013.

(11) Sgouros SN, Bergele C. Clinical outcome of patients with *Helicobacter pylori* infection: the bug, the host, or the environment? Postgrad Med J 2006 May;82(967):338-342.

(12) Hatakeyama M. The role of *Helicobacter pylori* CagA in gastric carcinogenesis. Int J Hematol 2006 Nov;84(4):301-308.

(13) Wada A, Yamasaki E, Hirayama T. *Helicobacter pylori* vacuolating cytotoxin, VacA, is responsible for gastric ulceration. J Biochem 2004 Dec;136(6):741-746.

(14) Gerhard M, Lehn N, Neumayer N, Boren T, Rad R, Schepp W, et al. Clinical relevance of the *Helicobacter pylori* gene for blood-group antigen-binding adhesin. Proc Natl Acad Sci U S A 1999 Oct 26;96(22):12778-12783.

(15) Censini S, Lange C, Xiang Z, Crabtree JE, Ghiara P, Borodovsky M, et al. *cag*, a pathogenicity island of *Helicobacter pylori*, encodes type I-specific and disease-associated virulence factors. Proc Natl Acad Sci U S A 1996 Dec 10;93(25):14648-14653.

(16) Segal ED, Lange C, Covacci A, Tompkins LS, Falkow S. Induction of host signal transduction pathways by *Helicobacter pylori*. Proc Natl Acad Sci U S A 1997 Jul 8;94(14):7595-7599.

(17) Covacci A, Censini S, Bugnoli M, Petracca R, Burroni D, Macchia G, et al. Molecular characterization of the 128-kDa immunodominant antigen of *Helicobacter pylori* associated with cytotoxicity and duodenal ulcer. Proc Natl Acad Sci U S A 1993 Jun 15;90(12):5791-5795.

(18) Argent RH, Kidd M, Owen RJ, Thomas RJ, Limb MC, Atherton JC. Determinants and consequences of different levels of CagA phosphorylation for clinical isolates of *Helicobacter pylori*. Gastroenterology 2004 Aug;127(2):514-523.

(19) Argent RH, Zhang Y, Atherton JC. Simple method for determination of the number of *Helicobacter pylori* CagA variable-region EPIYA tyrosine phosphorylation motifs by PCR. J Clin Microbiol 2005 Feb;43(2):791-795.

(20) Higashi H, Tsutsumi R, Fujita A, Yamazaki S, Asaka M, Azuma T, et al. Biological activity of the *Helicobacter pylori* virulence factor CagA is determined by variation in the tyrosine phosphorylation sites. Proc Natl Acad Sci U S A 2002 Oct 29;99(22):14428-14433.

(21) Hayashi T, Morohashi H, Hatakeyama M. Bacterial EPIYA effectors-where do they come from? What are they? Where are they going? Cell Microbiol 2013 Mar;15(3):377-385.

(22) Azuma T, Yamakawa A, Yamazaki S, Fukuta K, Ohtani M, Ito Y, et al. Correlation between variation of the 3' region of the *cagA* gene in *Helicobacter pylori* and disease outcome in Japan. J Infect Dis 2002 Dec 1;186(11):1621-1630.

(23) Lawrence BP, Brown WJ. Autophagic vacuoles rapidly fuse with pre-existing lysosomes in cultured hepatocytes. J Cell Sci 1992 Jul;102 (Pt 3)(Pt 3):515-526.

(24) Jafari F, Shokrzadeh L, Dabiri H, Baghaei K, Yamaoka Y, Zojaji H, et al. *vacA* genotypes of *Helicobacter pylori* in relation to *cagA* status and clinical outcomes in Iranian populations. Jpn J Infect Dis 2008 Jul;61(4):290-293.

(25) Atherton JC, Cover TL, Twells RJ, Morales MR, Hawkey CJ, Blaser MJ. Simple and accurate PCR-based system for typing vacuolating cytotoxin alleles of *Helicobacter pylori*. J Clin Microbiol 1999 Sep;37(9):2979-2982.

(26) Atherton JC, Cao P, Peek RM,Jr, Tummuru MK, Blaser MJ, Cover TL. Mosaicism in vacuolating cytotoxin alleles of *Helicobacter pylori*. Association of specific *vacA* types with cytotoxin production and peptic ulceration. J Biol Chem 1995 Jul 28;270(30):17771-17777.

(27) Miehlke S, Kirsch C, Agha-Amiri K, Gunther T, Lehn N, Malfertheiner P, et al. The *Helicobacter pylori vacA s1, m1* genotype and *cagA* is associated with gastric carcinoma in Germany. Int J Cancer 2000 Aug 1;87(3):322-327.

(28) Zambon CF, Navaglia F, Basso D, Rugge M, Plebani M. *Helicobacter pylori babA2, cagA*, and *s1 vacA* genes work synergistically in causing intestinal metaplasia. J Clin Pathol 2003 Apr;56(4):287-291.

(29) Rhead JL, Letley DP, Mohammadi M, Hussein N, Mohagheghi MA, Eshagh Hosseini M, et al. A new *Helicobacter pylori* vacuolating cytotoxin determinant, the intermediate region, is associated with gastric cancer. Gastroenterology 2007 Sep;133(3):926-936.

(30) Winter JA, Letley DP, Cook KW, Rhead JL, Zaitoun AA, Ingram RJ, et al. A role for the vacuolating cytotoxin, VacA, in colonization and *Helicobacter pylori*-induced metaplasia in the stomach. J Infect Dis 2014 Sep 15;210(6):954-963.

(31) Ogiwara H, Graham DY, Yamaoka Y. *vacA* i-region subtyping. Gastroenterology 2008 Apr;134(4):1267; author reply 1268.

(32) Ilver D, Arnqvist A, Ogren J, Frick IM, Kersulyte D, Incecik ET, et al. *Helicobacter pylori* adhesin binding fucosylated histo-blood group antigens revealed by retagging. Science 1998 Jan 16;279(5349):373-377.

(33) Boren T, Falk P, Roth KA, Larson G, Normark S. Attachment of *Helicobacter pylori* to human gastric epithelium mediated by blood group antigens. Science 1993 Dec 17;262(5141):1892-1895.

(34) Alm RA, Bina J, Andrews BM, Doig P, Hancock RE, Trust TJ. Comparative genomics of *Helicobacter pylori*: analysis of the outer membrane protein families. Infect Immun 2000 Jul;68(7):4155-4168.

(35) Mahdavi J, Sonden B, Hurtig M, Olfat FO, Forsberg L, Roche N, et al. *Helicobacter pylori* SabA adhesin in persistent infection and chronic inflammation. Science 2002 Jul 26;297(5581):573-578.

(36) Peck B, Ortkamp M, Diehl KD, Hundt E, Knapp B. Conservation, localization and expression of HopZ, a protein involved in adhesion of *Helicobacter pylori*. Nucleic Acids Res 1999 Aug 15;27(16):3325-3333.

(37) Odenbreit S, Till M, Hofreuter D, Faller G, Haas R. Genetic and functional characterization of the *alpAB* gene locus essential for the adhesion of *Helicobacter pylori* to human gastric tissue. Mol Microbiol 1999 Mar;31(5):1537-1548.

(38) Stolte M, Meining A. The updated Sydney system: classification and grading of gastritis as the basis of diagnosis and treatment. Can J Gastroenterol 2001 Sep;15(9):591-598.

(39) Reoch J. The Decomposition of Urea. J Anat Physiol 1875 May;9(Pt 2):368-385.

(40) MacFaddin JF. Biochemical tests for identification of medical bacteria. 3rd ed. ed. Philadelphia, PA: Lippincott Williams & Wilkins; 2000.

(41) Gordon, J. and McLeod, J.W. The practical application of the direct oxidase reaction in bacteriology. The Journal of Pathology and Bacteriology 1928;31(2):185.

(42) Barbas III CF, Burton, D.R. Scott, J.K. and Silverman, G.J. Quantification of DNA and RNA. Cold Spring Harbor Protocol 2007.

(43) Panayotopoulou EG, Sgouras DN, Papadakos K, Kalliaropoulos A, Papatheodoridis G, Mentis AF, et al. Strategy to characterize the number and type of repeating EPIYA phosphorylation motifs in the carboxyl terminus of CagA protein in *Helicobacter pylori* clinical isolates. J Clin Microbiol 2007 Feb;45(2):488-495.

(44) Oudbier JH, Langenberg W, Rauws EA, Bruin-Mosch C. Genotypical variation of *Campylobacter pylori* from gastric mucosa. J Clin Microbiol 1990 Mar;28(3):559-565.

(45) Heiner CR, Hunkapiller KL, Chen SM, Glass JI, Chen EY. Sequencing multimegabase-template DNA with BigDye terminator chemistry. Genome Res 1998 May;8(5):557-561.

(46) Jacobson K. The changing prevalence of *Helicobacter pylori* infection in Canadian children: should screening be performed in high-risk children? Can J Gastroenterol 2005 Jul;19(7):412-414.

(47) Sethi A, Chaudhuri M, Kelly L, Hopman W. Prevalence of *Helicobacter pylori* in a First Nations population in northwestern Ontario. Can Fam Physician 2013 Apr;59(4):e182-7.

(48) Goodman KJ, Jacobson K, Veldhuyzen van Zanten S. *Helicobacter pylori* infection in Canadian and related Arctic Aboriginal populations. Can J Gastroenterol 2008 Mar;22(3):289-295.

(49) Oliveira JG, Ferreira CH, Camerin AC, Rota CA, Meurer L, Silveira TR. Prevalence of infection with *cagA*-positive *Helicobacter pylori* strains among children and adolescents in southern Brazil. Arq Gastroenterol 2014 Sep;51(3):180-185.

(50) Pereira WN, Ferraz MA, Zabaglia LM, de Labio RW, Orcini WA, Bianchi Ximenez JP, et al. Association among *H. pylori* virulence markers *dupA*, *cagA* and *vacA* in Brazilian patients. J Venom Anim Toxins Incl Trop Dis 2014 Jan 23;20(1):1.

(51) Matos JI, de Sousa HA, Marcos-Pinto R, Dinis-Ribeiro M. *Helicobacter pylori* CagA and VacA genotypes and gastric phenotype: a meta-analysis. Eur J Gastroenterol Hepatol 2013 Dec;25(12):1431-1441.

(52) Kersulyte D, Bertoli MT, Tamma S, Keelan M, Munday R, Geary J, et al. Complete Genome Sequences of Two *Helicobacter pylori* Strains from a Canadian Arctic Aboriginal Community. Genome Announc 2015 Apr 16;3(2):10.1128/genomeA.00209-15.

(53) Fajardo CA, Quiroga AJ, Coronado A, Labrador K, Acosta N, Delgado P, et al. CagA EPIYA polymorphisms in Colombian *Helicobacter pylori* strains and their influence on disease-associated cellular responses. World J Gastrointest Oncol 2013 Mar 15;5(3):50-59.

(54) Ribeiro ML, Godoy AP, Benvengo YH, Mendonca S, Pedrazzoli J,Jr. Clinical relevance of the *cagA, vacA* and *iceA* genotypes of *Helicobacter pylori* in Brazilian clinical isolates. FEMS Immunol Med Microbiol 2003 May 25;36(3):181-185.

(55) Gatti LL, Fagundes e Souza EK, Leite KR, Bastos EL, Vicentini LR, Silva LC, et al. *cagA vacA* alelles and *babA2* genotypes of *Helicobacter pylori* associated with gastric disease in Brazilian adult patients. Diagn Microbiol Infect Dis 2005 Apr;51(4):231-235.

(56) Mattar R, dos Santos AF, Eisig JN, Rodrigues TN, Silva FM, Lupinacci RM, et al. No correlation of *babA2* with *vacA* and *cagA* genotypes of *Helicobacter pylori* and grading of gastritis from peptic ulcer disease patients in Brazil. Helicobacter 2005 Dec;10(6):601-608.

(57) Zhang Y, Liu H, Zhou K. Lack of correlation of *vacA* genotype, *cagA* gene of *Helicobacter pylori* and their expression products with various gastroduodenal diseases. Chin Med J (Engl) 2001 Jul;114(7):703-706.

(58) Maeda S, Ogura K, Yoshida H, Kanai F, Ikenoue T, Kato N, et al. Major virulence factors, VacA and CagA, are commonly positive in *Helicobacter pylori* isolates in Japan. Gut 1998 Mar;42(3):338-343.

(59) Torres LE, Melian K, Moreno A, Alonso J, Sabatier CA, Hernandez M, et al. Prevalence of *vacA*, *cagA* and *babA2* genes in Cuban *Helicobacter pylori* isolates. World J Gastroenterol 2009 Jan 14;15(2):204-210.

(60) Sahara S, Sugimoto M, Vilaichone RK, Mahachai V, Miyajima H, Furuta T, et al. Role of *Helicobacter pylori cagA* EPIYA motif and *vacA* genotypes for the development of gastrointestinal diseases in Southeast Asian countries: a meta-analysis. BMC Infect Dis 2012 Sep 21;12:223-2334-12-223.

(61) Zhang BB, Li Y, Liu XQ, Wang PJ, Yang B, Bian DL. Association between *vacA* genotypes and the risk of duodenal ulcer: a meta-analysis. Mol Biol Rep 2014 Nov;41(11):7241-7254.

(62) Faundez G, Troncoso M, Figueroa G. *cagA* and *vacA* in strains of *Helicobacter pylori* from ulcer and non-ulcerative dyspepsia patients. BMC Gastroenterol 2002 Sep 10;2:20.

(63) Chen MY, He CY, Meng X, Yuan Y. Association of *Helicobacter pylori babA2* with peptic ulcer disease and gastric cancer. World J Gastroenterol 2013 Jul 14;19(26):4242-4251.

(64) Gramley WA, Asghar A, Frierson HF,Jr, Powell SM. Detection of *Helicobacter pylori* DNA in fecal samples from infected individuals. J Clin Microbiol 1999 Jul;37(7):2236-2240.

(65) Tummuru MK, Cover TL, Blaser MJ. Cloning and expression of a high-molecular-mass major antigen of *Helicobacter pylori*: evidence of linkage to cytotoxin production. Infect Immun 1993 May;61(5):1799-1809.

(66) Tomasini ML, Zanussi S, Sozzi M, Tedeschi R, Basaglia G, De Paoli P. Heterogeneity of cag genotypes in *Helicobacter pylori* isolates from human biopsy specimens. J Clin Microbiol 2003 Mar;41(3):976-980.

(67) Sheu BS, Sheu SM, Yang HB, Huang AH, Wu JJ. Host gastric Lewis expression determines the bacterial density of *Helicobacter pylori* in *babA2* genopositive infection. Gut 2003 Jul;52(7):927-932.

(68) Miernyk K, Morris J, Bruden D, McMahon B, Hurlburt D, Sacco F, et al. Characterization of *Helicobacter pylori cagA* and *vacA* genotypes among Alaskans and their correlation with clinical disease. J Clin Microbiol 2011 Sep;49(9):3114-3121.

(69) Watada M, Shiota S, Matsunari O, Suzuki R, Murakami K, Fujioka T, et al. Association between *Helicobacter pylori cagA*-related genes and clinical outcomes in Colombia and Japan. BMC Gastroenterol 2011 Dec 22;11:141-230X-11-141.

(70) Reva I, Takano T, Higuchi W, Iwao Y, Taneike I, Nakagawa S, et al. Virulence genotypes and drug resistance of *Helicobacter pylori* from Vladivostok, Russia: another feature in the Far East. Microbiol Immunol 2012 Mar;56(3):198-202.

(71) Ghotaslou R, Milani M, Akhi MT, Nahaei MR, Hasani A, Hejazi MS, et al. Diversity of *Helicobacter pylori cagA* and *vacA* Genes and Its Relationship with Clinical Outcomes in Azerbaijan, Iran. Adv Pharm Bull 2013;3(1):57-62.

(72) Abdullah SM, Hussein NR, Salih AM, Merza MA, Goreal AA, Odeesh OY, et al. Infection with *Helicobacter pylori* strains carrying *babA2* and *cagA* is associated with an increased risk of peptic ulcer disease development in Iraq. Arab J Gastroenterol 2012 Dec;13(4):166-169.

(73) Aziz F, Chen X, Yang X, Yan Q. Prevalence and correlation with clinical diseases of *Helicobacter pylori cagA* and *vacA* genotype among gastric patients from Northeast China. Biomed Res Int 2014;2014:142980.

(74) Secka O, Antonio M, Berg DE, Tapgun M, Bottomley C, Thomas V, et al. Mixed infection with *cagA* positive and *cagA* negative strains of *Helicobacter pylori* lowers disease burden in The Gambia. PLoS One 2011;6(11):e27954.

(75) Kidd M, Lastovica AJ, Atherton JC, Louw JA. Heterogeneity in the *Helicobacter pylori vacA* and *cagA* genes: association with gastroduodenal disease in South Africa? Gut 1999 Oct;45(4):499-502.

(76) Biernat MM, Gosciniak G, Iwanczak B. Prevalence of *Helicobacter pylori cagA, vacA, iceA, babA2* genotypes in Polish children and adolescents with gastroduodenal disease. Postepy Hig M

ed Dosw (Online) 2014 Aug 22;68:1015-1021.

(77) Homan M, Luzar B, Kocjan BJ, Orel R, Mocilnik T, Shrestha M, et al. Prevalence and clinical relevance of *cagA, vacA*, and *iceA* genotypes of *Helicobacter pylori* isolated from Slovenian children. J Pediatr Gastroenterol Nutr 2009 Sep;49(3):289-296.

(78) Basso D, Zambon CF, Letley DP, Stranges A, Marchet A, Rhead JL, et al. Clinical relevance of *Helicobacter pylori cagA* and *vacA* gene polymorphisms. Gastroenterology 2008 Jul;135(1):91-99.

(79) Belda S, Saez J, Santibanez M, Rodriguez JC, Sola-Vera J, Ruiz-Garcia M, et al. Relationship between bacterial load, morbidity and *cagA* gene in patients infected by *Helicobacter pylori*. Clin Microbiol Infect 2012 Jul;18(7):E251-3.

(80) van Doorn LJ, Figueiredo C, Sanna R, Plaisier A, Schneeberger P, de Boer W, et al. Clinical relevance of the *cagA*, *vacA*, and *iceA* status of *Helicobacter pylori*. Gastroenterology 1998 Jul;115(1):58-66.

(81) Oliveira AG, Santos A, Guerra JB, Rocha GA, Rocha AM, Oliveira CA, et al. *babA2*- and *cagA*-positive *Helicobacter pylori* strains are associated with duodenal ulcer and gastric carcinoma in Brazil. J Clin Microbiol 2003 Aug;41(8):3964-3966.

(82) Paniagua GL, Monroy E, Rodriguez R, Arroniz S, Rodriguez C, Cortes JL, et al. Frequency of *vacA*, *cagA* and *babA2* virulence markers in *Helicobacter pylori* strains isolated from Mexican patients with chronic gastritis. Ann Clin Microbiol Antimicrob 2009 Apr 30;8:14-0711-8-14.

(83) Talebi Bezmin Abadi A, Taghvaei T, Mohabbati Mobarez A, Vaira G, Vaira D. High correlation of *babA 2*-positive strains of *Helicobacter pylori* with the presence of gastric cancer. Intern Emerg Med 2013 Sep;8(6):497-501.

(84) Yu J, Leung WK, Go MY, Chan MC, To KF, Ng EK, et al. Relationship between *Helicobacter pylori babA2* status with gastric epithelial cell turnover and premalignant gastric lesions. Gut 2002 Oct;51(4):480-484.

(85) Mizushima T, Sugiyama T, Komatsu Y, Ishizuka J, Kato M, Asaka M. Clinical relevance of the *babA2* genotype of *Helicobacter pylori* in Japanese clinical isolates. J Clin Microbiol 2001 Jul;39(7):2463-2465.

(86) Chomvarin C, Namwat W, Chaicumpar K, Mairiang P, Sangchan A, Sripa B, et al. Prevalence of *Helicobacter pylori vacA, cagA, cagE, iceA* and *babA2* genotypes in Thai dyspeptic patients. Int J Infect Dis 2008 Jan;12(1):30-36.

(87) Homan M, Sterbenc A, Kocjan BJ, Luzar B, Zidar N, Orel R, et al. Prevalence of the *Helicobacter pylori babA2* gene and correlation with the degree of gastritis in infected Slovenian children. Antonie Van Leeuwenhoek 2014 Oct;106(4):637-645.

Chapter 3

Strategies to inhibit *H. pylori* urease gene expression and urease activity required for colonization in the human stomach [2]

Chapter 2 discussed the association of certain *H. pylori* virulence factors (*cagA, vacA* and *babA2*) with gastric diseases that may put individuals at risk of developing gastric cancer. The community of Aklavik is concerned about effective treatment approaches for *H. pylori* infections to reduce their risk of developing gastric diseases such as gastric cancer. Current treatment approaches may fail to treat *H. pylori* infections in Northern Aboriginal Communities. In this chapter, I focused on developing alternative treatment approaches that specifically target the *H. pylori* urease genes that are universally present in all isolates and required for colonization of the human stomach.

3.1 Introduction

Helicobacter pylori infection is one of the most common bacterial infections as it is found worldwide but is more frequently observed in less developed regions (1). *H. pylori* infection has an elevated occurrence in Aklavik, NWT where 58% of residents were tested positive for *H. pylori* infection by urea breath test (2). The infection is usually acquired in early childhood and persists for lifetime unless treated. The infection may result in gastritis, gastric or duodenal ulcer. *H. pylori* may also cause gastric cancer (3).

Since its discovery in 1982 by Barry Marshall and Robin Warren (4,5), eradication was the ultimate goal for the treatment. Several antibiotics and treatment regiments were tried but 100% eradication rate has never been achieved (6). Treatment failure may be due to antimicrobial resistance (7), re-infection (8), patient compliance and the high cost of currently used drugs (6). Three antibiotics commonly used for *H. pylori* treatment are clarithromycin, metronidazole and amoxicillin (9). The resistance rates to clarithromycin have been reported to be from 2.5-48.2% (10,11). The resistance to metronidazole is higher than Clarithromycin and is continuously increasing. The resistance rates have been reported to be from 19-64% where the highest prevalence was observed in Iran and China (12,13). Resistance to amoxicillin was reported in

[2] The work presented in this chapter is in preparation for submission for publication.

China and Iran at low rates (0.3-2.5% respectively) (12,13). In Aklavik NWT, the eradication rates to triple and sequential therapies were 59% and 73% respectively (14). Treatment failure presents a need for new treatment strategies. Ideally, treatment targeted specifically to *H. pylori* is preferred to avoid disruption of commensal flora. Inhibiting the ability of *H. pylori* to neutralize the acidity of the stomach may impair its ability to survive in the stomach.

Urease is a vital enzyme for *H. pylori* colonization. It catalyzes the hydrolysis of urea to form carbon dioxide (CO_2) and ammonia (NH_3), which neutralizes the gastric acid, thereby allowing *H. pylori* to grow (15). Urease is encoded by urease genes which are localized to a 34 kb portion of *H. pylori* chromosome. The urease genes are composed of structural (*ureA*, *ureB*), regulatory (*ureC*, *ureD*) and accessory (*ureI*, *ureE*, *ureF*, *ureG*, *ureH*) genes. UreB subunit is the active site of the enzyme (16). A urease-negative mutant of *H. pylori,* constructed by allelic exchange mutagenesis, lacked the ability to colonize the stomach of nude mice (17). Similar results were observed for gnotobiotic piglets infected with urease negative *H. pylori* which illustrate the importance of urease for *H. pylori* colonization (18). Urease inhibitors, such as acetohydroxamic acid, have been studied for possible use in treating *H. pylori* infections (19).

Gene silencing is a rapidly developing field with potential therapeutic applications (20). RNA interference was recently studied in *E. coli* using antisense RNA expressed from a doxycycline-inducible vector (21). Downregulation of *yidC* in *E. coli* was possible by antisense RNA resulting in sensitization to antibacterial essential oils eugenol and carvacrol (22). Targeting methicillin-resistant *Staphylococcus aureus* (MRSA) coagulase with siRNA, delivered by natural competence, inhibited both mRNA expression and the activity of MRSA coagulase *in vitro* (23). An antisense peptide nucleic acid (PNA) targeting the CmeABC multidrug efflux transporter in *Campylobacter jejuni* rendered *C. jejuni* isolates more susceptible to ciprofloxacin and erythromycin (24). Gene silencing by antisense DNA may be an effective strategy for the suppression of genes required for the colonization of *H. pylori* and thereby provide an alternate strategy for its eradication.

Liposomes have been designed to improve the delivery of antimicrobials to *H. pylori (25)*. However, this approach may not be successful for the eradication of antimicrobial-resistant *H. pylori*. Liposomes have been studied as a possible means to improve the electroporation efficiency of *E. coli*. DOTAP® [1,2-bis(oleoyloxy)-3-(trimethylammonio) propane], a cationic liposome, successfully induced *E.coli* transformation without electroporation (26). Although liposome-

mediated delivery of siRNAs into eukaryotic cells has been described (27), no investigations have been carried out in bacterial cells.

In chapter 2, I characterized *H. pylori* isolates from Aklavik, NWT. The urease enzyme was detected in all Aklavik isolates while the *babA2* gene was detected in 97% of the isolates. While interfering with urease production can be used for the treatment, *babA2* can be used to target novel treatments to only *H. pylori* and not to other commensal bacteria in GIT. This study investigated three different strategies for urease gene silencing: 1) the use of liposomes to deliver a plasmid with a defective urease gene to *H. pylori* for transformation by allelic exchange, 2) the use of liposomes loaded with *ureB* antisense DNA to *H. pylori* to knockdown urease expression, and 3) the use of liposomes to deliver siRNA to knockdown urease expression. Each strategy offers the potential to impair urease production and interfere with the ability of *H. pylori* to survive and colonize the acid environment of the stomach. This approach would offer a novel way to the use of current antimicrobials to treat *H. pylori* infections, and decrease rates of antimicrobial resistance. In this chapter, I investigated the effect of the three approaches on the urease activity of Aklavik *H. pylori* isolates. Since the variation in urease activity among *H. pylori* isolates was previously reported (28). An isolate with high urease activity was a good target to test the effect of interfering molecules (antisense DNA or siRNA) on urease activity.

Hypothesis: Liposome-mediated delivery of antisense DNA or siRNA against the urease gene of *H. pylori* will specifically disrupt the ability of *H. pylori* isolates (including those from Aklavik) to produce urease and thereby impair *H. pylori* survival in the stomach.

3.2 Materials and Methods

Unless otherwise stated, chemicals, disposables and glassware were purchased from Fisher Scientific (Ottawa, ON, Canada), antibiotics were purchased from Sigma-Aldrich (Oakville, ON, Canada), and enzymes were purchased from Invitrogen (Carlsbad, CA, USA).

3.2.1 *H. pylori* transformation studies

3.2.1.1 Bacterial strains for transformation studies

The reference *H. pylori* strains; J99, 26695, G27 and SS1 (ATCC) were used in transformation experiments along with the Aklavik *H. pylori* isolates A46, A31, A62 and A75.

The clinical isolate *H. pylori* UA802 was previously isolated and characterized and was used to study *H. pylori* transformation. The rationale behind using this particular isolate is that UA802 was previously transformed with pUOA26, a modified plasmid isolated from NCTC 11639. UA802 is a plasmid-free isolate that was previously reported to be completely resistant to natural transformation and could only be successfully transformed by electroporation with a transformation frequency < 1.0×10^{-8} transformants/µg DNA (29). *E. coli* SE 5000 containing the plasmid pHP808 was kindly supplied by the Mobley Research Laboratory (Department of Microbiology and Immunology, University of Michigan Medical School, USA).

3.2.1.2 Plasmids

The plasmid pHP808 is a pACYC184 derivative (**Figure 3.1**) containing the entire urease gene cluster in an 11 kb insert and a chloramphenicol resistance selective marker (30). A novel plasmid, pHP809, was created by treating pHP808 with *Bam*HI resulting in the removal of a 1.3 kb portion of the urease gene that encodes for the *ureAB* gene. Following electrophoresis, the larger fragment (13 kb) was purified and ligated to produce the plasmid pHP809 containing a defective urease gene (**Figure 3.2**). The plasmid pHP809 was transformed into *E. coli* TOP 10 cells (One Shot®Top 10 cells, Invitrogen, Carlsbad, CA, USA), which are chemically competent cells. On vial of the cells was thawed on ice then 10 ng of pHP809 in 5 µL sterile deionized water was added. The mixture was incubated on ice for 30 minutes and then heat-shocked for 30 seconds at 42°C without shaking. After heat-shock, the tube was transferred immediately to ice and 250 µL of room temperature S.O.C. medium was added. The tube was incubated at 37°C for 30 minutes with shaking (200 rpm). The entire transformation reaction was added to 10 mL LB broth containing 20 µg/ml chloramphenicol and grown overnight at 37°C with shaking (200 rpm).

Plasmid pHP809 was extracted from *E. coli* Top10 by using the QIAprep® Spin Midiprep Kit (Qiagen Sciences, Maryland, USA) and was used for subsequent transformation of *H. pylori*. *E. coli* TOP10 was tested for urease activity by incubating in urease test medium (Chapter 2, section 2.2.2.1) before and after being transformed with pHP809. Urease activity was assessed at 24 h incubation. The deletion of the *ureAB* portion of the urease gene was confirmed by *ureAB* PCR before and after mutation. PCR was performed as previously described in chapter 2 section 2.2.4.3 using *ureAB-F* (5'-AGGAGAATGAGATGA-3') as forward primer and *ureAB-R* (5'-ACTTTATTGGCTGGT-3') as reverse primer. PCR reaction was done for 40 cycles of

denaturation at 94°C for 1 minute, annealing at 45°C for 90 minutes and extension at 72°C for 1 minutes (31).

3.2.1.3 Natural transformation

H. pylori was cultured from frozen stock cultures onto BHI/YE/HS agar containing vancomycin (15 µg/mL), amphotericin (15 µg/mL) for 5 days and then subcultured for 24 h on fresh agar plate. Fresh 24 h culture was plated at high density on BHI/YE/HS agar on areas of 8-10 mm in diameter, and then incubated for 5 h at 37˚C under microaerobic conditions. Aliquots of 2 µg plasmid DNA (pHP809) in 8 µL TE buffer (10 mM-Tris, 1 mM-EDTA, pH 8) were spotted directly onto the inoculated agar, and incubation was continued for 18 h. The DNA-treated cells were collected with a loop in 50 µL TE buffer and were then spread on BHI/YE/HS agar containing 20 µg/ml chloramphenicol to select transformants. Cultures were checked every 48 h for up to 21 days to detect the growth and supplemented with 100 µL BHI/YE broth when no growth was detected (29).

3.2.1.4 Electroporation

H. pylori was cultured from frozen stock cultures onto BHI/YE/HS agar plates containing vancomycin (15 µg/mL), amphotericin (15 µg/mL) for 5 days and then subcultured for 24 h on a fresh agar plate. The growth was suspended in 5 ml BHI/YE/HS in a 50 mL tissue culture flask to achieve OD_{600} = 0.1 and then grown with agitation (80 rpm) for 18 h at 37˚C under microaerobic conditions. At the end of the incubation period the OD_{600} was determined and adjusted to OD_{600} = 0.5 corresponding to 1 x 10^9 – 1 x 10^{10} cfu/mL by adding BHI/YE/HS broth. For each electroporation, 1 ml of culture was pelleted by centrifugation (1500 x *g*, 3 min), washed three times in 500 µL electroporation buffer (272 mM sucrose, 15 % glycerol, 2.43 mM K_2HPO_4 and 0.57 mM KH_2PO_4, stored at 4°C), and suspended in a final volume of 40 µL of electroporation buffer. The bacteria were put into prechilled 0.2 cm electroporation cuvettes, and 2 µg of plasmid was added. Electroporation was done using 2500 V, 25 µF capacitor and 200 Ώ (32). After electroporation, the volume was brought to 1 ml by the addition of phosphate buffered saline, pH 7.4, and then 100 µL aliquots were poured onto BHI/YE/HS agar plates. Following an overnight expression period, the growth was subcultured onto BHI/YE/HS agar containing 20 µg/mL

chloramphenicol to select transformants. Cultures were checked every 48h to detect the growth and supplemented with 100 µL BHI/YE broth when no growth was detected (32).

3.2.1.5 Transformation using DOTAP® Liposomes

DOTAP® is a commercially available cationic liposome that was purchased from Roche applied Science. *H. pylori* was cultured from frozen stock cultures onto BHI/YE/HS agar and then subcultured for 24 h on a fresh agar plate. Fresh culture was plated at high density on BHI/YE/HS agar on areas of 8-10 mm in diameter, and then incubated for 5 h at 37˚C under microaerobic conditions. Then, 2 µg plasmid DNA (pHP809) in 50 µL TE buffer was mixed with 30 µg DOTAP® (Roche Applied Science, Gaithersburg, MD, USA) and 70 µL HEPES buffered saline (HBS, 20 mM HEPES, 150 mM NaCl, pH 7.4) and allowed to stand for 15 minutes at room temperature. The mixture was spotted directly onto the inoculated agar, and incubation was continued for 18h. The transformants were selected using the same protocol as for natural transformation (section 3.2.1.3). Electroporation in presence of DOTAP® was performed using the same electroporation protocol mentioned above (section 3.2.1.4) but in the presence of 2 µg plasmid in 50 µL TE buffer, 30 µg DOTAP® and 70 µL HBS.

3.2.1.6 Determination of transformation frequency and efficiency

The transformation frequencies were determined for natural transformation, electroporation and transformation in presence of DOTAP®. The plasmid-treated cells were collected with a loop in 100 µL of TE buffer and were then spread on BHI/YE/HS agar plates with and without 20 µg/mL chloramphenicol after 10-fold serial dilutions up to 10^{-8}, to select transformants. Transformation frequencies were calculated from the number of transformants divided by the number of colonies obtained by plating the same suspension on BHI/YE/HS agar plates without chloramphenicol (29).

Transformation efficiency is defined as the number of transformants obtained by using 1 µg of plasmid DNA in a transformation reaction. It is calculated using the following formula:
Transformation efficiency = # colonies on plate/ng of DNA plated X 1000 (transformants/µg DNA) (33)

3.2.1.7 Detection of urease activity in transformed *H. pylori*

H. pylori transformed with pHP809 was streaked on BHI/YE/HS agar plates containing 20 µg/mL chloramphenicol. Single colonies were subcultured on fresh agar plates and the growth was enhanced by the addition of BHI/YE broth then tested for urease activity by urea test medium as previously explained (2.2).

3.2.2 Suppression of urease expression

3.2.2.1 Culture of Aklavik isolates for urease activity assay

Urease activity was evaluated for the reference strain *H. pylori* 26695 and 18 Aklavik *H. pylori* isolates (A20, A35, A36, A42, A43, A44, A52, A64, A70, A88, A96, A135, A138, A143, A149, A156, A185, A214) resistant to at least one antibiotic. *H. pylori* isolates were cultured from frozen stock cultures and then subcultured for 24 h. The cultures were suspended in BHI/YE/HS broth to achieve an OD_{600} = 0.1 (equivalent to 10^6-10^7 cfu/ml). The cultures were incubated in 6-well tissue culture plates in a shaking incubator (120 rpm) for 18 h at 37°C under microaerobic conditions. At the end of incubation period, the OD_{600} of the culture was adjusted to 0.4. One set of 50 µL aliquots were taken to determine # cfu/mL. Another set of 250 µL aliquots were taken and used for assessing urease activity. Serial 10-fold dilutions of cell suspensions in saline were prepared in duplicate and 25 µL were plated in triplicate and incubated for 5 days to determine the cfu/ml.

3.2.2.2 Urease activity assay

Aliquots (250 µL) of cell suspensions (OD_{600} = 0.4) obtained from 2.8.1 were centrifuged (12000 x *g*, 10 min, 4°C) to pellet the cells. Cell pellets were washed with saline and resuspended in 250 µL sterile MQ water. The cells were disrupted by sonication for 20 seconds using Braun sonic 2000 sonicator, USA to liberate the cell contents (urease), then centrifuged (12000 x *g*, 10 min, 4°C). The urease activity of the supernatants was measured by kinetic assay over 150 min at A_{560} using urease test reagent (50 mM phosphate buffer pH 6.8 containing 500 mM urea, 0.02% phenol red and 0.1 mM dithiothreitol) (34). The urease activity was plotted on the y-axis vs incubation time on the x-axis.

3.2.3 Urease gene silencing studies

3.2.3.1 *H. pylori* A64 growth curve

H. pylori A64 was cultured from frozen stock culture and then subcultured for 24 h. The culture was suspended in BHI/YE/HS broth to achieve an OD_{600} = 0.1 (equivalent to 10^6-10^7 cfu/ml). The culture was incubated in 6-well tissue culture plates in a shaking incubator (120 rpm) for 24 h at 37°C under microaerobic conditions. Aliquots of 50 µL were taken at 3, 6, 12 and 24 h and added to 450 µL saline. Serial 10 fold dilutions were made up to 10^{-8} and 25 µL aliquots were plated on BHI/YE/HS agar plates in triplicate to determine # cfu/mL. The growth curve of *H. pylori* A64 was made by plotting Log_{10} cfu/mL versus incubation period in hours. The experiment was done in triplicates on two different days.

3.2.3.2 Antisense DNA design for *ureB* gene

Antisense DNA was designed against the *ureB* gene (UBA). A sequence of 21 bases were designed to be complimentary to the open reading frame of *ureB* gene downstream from the start codon (35). The specificity of the designed sequence was confirmed by blasting the sequence against all sequences in Genbank. A scrambled (scr) sequence was also designed as a negative control as it has the same nucleotide content of the antisense DNA but in different order (**Table 3.1**). The designed sequences were analyzed for hairpin and secondary structures using the Integrated DNA Technology design tool (36).

3.2.3.3 *ureB* antisense DNA treatments

H. pylori A64 (high urease producer) was cultured on BHI/YE/HS plates for 24 h. The culture was suspended in BHI/YE/HS broth to achieve OD_{600} = 0.1. A64 was grown in 6-well tissue culture plates in a shaking incubator (120 rpm) for 24 h at 37°C under microaerobic conditions. The designed oligos were added at a concentration of 20 nM in the absence and presence of 1.6 µM DOTAP®. Treatments were added at 0, 6 and 10 h. At the end of incubation period the cell lysates for urease assay were prepared as previously described. The number of cfu/ml was also determined to assess the effect of various treatments on *H. pylori* growth. The effect of increasing concentrations of UBA (1-5 µM) on the urease activity of *H. pylori* A64 was examined in the absence and the presence of 1.6 µM DOTAP®. The effect 20 nM UBA+ DOTAP® (0.4-6.4 µM) was also tested. The effect of 20 nM UBA + DOTAP® on the urease activity of 4

other Aklavik isolates was also investigated with high (A70), intermediate (A20, A135) and low (A96) urease activity. Each experiment was done in triplicate and repeated three times.

3.2.3.4 *ureB* gene expression

Real-time reverse-transcriptase PCR was performed to verify the inhibition in urease activity caused by 20 nM UBA + DOTAP® was due to a decrease in urease gene expression. *H. pylori* A64 was cultured and treated with DOTAP®, UBA and scr UBA as described above. At the end of incubation period 800 µL aliquots containing 3.2 x 10^8 cfu were taken for RNA extraction using the TRIzol® reagent as previously described (Chapter 2: section 2.2.5.1). RNA concentrations and purity were assessed using the NanoVue, GE Healthcare UK, where 1.0 unit at OD_{260} = mg RNA. The quality of RNA was assessed by formamide denaturation and running through 1.5% agarose gel (37). RNA samples were treated with DNase I (Invitrogen, USA) to degrade any contaminating DNA. cDNA was synthesized using High Capacity Reverse Transcriptase kit (Applied Biosystem, USA) as previously described (Chapter 2; section 2.2.5), for urease gene expression. A non-RT negative control (without reverse transcriptase) confirmed the absence of any contaminating genomic DNA. Relative quantitation (RQ) of urease mRNA levels (converted to cDNA) were measured by q-PCR analysis (Step One Plus (Applied Biosystem, USA) relative to 16S rRNA mRNA levels (converted to cDNA), the endogenous control whose expression is constant and not affected by UBA treatment (38). PCR was performed in a 10 µL reaction volume containing 1 µL cDNA, 2 µL primer (3.2 µM), 2 µL RNase free water and 5 µL SYBR® green master mix (Qiagen, USA). PCR was carried out at 95°C for 5 min, followed by 30 cycles at 95°C for 10 s, 60°C for 30 s. A further melting curve step analyzing the purity of PCR products were performed at 95°C for 15 s, 60°C for 1 min and 95°C for 15 s to ensure the specificity of the primers used.

3.2.3.5 siRNA design and treatment

siRNAs were designed against *ureA* and *ureB* gene using the Applied Biosystems Design Tool (39). The specificity of the designed sequence was confirmed by blasting the sequence against the entire Genbank database. Scrambled sequences were also designed as a negative control (**Table 3.1**). The oligonucleotides were dissolved in RNase free duplex buffer (100 mM potassium acetate, 30 mM HEPES, pH 7.5). To prepare siRNA duplexes, equimolar amounts of complementary oligonucleotides were mixed and then annealed by heating to 90°C for 1 min followed by reducing

the temperature by 1°C per min until the temperature reached 5°C (40). The effect of 20 nM of ureA siRNA or ureB siRNA and the combined effect of both of them on urease activity were determined in the absence and presence of 1.6 μM DOTAP®. Each experiment was done in triplicate and repeated three times.

3.2.3.6 Statistic analysis

Data are expressed as mean ± SEM. Significant differences among more than two groups were detected by one-way ANOVA. When a sigificance was found by one-way ANOVA, the Turkey post-hoc test was performed to identify which groups were significantly different and to avoid a type I error. However, when no significance was found by one-way ANOVA, the Duncan test was performed to avoid a type II error. A *p*-value of < 0.05 was considered statistically significant. All analyses were performed using IBM SPSS® version 20 (New York, USA).

3.3 Results

3.3.1 *H. pylori* transformation

3.3.2 Construction of pHP809 carrying a mutant urease gene

As expected, no urease activity was detected for *E. coli* TOP10 before transformation. *E. coli* TOP10 transformed with pHP808 was urease positive. On the other hand, *E. coli* TOP10 transformed with pHP809 was urease negative after 72 h incubation. PCR amplification of pHP808 and pHP809 using *ureAB* primers gave amplicon sizes of 2.4 kb (entire *ureAB* gene) and 1.1 kb (*ureAB* gene with 1.3 kb deletion) respectively (**Figure 3.3**).

3.3.3 Transformation of *H. pylori* UA802 with pHP809

UA802 was transformed by pHP809 using DOTAP® liposomes and electroporation in presence of DOTAP®. Neither natural transformation nor electroporation alone was successful in mediating UA802 transformation. Attempts that were done to determine the transformation frequency of UA802 by pHP809 using DOTAP® alone and DOTAP® + electroporation were not successful. Dilutions were prepared up to 10^{-14} in 3 sets.

3.3.4 Urease activity of transformed *H. pylori* UA802

Although pHP809 carries a mutant urease gene, all recovered transformants were urease positive. UA802 is a plasmid-free *H. pylori*. After transformation using DOTAP® alone and DOTAP® + electroporation, plasmid isolation was performed. The isolated plasmid was pHP809. The transformation of *H. pylori* with pHP809 did not achieve the required mutation.

3.3.5 Transformation of reference and Aklavik *H. pylori* isolates

DOTAP® alone transformed 7 out of 8 *H. pylori* isolates with pHP809. DOTAP® alone was the most successful method to induce *H. pylori* transformation, followed by electroporation in the presence of DOTAP® with 6 of 8 strains tranformed. Electroporaton alone transformed only 3 of 8 strains. Natural transformation was the least successful method in inducing transformation of only 2 out of 8 isolates (**Table 3.2**).

3.3.6 Urease activity of Aklavik isolates

All of the Aklavik isolates (A20, A35, A36, A42, A43, A44, A52, A64, A70, A88, A96, A135, A138, A143, A149, A156, A185, A214) showed much higher urease activity than the reference strain 26695, which was barely detectable (**Figure 3.4**). *H. pylori* isolate A64 had the highest urease activity. Four isolates were classified as moderate urease producers, and the remaining isolates were classified as low urease producers.

3.3.7 Growth rate of *H. pylori* A64

The growth rate of *H. pylori* A64 was investigated over 24 hours. Different growth rates were observed when the growth rate was assessed at different days even when the starting inoculum size was the same ($OD_{600} = 0.1$). Two representative growth curves are shown in **Figure 3.5**.

3.3.8 Effect of interfering oligonucleotides treatment on urease activity

20 nM UBA + DOTAP® inhibited the urease activity by 40% ± 1.3 ($p < 0.008$). In absence of DOTAP®, UBA was not able to induce any inhibition in urease activity indicating that DOTAP® was essential for UBA delivery into *H. pylori* cells. The scrambled structure of UBA did not affect the urease activity, which means that the inhibition was due to the specific structure of UBA

(**Figure 3.6**). All treatments did not have any significant effect on *H. pylori* growth when compared to untreated control.

The inhibition of urease transcription was verified by real-time reverse-transcriptase PCR. RNA samples were intact as illustrated by the presence of two sharp bands on the agarose gel corresponding to the 16S and 23S rRNA. The intensity of the 23S band was about double that of 16S (**Figure 3.7**). A single product was by q-PCR obtained as illustrated by single peak of fluorescence for each primer pair (**Figure 3.8**). UBA+ DOTAP® suppressed the urease expression by 36.4% ± 1.9 ($p = 0.0078$) (**Figure 3.9**).

UBA 4 μM was the optimum concentration that caused 59% ± 3 ($p < 0.05$) inhibition in urease activity. Further increases in UBA concentration did not decrease urease inhibition (**Figure 3.10**). DOTAP® 1.6 μM was the optimum concentration to achieve maximum urease inhibition. Higher concentrations in DOTAP® did not further inhibit urease activity (**Figure 3.11**).

UBA + DOTAP® suppressed urease activity of A70 by 26% ± 1.5 ($p = 0.01$) although A70 had lower urease activity than A64 (**Figure 3.12**). The growth rate of A70 was much higher than that of A64. Although the starting OD_{600} was the same for both isolates (0.1), the OD_{600} after 24 h incubation was 2.2 for A70, while that of A64 was in the range of 0.6-0.8. UBA + DOTAP® suppressed the urease activity of A20 and A135 by 35.8% ± 0.6 and 38.8% ± 1.4 ($p < 0.0001$) respectively (**Figures 3.13 and 3.14**). The two isolates had intermediate urease activity but also different growth rate ($OD_{600} = 1.2$ and 0.7 respectively). The same treatment was able to suppress the urease activity of A96 by 48% ± 1.2 ($p < 0.0001$) (**Figure 3.15**). A96 is a week urease producer but had a high growth rate ($OD_{600} = 1.3$ after 24 h growth). None of the treatments had any effect on the growth rate of *H. pylori* isolates under the neutral pH conditions of the experiment when compared to the untreated control.

20 nM ureA siRNA or ureB siRNA did not inhibit the urease activity of *H. pylori* A64 in presence or absence of DOTAP®. The simultaneous addition of both ureA siRNA and ureB siRNA did not inhibit the urease activity of A64.

3.4 Discussion

3.4.1 *H. pylori* transformation

Although cationic liposomes have wide applications in delivering interfering molecules to eukaryotic cells (41), they have not been widely investigated as vectors for bacterial transformation. In the present study, DOTAP® was successful to induce *H. pylori* transformation with a plasmid carrying a chloramphenicol resistance marker. DOTAP® could transform *H. pylori* isolates that are resistant to natural transformation and/or electroporation. Similar results were reported for *E.coli* where DOTAP® alone without electroporation was successful to induce its transformation with plasmids (2.2-7.2 kb) in size. The transformation efficiency was $2 \times 10^4 - 2 \times 10^5$ transformants/µg plasmid DNA and no colonies were observed for control without DOTAP® (42).

In this study, I could not determine the transformation efficiency for any of the transformed isolates. This can be attributed to the slow growth nature of *H. pylori* and the low transformation frequency due to the bigger size of the plasmid used (13 kb). It was previously reported that as the plasmid size increases, the transformation efficiency decreases (42). Although some *H. pylori* strains are naturally competent for transformation by plasmid, there is significant variations in transformation frequencies among different *H. pylori* strains with some strains completely resistant to transformation. It has been proposed that variation in plasmid transformation frequency may be due to the presence of certain DNA restriction and modification (R-M) mechanisms which consist of two contrasting enzymatic activities: a restriction endonuclease (REase) and a methyltransferase (MTase). The REase protects bacteria from foreign DNA by recognizing and cleaving foreign DNA sequences at specific sites, while MTase activity allows for the discrimination between self and nonself DNA, by transferring methyl groups to the same specific DNA sequence within the bacterial genome and so preventing its cleavage (44).

The plasmid used in this study carried a mutant urease gene to induce knock out of urease gene by allelic exchange mutagenesis. However, none of the transformants were urease negative. The absence of allelic exchange mutagenesis may be due to the low transformation efficiencies and high frequencies of illegitimate recombination (43). In addition, the plasmid used may have a low copy number which hinder efficient allelic exchange mutagenesis. Cleavage of the introduced plasmid by the R-M system may have also contributed to failure of allelic exchange mutagenesis.

DOTAP® may enhance the transformation of *H. pylori* by protecting the DNA from degradation. *H. pylori* produces DNase that rapidly degrades plasmids used to transform *H. pylori*. Liposomes may decrease the accessibility of plasmid to DNase, decrease their degradation and hence enhance transformation. The most common mechanism of delivery of liposomal content into cells is proposed to be mediated by liposome adsorption onto the cell surface followed by endocytosis, on the other hand liposome fusion with cells seems to occur at low frequency (45). The adsorption of loaded liposomes onto the cell surface depends mainly on the overall charge of the loaded liposomes. Positively charged liposomes adhere to the cell surface more readily than negatively charged or neutral liposomes. One of the limitation of the recent study that I did not determine the overall charge of the loaded liposomes. I used DNA/liposomes ratio recommended by the manufacturer, however, this ratio is constant and does not account for the variability in the overall charge of loaded liposomes.

3.4.2 Urease activity of *H. pylori* 26695 and Aklavik isolates

The variability in urease activity among *H. pylori* isolates was previously reported (28). *H. pylori* isolates from cases of gastric cancer were found to be of higher urease activity than that of controls or duodenal ulcer patients (28). Aklavik *H. pylori* isolates showed high variability in their urease activities. For all tested isolates, the urease activity was much higher than for the reference strain 26695, which is a lab-adapted strain that has been cultured in the lab under neutral pH conditions. Aklavik *H. pylori* isolates were sub-cultured a maximum of 3 times before being tested. *H. pylori* A64, which had the highest urease activity, was associated with moderate chronic and acute gastritis with no atrophy or metaplasia, however, the number of isolates tested was not enough to draw conclusions. Histopathology data for the five Aklavik *H. pylori* isolates that were utilized in urease inhibition assay are presented in **Table 3.3**.

3.4.3 Inhibition of *H. pylori* urease activity by antisense DNA

The increased reports of antibiotic resistance and treatment failure for *H. pylori* infection has become a serious problem raising the urgent need for alternative treatment approaches. Antisense oligonucleotide can specifically inhibit gene expression by binding to complementary mRNA strand (46). Because antisense oligonucleotides are highly susceptible to degradation by DNases, considerable efforts have been made to improve their stability and delivery (47).

This study revealed that *ureB* antisense DNA encapsulated into DOTAP® inhibited urease expression by 40% without being toxic to *H. pylori.* In the absence of DOTAP®, 50 times more of antisense oligonucleotide is needed to induce the same level of inhibition. Fillion *et al.,* reported 42% reduction in β-galactosidase activity upon treating *E.coli* with anti-*lacZ* oligonucleotide encapsulated into anionic liposomes. The author rationalized the use of anionic liposomes to avoid possible toxicity of cationic liposomes and enhance the release of interfering oligonucleotide from liposomes after delivery (47). An antisense peptide nucleic acid (PNA) targeting the CmeABC multidrug efflux transporter in *Campylobacter jejuni,* a bacterium closely related to *H. pylori,* rendered *C. jejuni* isolates more susceptible to ciprofloxacin and erythromycin (24). Gene silencing was also reported for Gram positive bacteria. Targeting methicillin-resistant *Staphylococcus aureus* (MRSA) coagulase with siRNA, delivered by natural competence, inhibited both mRNA expression and the activity of MRSA coagulase *in vitro* (40). The inhibition in urease activity was not the same for other Aklavik isolates due to the variability in both urease activity and growth rate. The antisense oligonucleotide used in this study was unmodified and so no inhibition of urease activity was observed at low concentration due to its rapid degradation. The use of phosphorothioate oligonucleotides, in which the non-bridging oxygen atoms are replaced by sulfur in the DNA backbone of the molecule, will increase resistance to DNases, and may increase the degree of inhibition. In addition, modification of lipid composition and charge of liposomes may also play a role in enhancing stability delivery and actions of loaded oligonucleotides.

Although gene silencing by siRNA was possible in Gram positive bacteria (40), no previous reports of its success in Gram negative bacteria. RNA interference is induced by 21–25 nucleotide double-stranded RNA fragment which is cleaved by RNase III like enzymes, called Dicer, making RNA-induced silencing complexes (RISCs) which finally leads to endonucleolytic cleavage of the complementary target mRNA (48). In the present study, siRNA against *ureAB* gene of *H. pylori* was not successful in inducing complete urease knockdown. The RNase III like enzymes required for the activation of siRNA may be absent in *H. pylori*. Furthermore, siRNA is well known for its short duration of action, which may lead to its inactivity in *H. pylori* due to the high abundance of urease.

DOTAP liposomes alone was successful to induce transformation of eukaryotic cells (49). Doh *et al.,* labelled DOTAP with a fluorescent dye (NBD) to investigate the intracellular

trafficking of DOTAP. The transfection efficiency of DT-NBD liposome was comparable to commercial NBD PE liposome with no additional cytotoxicity. Others reported that the use of 100% DOTAP for gene delivery is inefficient due to the density of positive charges on the liposome surface, which possibly prevents counter ion exchange. DOTAP is completely protonated at pH 7.4 (which is not the case for all other cationic lipids), so it is possible that more energy is required to separate the DNA from the lipoplex for successful transfection (42). Thus, for DOTAP to be more effective in gene delivery, it should be combined with a helper lipid, as seems to be the case for most cationic lipid formulations.

In conclusion, antibiotic approaches are often ineffective to treat *H. pylori* infections in northern communities. Since urease is required for colonization, imparing urease production may reduce *H. pylori* infection in the stomach. In chapter 3, I investigated the possibility of using three strategies to interfere with urease production by *H. pylori*. The first strategy was to knockout urease expression by using a plasmid carrying a mutant urease gene. However, the plasmid was not successful to induce knockout of urease expression.

The second strategy was to interfere with *ureB* gene expression using antisense DNA. The *ureB* antisense DNA encapsulated into DOTAP® cationic liposomes inhibited urease expression by 40% without being toxic to *H. pylori* when experiments were performed at neutral pH values but was not successful to induce complete suppression of urease expression. Further modification of lipid content of cationic liposomes and/or interfering molecules may be needed to achieve complete suppression of urease inhibition. The third strategy was to interfere with urease gene expression using siRNA but this strategy was not successful to induce any inhibition in urease activity.

The work presented in this chapter demonstrated the possibility of interfering with an important *H. pylori* enzyme that is essential for gastric colonization and may offer a novel and specific approach to treat *H. pylori* infections. Interfering with the ability of the pathogen to survive in its host may present a specific treatment approach that can be applied not only to *H. pylori* but also to other organisms with multiple drug resistance. This technique if well optimized to completely suppress urease production may offer alternative treatment approach for *H. pylori* infections that fail standard therapies such as those from Aklavik.

Table 3.1 Sequences of the designed antisense DNA, siRNAs and the corresponding scrambled oligonucleotides

Oligo	Sequence (5' →3')	Position in gene sequence*
UBA	GCC CAA TCT CAC TTT ATC GCC	547
Scr UBA	CCC ACT CTC ACC CAT AGT TGT	NA
ureAsiRNA(+)	CCGUGCAUACCCCUAUUGAUU	57
ureAsiRNA(-)	UCAAUAGGGGUAUGCACGGUU	
Scr ureAsiRNA(+)	GCUCAAGUCCUCACUCUAGUU	NA
Scr ureAsiRNA(-)	CUAGAGUGAGGACUUGAGCUU	
ureBsiRNA(+)	AGUGAGAUUGGGCGAUACAUU	553
ureBsiRNA(-)	UGUAUCGCCCAAUCUCACUUU	
Scr ureBsiRNA(+)	UGAUAGGAGGAGUGUCCAAUU	NA
Scr ureBsiRNA(-)	UUGGACACUCCUCCUAUCAUU	

* *H. pylori* 26695 *ureAB* (AF507994)

Table 3.2 Transformation of 8 reference and Aklavik *H. pylori* isolates

H. pylori	**Transformation method**			
	Natural transformation	Electroporation	DOTAP®	Electroporation + DOTAP®
G27	-	-	-	-
J99	-	+	+	+
26695	-	+	+	+
SS1	-	-	+	-
A46	-	-	+	+
A31	+	-	+	+
A62	-	-	+	+
A75	+	+	+	+

Transformation of reference and Aklavik *H. pylori* strains was performed as described in sections 3.2.1.3-3.2.1.6.

H. pylori reference strains: G27, J99, 26695, SS1

H. pylori Aklavik isolates: A46, A31, A62, A75

Table 3.3 Histopathology scores for 5 Aklavik *H. pylori* isolates with low, intermediate and high urease activity

H. pylori isolate	Histopathology score
A64	Moderate acute and chronic gastritis
A70	Moderate acute, chronic gastritis & mild intestinal metaplasia
A20	Mild acute gastritis, moderate chronic gastritis & moderate metaplasia
A135	Mild acute and chronic gastritis
A96	Moderate acute gastritis, severe chronic gastritis & mild atrophy

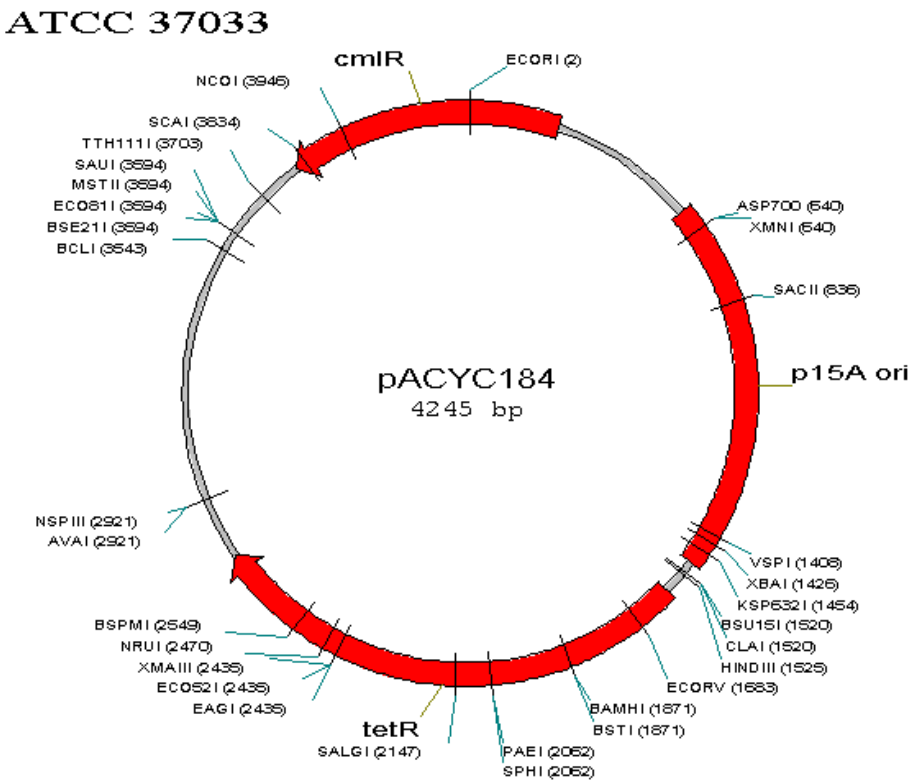


Figure 3.1 Restriction map of plasmid pACYC184

Figure reproduced with permission (http://www.atcc.org/products/all/37033.aspx)

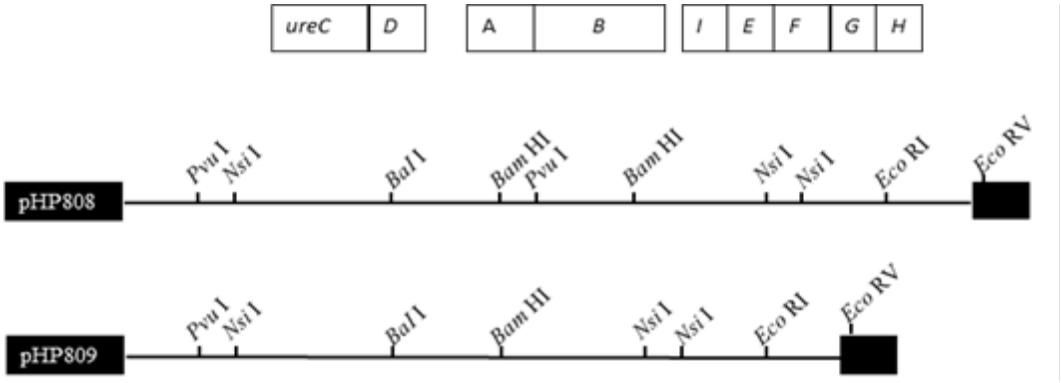


Figure 3.2 Restriction map of plasmids pHP808 and pHP809

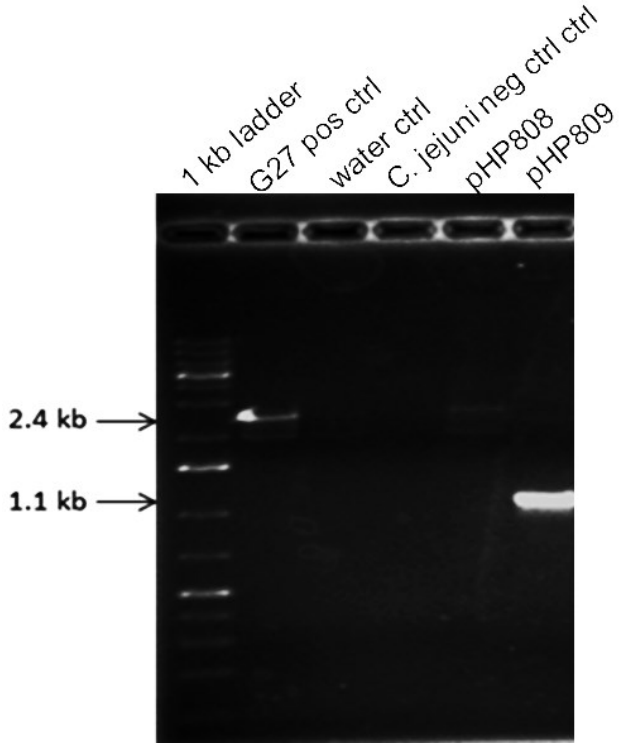


Figure 3.3 *ureAB* PCR of pHP808 and pHP809

The ureAB PCR was performed on plasmids pHP808 and pHP809 as described in section 3.2.1.2.

2.4 kb amplicon represents the entire *ureAB* gene.

1.1 kb amplicon represents the *ureAB* gene with 1.3 kb deletion.

H. pylori G27 is a positive urease gene control. *C. jejuni* CO848 serves as a non-*H. pylori* DNA negative control. Water serves as a no DNA negative control.

A 1 Kb plus DNA ladder served as reference to estimate amplicon size.

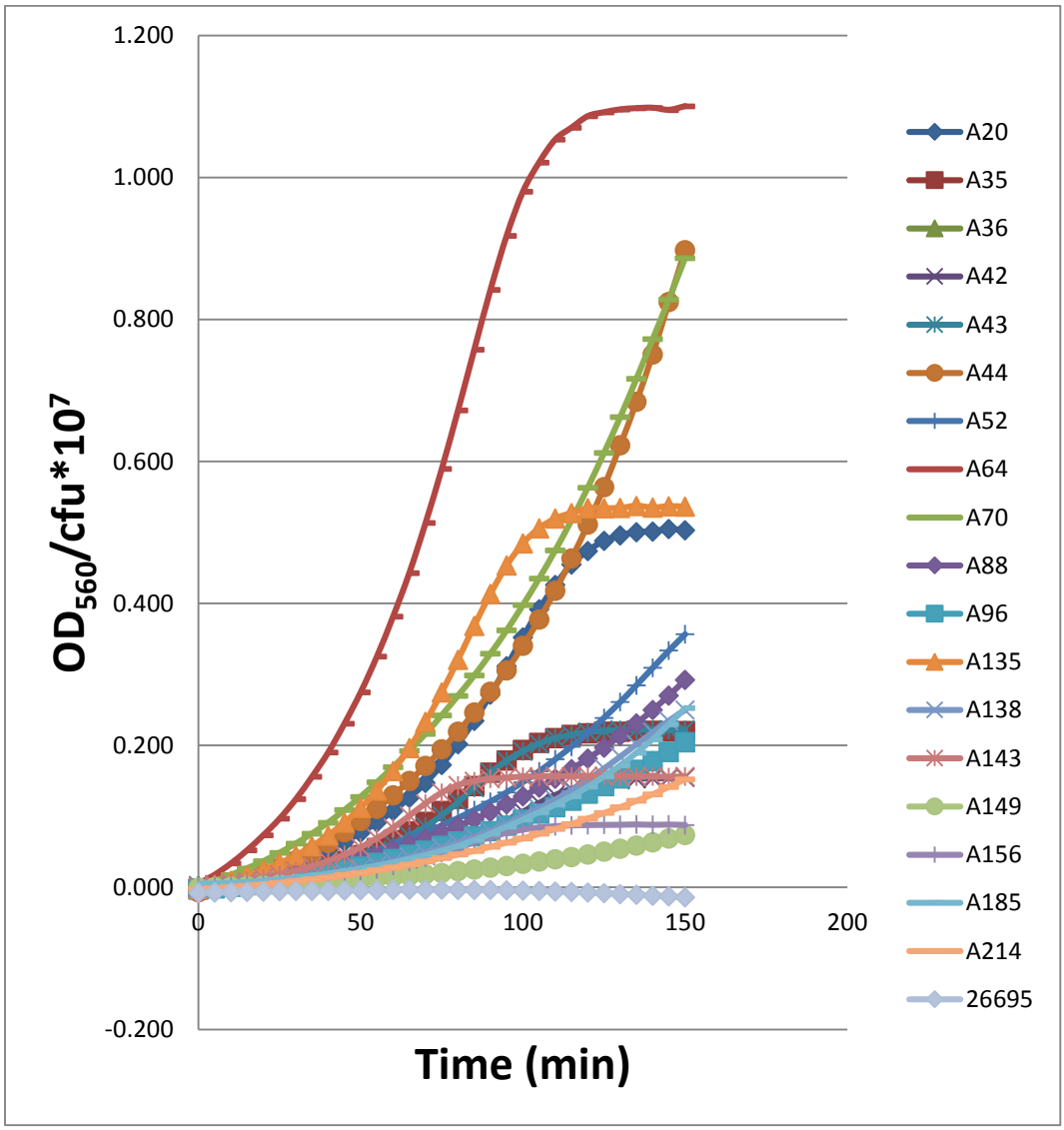


Figure 3.4 Urease activity of *H. pylori* 26695 and 16 Aklavik isolates

Urease activity was measured over 150 min and reported as OD_{560}/cfu x 10^6 as described in section 3.2.2.2.

n = 2 per *H. pylori* studied

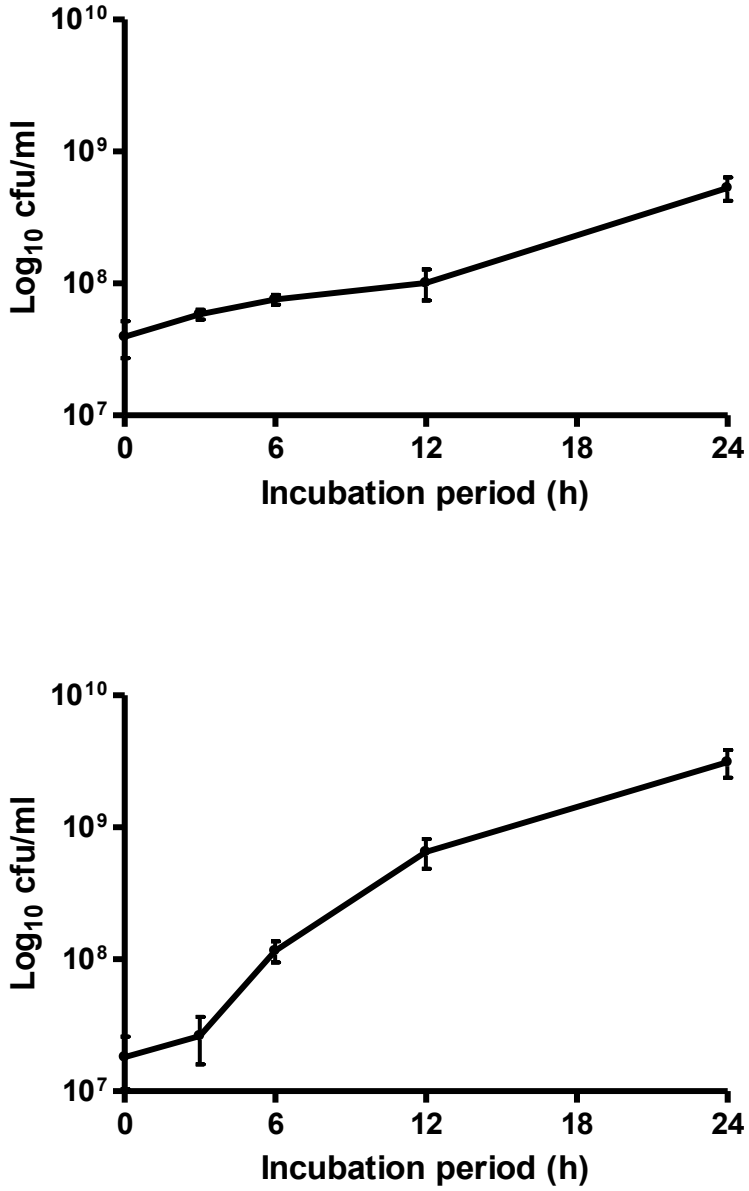


Figure 3.5 Growth curves of *H. pylori* A64 over 24 h

Two representative growth curves were generated for Aklavik *H. pylori* A64, with a starting inoculum of OD_{600} = 0.1, and measured at 3, 6,12, and 24 h as described in section 3.2.3.1. n = 3 for each curve

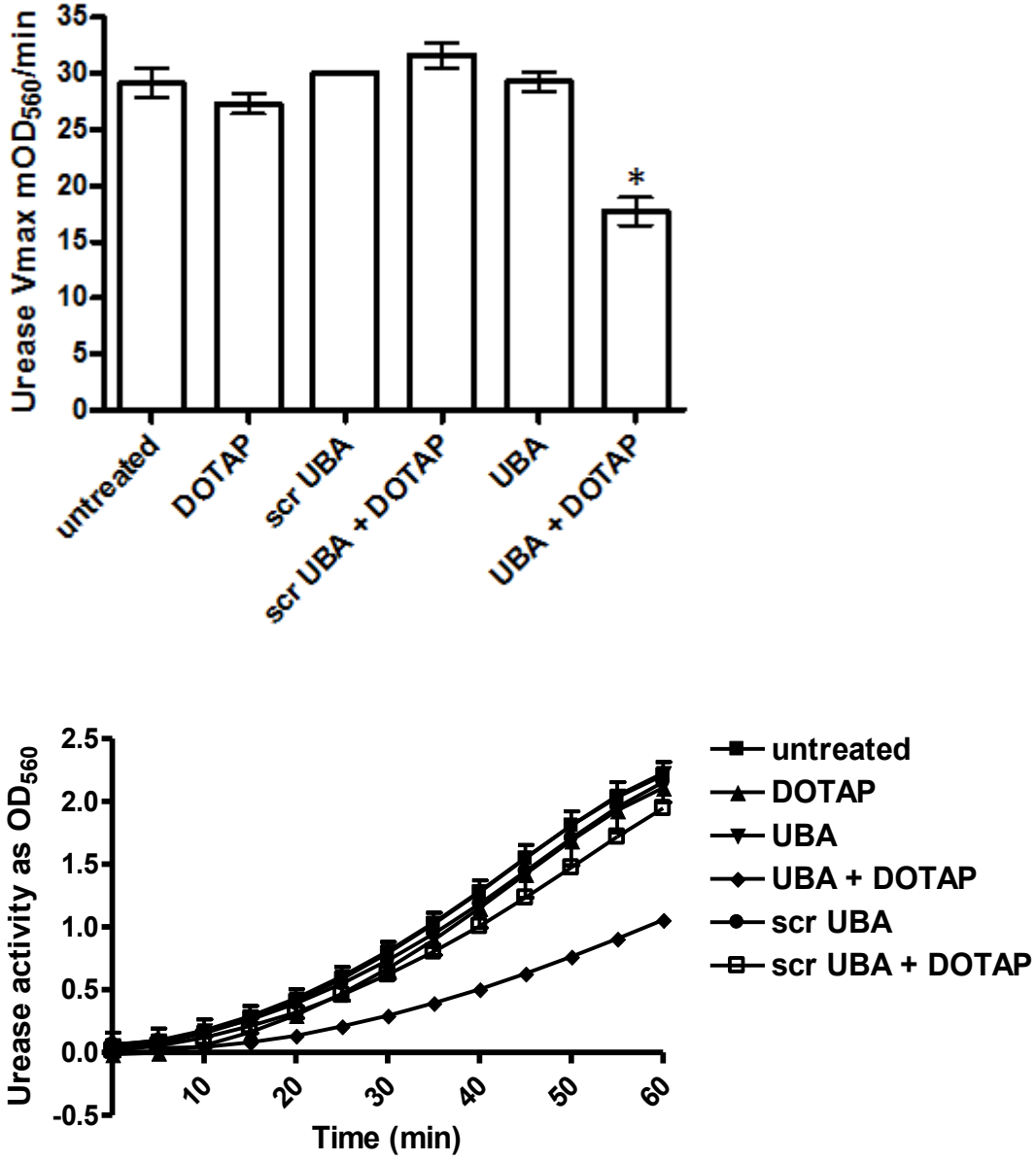


Figure 3.6 *H. pylori* A64 urease activity with ±20 nM UBA± DOTAP®

Urease activity of *H. pylori* A64 is measured as Vmax mOD_{560}/min at 60 min (upper panel), and every 5 min up to 60 min (lower panel) as described in section 3.2.2.2 for urease antisense DNA treatments (UBA) with or without liposomes (DOTAP®) as described in 3.2.3.3. scr UBA refers to scrambled urease antisense treatment that serves as a negative antisense DNA control.

* $p = 0.008$, UBA+DOTAP® vs other treatment groups

n = 3 per treatment group

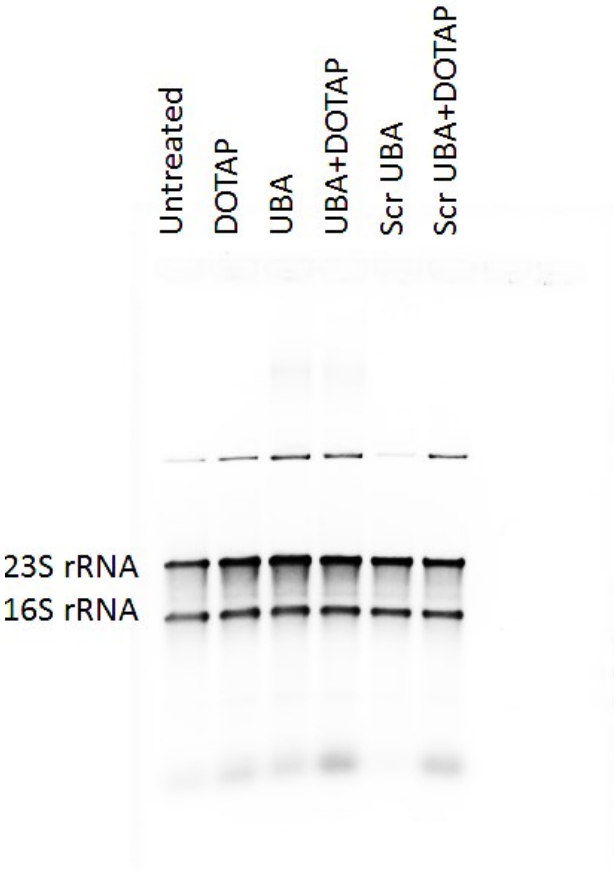


Figure 3.7 Agarose gel electrophoresis of *H. pylori* RNA denatured by formamide

RNA quality is assessed as described in section 3.2.3.4 prior to performing real-time reverse-transcriptase PCR to assess urease expression following treatment with antisense DNA (UBA) with or without liposomes (DOTAP®) as described in 3.2.3.3. scr UBA refers to a scrambled urease antisense DNA sequence treatment that serves as a negative antisense DNA control.

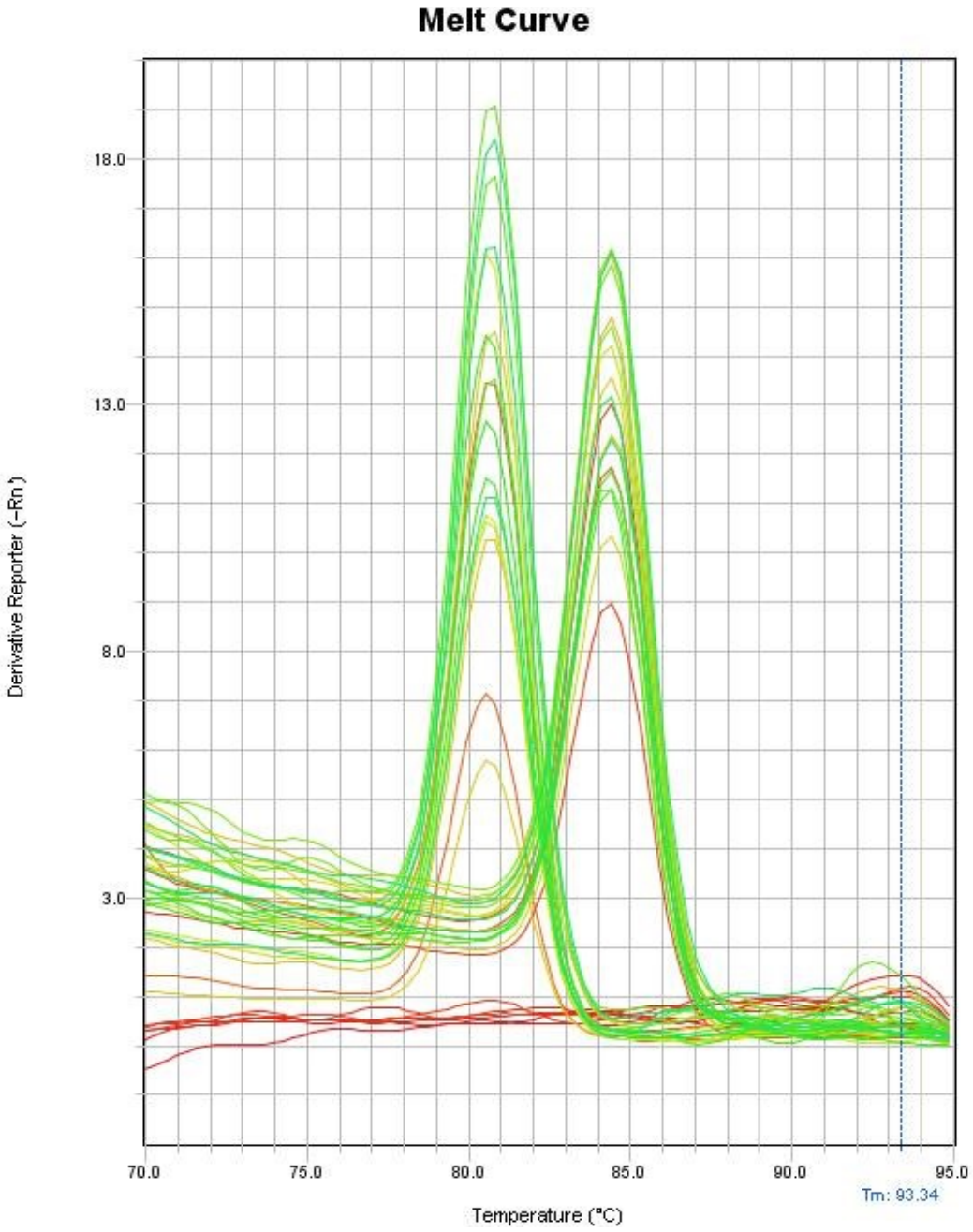


Figure 3.8 Melt curves for urease and 16S rRNA primer pairs.

Real-time reverse-transcriptase PCR was performed as described in 3.2.3.4.

Melt curves demonstrate specificity of urease gene and 16S rRNA amplicons.

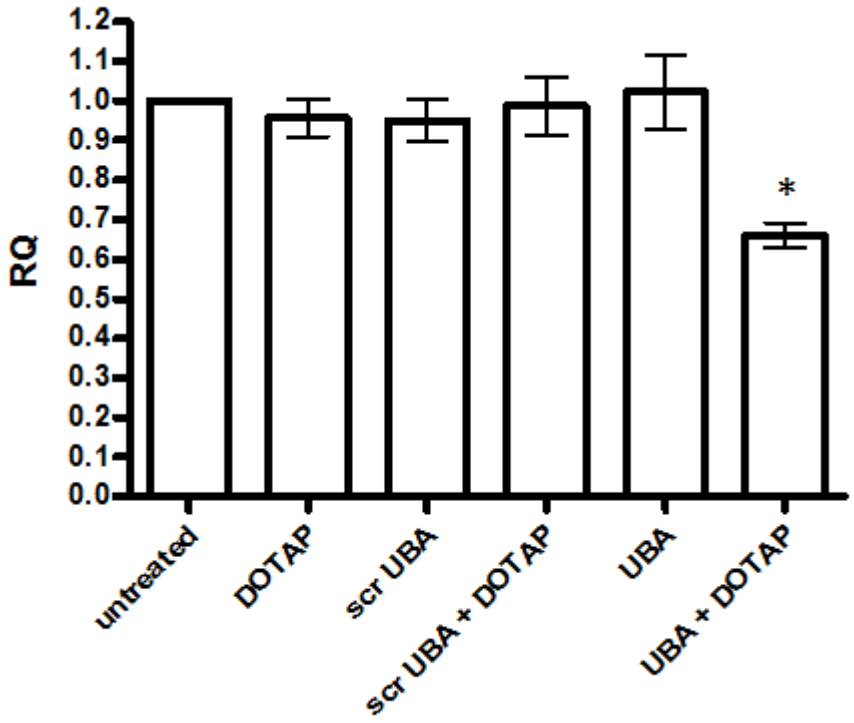


Figure 3.9 Real-time reverse-transcriptase PCR confirmation of urease transcription

RQ: Relative Quantification represents the change in urease mRNA expression as compared with the mRNA expression of the 16S rRNA internal control as described in section 3.2.3.4.

Urease expression was assessed following treatment with antisense DNA (UBA) with or without liposomes (DOTAP®) as described in 3.2.3.3. scr UBA refers to a scrambled urease antisense DNA sequence treatment that serves as a negative antisense DNA control.

* $p = 0.0078$, UBA+DOTAP® vs other groups

n = 3 per treatment group

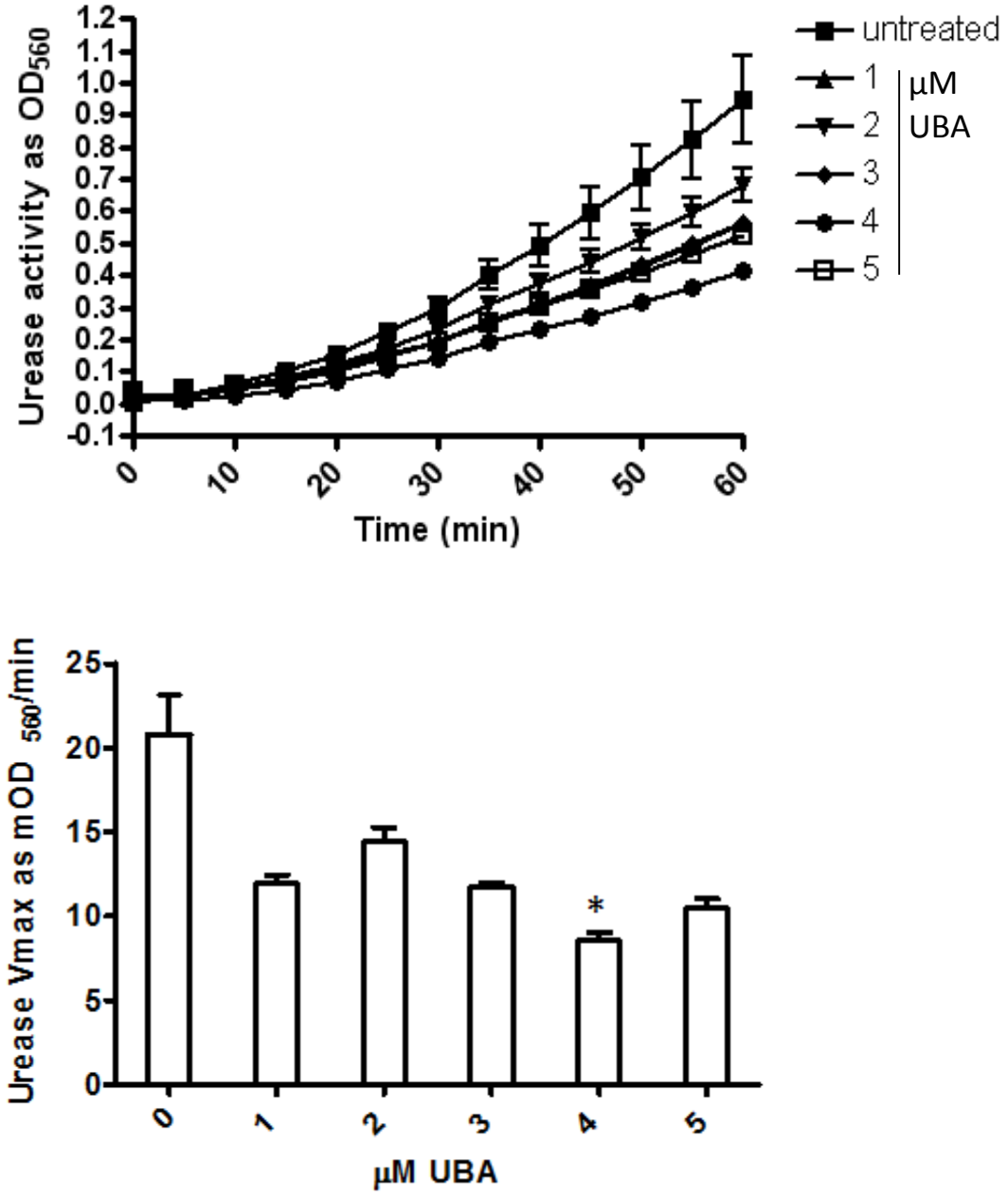


Figure 3.10 Effect of increasing concentrations of UBA in the absence of DOTAP® for *H. pylori* A64

Urease expression of *H. pylori* A64 was assessed following treatment with increasing concentrations of antisense DNA (UBA) in the absence of liposomes (DOTAP®) as described in 3.2.3.3.

*$p < 0.05$, 4 µm UBA vs other concentrations

n = 3 per UBA concentration

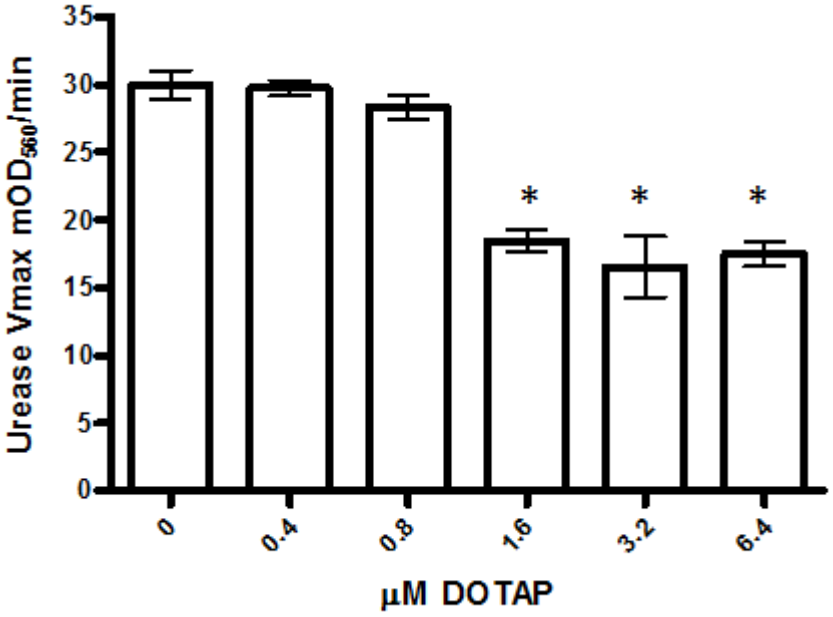


Figure 3.11 Effect of increasing concentrations of DOTAP® in the presence of 20 nM UBA on the urease activity of *H. pylori* A64

*$p < 0.0001$, 1.6 µm DOTAP® vs DOTAP® concentrations $\leq$ 0.8 µm DOTAP

3.2 µm DOTAP® vs DOTAP® concentrations $\leq$ 0.8 µm DOTAP

6.4 µm DOTAP® vs DOTAP® concentrations $\leq$ 0.8 µm DOTAP

n = 3 per DOTAP® concentration

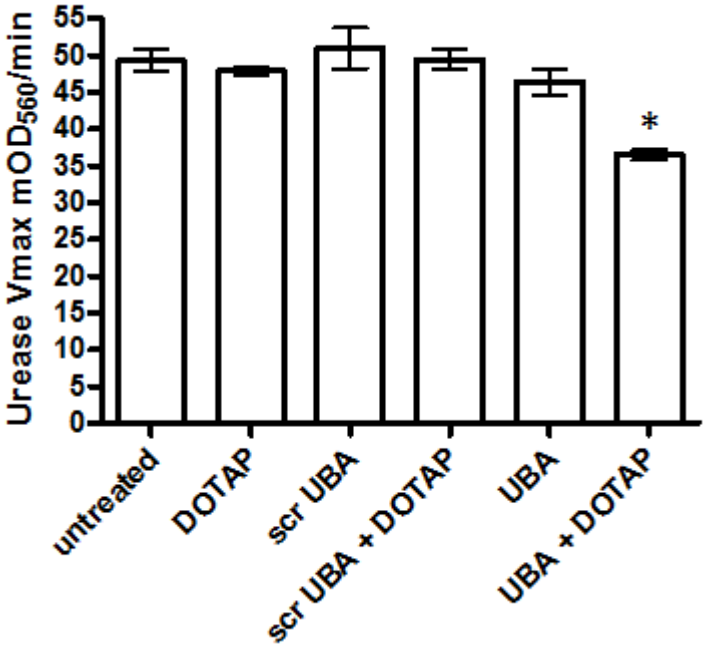


Figure 3.12 Effect of UBA ± DOTAP® on the urease activity of *H. pylori* A70

Urease activity of *H. pylori* A70 is measured as Vmax mOD_{560}/min at 60 min as described in section 3.2.2.2 for urease antisense DNA treatments (UBA) with or without liposomes (DOTAP®) as described in 3.2.3.3. scr UBA refers to scrambled urease antisense treatment that serves as a negative antisense DNA control.

*p = 0.01, UBA+DOTAP® vs other groups

n = 3 per treatment group

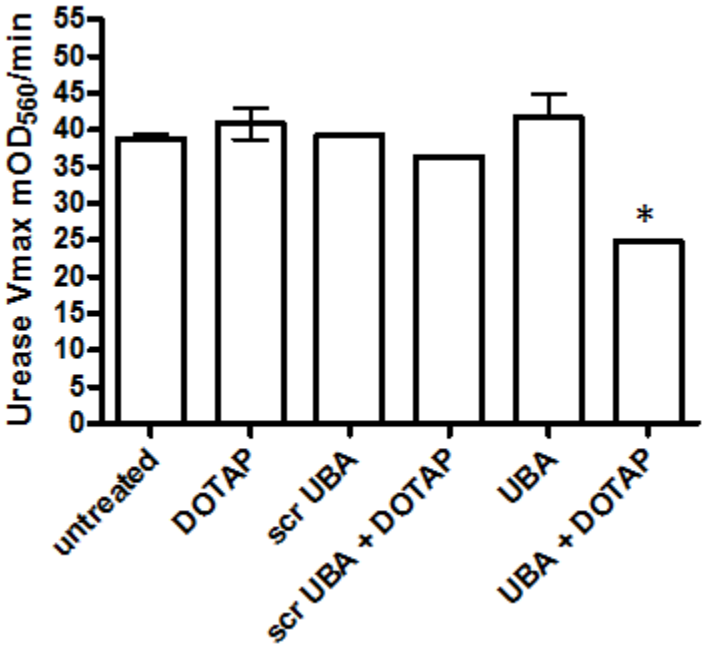


Figure 3.13 Effect of UBA ± DOTAP® on the urease activity of A20

Urease activity of *H. pylori* A20 is measured as Vmax mOD_{560}/min at 60 min as described in section 3.2.2.2 for urease antisense DNA treatments (UBA) with or without liposomes (DOTAP®) as described in 3.2.3.3. scr UBA refers to scrambled urease antisense treatment that serves as a negative antisense DNA control.

*$p < 0.0001$, UBA+DOTAP vs other groups

n = 3 per treatment group

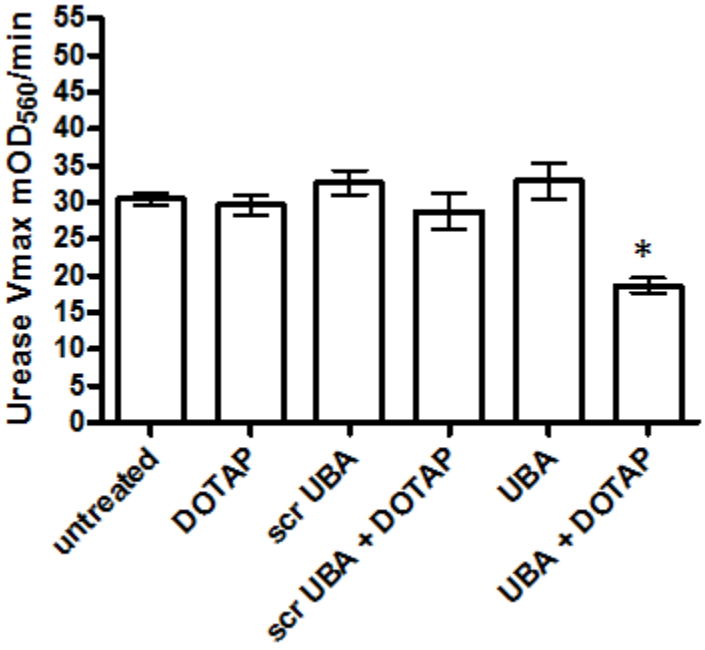


Figure 3.14 Effect of UBA ± DOTAP® on the urease activity of A135

Urease activity of *H. pylori* A70 is measured as Vmax mOD_{560}/min at 60 min as described in section 3.2.2.2 for urease antisense DNA treatments (UBA) with or without liposomes (DOTAP®) as described in 3.2.3.3. scr UBA refers to scrambled urease antisense treatment that serves as a negative antisense DNA control.

*$p < 0.0001$, UBA+DOTAP vs other groups

n = 3 per treatment group

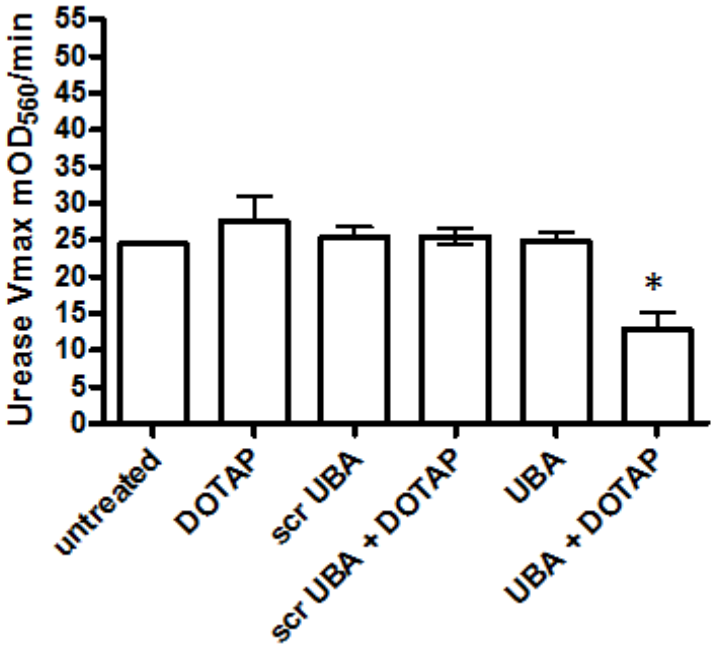


Figure 3.15 Effect of UBA ± DOTAP® on the urease activity of A96

Urease activity of *H. pylori* A96 is measured as Vmax mOD_{560}/min at 60 min as described in section 3.2.2.2 for urease antisense DNA treatments (UBA) with or without liposomes (DOTAP®) as described in 3.2.3.3. scr UBA refers to scrambled urease antisense treatment that serves as a negative antisense DNA control.

*$p < 0.0001$, UBA+DOTAP vs other groups

n = 3 per treatment group

3.5 References

(1) Brown LM. *Helicobacter pylori*: epidemiology and routes of transmission. Epidemiol Rev 2000;22(2):283-297.

(2) Cheung J, Goodman K, Munday R, Heavner K, Huntington J, Morse J, et al. *Helicobacter pylori* infection in Canada's arctic: searching for the solutions. Can J Gastroenterol 2008 Nov;22(11):912-916.

(3) Kusters JG, van Vliet AH, Kuipers EJ. Pathogenesis of *Helicobacter pylori* infection. Clin Microbiol Rev 2006 Jul;19(3):449-490.

(4) Warren JR, Marshall BJ. Unidentified curved bacilli on gastric epithelium in active chronic gastritis. Lancet 1983 Jun 4;1(8336):1273-1275.

(5) Marshall BJ, Warren JR. Unidentified curved bacilli in the stomach of patients with gastritis and peptic ulceration. Lancet 1984 Jun 16;1(8390):1311-1315.

(6) Al-Eidan FA, McElnay JC, Scott MG, McConnell JB. Management of *Helicobacter pylori* eradication-the influence of structured counselling and follow-up. Br J Clin Pharmacol 2002 Feb;53(2):163-171.

(7) Meyer JM, Silliman NP, Wang W, Siepman NY, Sugg JE, Morris D, et al. Risk factors for *Helicobacter pylori* resistance in the United States: the surveillance of *H. pylori* antimicrobial resistance partnership (SHARP) study, 1993-1999. Ann Intern Med 2002 Jan 1;136(1):13-24.

(8) Cameron EA, Bell GD, Baldwin L, Powell KU, Williams SG. Long-term study of re-infection following successful eradication of *Helicobacter pylori* infection. Aliment Pharmacol Ther 2006 May 1;23(9):1355-1358.

(9) Georgopoulos SD, Papastergiou V, Karatapanis S. Current options for the treatment of *Helicobacter pylori*. Expert Opin Pharmacother 2013 Feb;14(2):211-223.

(10) Onder G, Aydin A, Akarca U, Tekin F, Ozutemiz O, Ilter T. High *Helicobacter pylori* resistance rate to clarithromycin in Turkey. J Clin Gastroenterol 2007 Sep;41(8):747-750.

(11) Suzuki RB, Lopes RA, da Camara Lopes GA, Hung Ho T, Speranca MA. Low *Helicobacter pylori* primary resistance to clarithromycin in gastric biopsy specimens from dyspeptic patients of a city in the interior of Sao Paulo, Brazil. BMC Gastroenterol 2013 Dec 4;13:164-230X-13-164.

(12) Gao W, Cheng H, Hu F, Li J, Wang L, Yang G, et al. The evolution of *Helicobacter pylori* antibiotics resistance over 10 years in Beijing, China. Helicobacter 2010 Oct;15(5):460-466.

(13) Tomatari, F. Haghi, A. Mohabbati Mobarez, M. Amini, D. Hosseini, and A. Talebi Bezmin Abadi. Resistance to Metronidazole and Clarithromycin in Dyspeptic Patients in Iran. Iranian Red Crescent Medical Journal 2010;12.

(14) Morse AL, Goodman KJ, Munday R, Chang HJ, Morse JW, Keelan M, et al. A randomized controlled trial comparing sequential with triple therapy for *Helicobacter pylori* in an Aboriginal community in the Canadian North. Can J Gastroenterol 2013 Dec;27(12):701-706.

(15) Marshall BJ, Barrett LJ, Prakash C, McCallum RW, Guerrant RL. Urea protects *Helicobacter (Campylobacter) pylori* from the bactericidal effect of acid. Gastroenterology 1990 Sep;99(3):697-702.

(16) Mobley HL, Cortesia MJ, Rosenthal LE, Jones BD. Characterization of urease from *Campylobacter pylori.* J Clin Microbiol 1988 May;26(5):831-836.

(17) Tsuda M, Karita M, Morshed MG, Okita K, Nakazawa T. A urease-negative mutant of *Helicobacter pylori* constructed by allelic exchange mutagenesis lacks the ability to colonize the nude mouse stomach. Infect Immun 1994 Aug;62(8):3586-3589.

(18) Eaton KA, Brooks CL, Morgan DR, Krakowka S. Essential role of urease in pathogenesis of gastritis induced by *Helicobacter pylori* in gnotobiotic piglets. Infect Immun 1991 Jul;59(7):2470-2475.

(19) Kosikowska P, Berlicki L. Urease inhibitors as potential drugs for gastric and urinary tract infections: a patent review. Expert Opin Ther Pat 2011 Apr 4.

(20) Isaka Y, Imai E, Takahara S, Rakugi H. Oligonucleotidic therapeutics. Expert Opin Drug Discov 2008 Sep;3(9):991-996.

(21) Nakashima N, Tamura T. Gene silencing in *Escherichia coli* using antisense RNAs expressed from doxycycline-inducible vectors. Lett Appl Microbiol 2013 Mar 11.

(22) Patil SD, Sharma R, Srivastava S, Navani NK, Pathania R. Downregulation of *yidC* in *Escherichia coli* by Antisense RNA Expression Results in Sensitization to Antibacterial Essential Oils Eugenol and Carvacrol. PLoS One 2013;8(3):e57370.

(23) Yamaoka Y. Roles of *Helicobacter pylori* BabA in gastroduodenal pathogenesis. World J Gastroenterol 2008 Jul 21;14(27):4265-4272.

(24) Jeon B, Zhang Q. Sensitization of *Campylobacter jejuni* to fluoroquinolone and macrolide antibiotics by antisense inhibition of the CmeABC multidrug efflux transporter. J Antimicrob Chemother 2009 May;63(5):946-948.

(25) Jain P, Jain S, Prasad KN, Jain SK, Vyas SP. Polyelectrolyte coated multilayered liposomes (nanocapsules) for the treatment of *Helicobacter pylori* infection. Mol Pharm 2009 Mar-Apr;6(2):593-603.

(26) Kawata Y, Yano S, Kojima H. *Escherichia coli* can be transformed by a liposome-mediated lipofection method. Biosci Biotechnol Biochem 2003 May;67(5):1179-1181.

(27) Ladewig K, Niebert M, Xu ZP, Gray PP, Lu GQ. Efficient siRNA delivery to mammalian cells using layered double hydroxide nanoparticles. Biomaterials 2010 Mar;31(7):1821-1829.

(28) Ito S, Kohli Y, Kato T, Murakita H, Ohotaki Y, Hirai M, et al. Differences in urease activity in live *Helicobacter pylori* cultured from patients with gastroduodenal diseases. Eur J Gastroenterol Hepatol 1995 Aug;7 Suppl 1:S83-8.

(29) Wang Y, Roos KP, Taylor DE. Transformation of *Helicobacter pylori* by chromosomal metronidazole resistance and by a plasmid with a selectable chloramphenicol resistance marker. J Gen Microbiol 1993 Oct;139(10):2485-2493.

(30) Hu LT, Mobley HL. Expression of catalytically active recombinant *Helicobacter pylori* urease at wild-type levels in *Escherichia coli*. Infect Immun 1993 Jun;61(6):2563-2569.

(31) Burucoa C, Lhomme V, Fauchere JL. Performance criteria of DNA fingerprinting methods for typing of *Helicobacter pylori* isolates: experimental results and meta-analysis. J Clin Microbiol 1999 Dec;37(12):4071-4080.

(32) Segal ED. Electroporation of *Helicobacter pylori*. Methods Mol Biol 1995;47:179-184.

(33) Tu Z. An improved system for competent cell preparation and high efficiency plasmid transformation using different *Escherichia coli* strains. Electronic Journal of Biotechnology 2005;8(1).

(34) Nakao M, Malfertheiner P. Growth inhibitory and bactericidal activities of lansoprazole compared with those of omeprazole and pantoprazole against *Helicobacter pylori*. Helicobacter 1998 Mar;3(1):21-27.

(35) Walder J. Antisense DNA and RNA: progress and prospects. Genes Dev 1988 May;2(5):502-504.

(36) Integrated DNA Technology. IDT SciTools® Web Tools. 2012; Available at: https://www.idtdna.com/pages/scitools. Accessed 09, 2011.

(37) Masek T, Vopalensky V, Suchomelova P, Pospisek M. Denaturing RNA electrophoresis in TAE agarose gels. Anal Biochem 2005 Jan 1;336(1):46-50.

(38) Wang J, Wang WH, Li J, Liu FX. Celecoxib inhibits *Helicobacter pylori* colonization-related factors. World J Gastroenterol 2010 Feb 21;16(7):846-853.

(39) Life technologies. http://www.appliedbiosystems.com/absite/us/en/home.html. Accessed 09, 2011.

(40) Yanagihara K, Tashiro M, Fukuda Y, Ohno H, Higashiyama Y, Miyazaki Y, et al. Effects of short interfering RNA against methicillin-resistant *Staphylococcus aureus* coagulase in vitro and in vivo. J Antimicrob Chemother 2006 Jan;57(1):122-126.

(41) Rocha A, Ruiz S, Coll JM. Improvement of DNA transfection with cationic liposomes. J Physiol Biochem 2002 Mar;58(1):45-56.

(42) Kawata Y, Yano S, Kojima H. *Escherichia coli* can be transformed by a liposome-mediated lipofection method. Biosci Biotechnol Biochem 2003 May;67(5):1179-1181.

(43) Pelicic V, Jackson M, Reyrat JM, Jacobs WR,Jr, Gicquel B, Guilhot C. Efficient allelic exchange and transposon mutagenesis in Mycobacterium tuberculosis. Proc Natl Acad Sci U S A 1997 Sep 30;94(20):10955-10960.

(44) Vasu K, Nagaraja V. Diverse functions of restriction-modification systems in addition to cellular defense. Microbiol Mol Biol Rev 2013 Mar;77(1):53-72.

(45) Manconi M, Isola R, Falchi AM, Sinico C, Fadda AM. Intracellular distribution of fluorescent probes delivered by vesicles of different lipidic composition. Colloids Surf B Biointerfaces 2007 Jun 15;57(2):143-151.

(46) Matzke M, Matzke AJ, Kooter JM. RNA: guiding gene silencing. Science 2001 Aug 10;293(5532):1080-1083.

(47) Fillion P, Desjardins A, Sayasith K, Lagace J. Encapsulation of DNA in negatively charged liposomes and inhibition of bacterial gene expression with fluid liposome-encapsulated antisense oligonucleotides. Biochim Biophys Acta 2001 Nov 1;1515(1):44-54.

(48) Elbashir SM, Harborth J, Lendeckel W, Yalcin A, Weber K, Tuschl T. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. Nature 2001 May 24;411(6836):494-498.

(49) Doh KO, Kim BK, Lee TJ, Park JW, Seu YB. Synthesis of NBD-labeled DOTAP analog to track intracellular delivery of liposome. J Microbiol Biotechnol 2013 Jan;23(1):131-135.

Chapter 4

Antibacterial activity of antisense DNA loaded and unloaded stearylamine-containing liposomes [3]

In chapter 3, I investigated the possibility of interfering with urease production as a novel approach to treat *H. pylori* infections with or without liposomes to enhance delivery to *H. pylori*. Chapter 2 genotypic studies provided evidence to support urease as the target gene. In this chapter, I aimed to alter the liposome composition to achieve maximum inhibition of urease activity.

4.1 Introduction

In the era of increased antimicrobial resistance to commonly used antibiotics, there is a great need for developing alternative treatment approaches. Liposomes are nanosized artificial spherical vesicles that consist of phospholipid bilayers. Since their first discovery by Alec D. Bangham, they have attracted attention as potential carriers for many bioactive molecules (1). Liposomes have been extensively studied to improve the delivery of antibiotics such as aminoglycosides, quinolones, polypeptides, and betalactames to target sites (2). However, this strategy may not be useful to treat infections caused by multi-drug resistant bacteria.

In the literature, there are few reports on the use of unloaded liposomes as antimicrobial agents. Cationic liposomes have antimicrobial activity against bacteria, fungi and protozoan (3-5). Cationic liposomes may react with phospholipids in cytoplasmic membranes, causing membrane distortion and protoplast lysis under osmotic stress. Moreover, cationic liposomes may be adsorbed on the surface of microbial cells causing a change in overall charge from negative to positive (6). The positive charge on microbial cells has been linked to the biocidal action as proteins might be aggregated and/or precipitated (4). Liposomes bearing phosphatidylcholine and stearylamine killed Leishmania *in vitro* and *in vivo*, without having any adverse effect to the host (5).

A recent study has reported antibacterial activity for liposomal linolenic acids against antibiotic-resistant *H. pylori*. The prepared liposomes were able to disrupt membranes of both *H. pylori* spiral and coccoid forms (7). Free fatty acids such as lauric acid, myristoleic acid, linoleic acid, and linolenic acid have antibacterial activities against wide range of bacteria including *H.*

[3] The work presented in this chapter is in preparation for submission for publication.

pylori. Since these lipid-like molecules are naturally present everywhere, they are less harmful than conventional antibiotics. Incorporation of free fatty acids into liposomes may have potent antimicrobial effect (8). The study investigates the impact of changing liposome lipid composition on the delivery of urease antisense DNA to impair urease activity, as well as the antimicrobial effects of varying stearylamine concentration in phosphatidylcholine/cholesterol liposomes.

4.2 Materials and Methods

Unless otherwise stated, chemicals, disposables and glassware were purchased from Fisher Scientific (Ottawa, ON, Canada), antibiotics were purchased from Sigma-Aldrich (Oakville, ON, Canada), and enzymes were purchased from Invitrogen (Carlsbad, CA, USA).

4.2.1 Liposome preparation and characterization

4.2.1.1 Preparation of stearylamine-containing liposomes

Liposomes were prepared by mixing egg phosphatidylcholine (PC) (100 mg/mL), cholesterol (CH) (20 mg/mL) and stearylamine (SA) (10 mg/ml) (Sigma- Aldrich, Oakville, ON, Canada) to achieve a molar ratio of 7:3:1 (**F1 liposomes**) and 7:3:2 (**F2 liposomes**) in chloroform in a round-bottom glass tube. The solvent was evaporated under nitrogen gas. Lipid film hydration was done using HEPES-NaCl buffer (20 mM HEPES, 135 mM NaCl, pH 7.4, osmolality~290 mOsm) at 60˚C. The lipid solution was transferred to a 5 ml cryo vial (Nalgene® cryogenic vial, Sigma-Aldrich, Oakville, ON, Canada) and vortexed vigorously to agitate and resuspend the liposomes for 3 min followed by heating the lipid solution in a water bath adjusted at 60˚C for another 3 min. The vortex/heat cycle was repeated 5 times. The lipid solution was frozen by immersing the cryo tube into liquid nitrogen, thawed by placing the tube in 60˚C water bath and vortexed vigorously for 3 min. The freeze/thaw/vortex cycle was repeated 5 times. The product of hydration is large multilamellar vesicles which can be stored at 4˚C under nitrogen gas for up to 3 days. Small unilamellar vesicles were obtained by extruding the large multilamellar vesicles 2 times through a 0.4 μm filter and 8 times through 0.2 μm filter (Nuclepore, Whatman, New Jersey, USA) under nitrogen gas using the Lipex Biomembranes extrusion device (Vancouver, British Columbia, Canada). Liposomes (F1 & F2) were sterilized by filtration through 0.45 μm filter (Merck KGaA, Darmstadt, Germany). A stream of nitrogen gas was bubbled in the sample to remove any traces of oxygen using a sterile Pasteur pipette and the container was tightly caped

and wrapped with parafilm to prevent lipid oxidation. Liposomes (F1 & F2) were stored at 4˚C for up to 4 weeks (9,10).

4.2.1.2 Assessment of liposome phospholipid content

The phospholipid content of liposomes was assessed using the Stewart assay (11). Briefly, the Stewart reagent (ferrothiocyanate) was prepared by dissolving 27.03 g of ferric chloride hexahydrate and 30.4 g of ammonium thiocyanate in double distilled water and the volume adjusted to 1 liter. Aliquots of 2.5 µL of liposomes were added to 1.5 ml Eppendorf tubes in triplicate, evaporated under nitrogen gas, and dissolved in 500 µL chloroform. Then, 500 µL of Stewart reagent was added followed by vortexing for 20 seconds. The tubes were centrifuged at 2000 x *g* for 10 min and the organic lower layer was removed by using a Pasteur pipette. The optical density of the developed yellowish brown colour was measured at 485 nm and compared to that of serial dilutions of standard egg PC (0, 5, 10, 20, 30, 40, 50, 100 µg/ml) (**Figure 4.1**) (11).

4.2.1.3 Liposome sizing

Liposome diameter was determined using the Malvern Zetasizer Nano S (Worcestershire, UK). The principle of the Zetasizer Nano S is based on two techniques that measure particles simultaneously. Dynamic light scattering measures the diffusion of particles moving under Brownian motion and converts this to the size and size distribution of particles. The second technique, static light scattering is used to determine the molecular weight of proteins and polymers, but this measure was not utilized in this study. Two sizes of latex beads were used as standards 120 nm and 200 nm to calibrate the instrument. Each liposome sample was sized twice.

4.2.2 Effect of unloaded liposomes on the growth of *H. pylori*

H. pylori A64 was cultured from a frozen stock on BHI/YE/HS agar plate and then subcultured onto a fresh plate for 24 h. The growth was suspended in BHI/YE/HS broth to achieve OD_{600} = 0.1. A64 was then cultured in 6-well tissue culture plates with shaking (120 rpm) for 24 h at 37°C under microaerobic conditions in the presence of different concentrations of liposomes (0, 25, 50, 75, 100, 125 µg/ml). The effect of liposome treatment on the growth of A64 was assessed after 3, 6, 12 and 24 h incubation by determining the number of cfu/ml.

4.2.3 Loading liposomes with UBA or scr UBA

4.2.3.1 Liposome loading with UBA and assessment of loading efficiency

Liposomes were mixed with either UBA or scr UBA in a weight ratio of 5:1 and then incubated at room temperature for 15 minutes in duplicate. One aliquot was treated with 5 µL of 0.5% SDS and then incubated in a water bath at 37°C for 10 minutes. The loading efficiency was assessed by a membrane impermeability assay in which the intensity of UBA + liposomes was compared to SDS treated UBA + liposomes, each stained with SYBR® Gold. The SDS disrupts liposomes, exposing UBA to SYBR® Gold stain. The increased intensity of the band observed after SDS treatment supports the presence of UBA/scr UBA loaded liposomes (12). Free UBA served as a positive control. HEPES-buffered saline and unloaded liposomes served as negative controls.

A constant amount of UBA was mixed with liposomes in an increasing weight ratio from 1:1 to 1:10, run through a 2% agarose gel, and then strained using SYBR® Gold to assess the effect of increasing concentration of liposomes on UBA staining intensity.

4.2.3.2 Preparation of UBA and scr UBA loaded F1 liposomes

The lipid mixture of F1 liposomes (PC:CH:SA, 7:3:1) was first dried under nitrogen gas as previously described previously in section 4.2.1.1. To load F1 liposomes, UBA or scr UBA were dissolved in hydration buffer before the lipid film hydration step to achieve a final UBA or scr UBA concentration of 0.09 mM. The prepared liposomes were characterized for size and phospholipid content as previously described in previously in sections 4.2.1.1 and 4.2.1.2.

4.2.3.3 Effect of UBA and scr UBA loaded F1 liposomes on *H. pylori* urease activity

The effect of 20 nM UBA or scr UBA loaded F1 liposomes on the urease activity of *H. pylori* A64 was investigated as previously described in Chapter 3, section 3.2.2.2. Since inhibition of *H. pylori* growth may affect UBA incorporation, the inhibitory effect of unloaded F1 liposomes (2.5 µg/mL) was also investigated by determining the number of cfu/ml at the end of incubation period.

4.2.4 Statistical analysis

Data are expressed as mean ± SEM. Significant differences among more than two groups were detected by one-way ANOVA. When a sigificance was found by one-way ANOVA, the Turkey post-hoc test was performed to identify which groups were significantly different and to avoid a type I error. However, when no significance was found by one-way ANOVA, the Duncan test was performed to avoid a type II error. A p-value of < 0.05 was considered statistically significant. All analyses were performed using IBM SPSS® version 20 (New York, USA).

4.3 Results

4.3.1 Characterization of stearylamine-containing liposomes

Liposome formulations contained 82-88.6% of total phospholipids from the egg PC aliquots used for preparation. The 120 nm and 200 nm standard latex beads had a narrow particle size distribution with the calculated standard deviations of 27.6 and 48.4 respectively (**Figures 4.2 and 4.3**). A uniform but broader distribution was observed for individual liposome preparations (**Figures 4.4-4.7**). Altering liposome composition did not affect liposome size as seen for F1 liposomes (**Figure 4.4**) versus F2 liposomes (**Figure 4.5**). Similarly, loading of the liposomes with UBA (**Figure 4.6**) or scr UBA (**Figure 4.7**) did not affect the size. Each liposome formulation was prepared on 3 different days and were of similar average size, regardless the composition or loading with UBA or scr UBA (**Figure 4.8**).

4.3.2 Antimicrobial activity of F1 and F2 liposomes

F1 liposomes inhibited the growth of *H. pylori* A64 in a concentration dependent manner. Inhibition of growth was observed at 12 h incubation for 75, 100 and 125 μg/ml relative to untreated *H. pylori*. (**Figures 4.9**). The greatest inhibition (95%) was observed with 125 μg/ml liposomes at 12 h post-treatment relative to untreated *H. pylori*. (**Figure 4.10**). However, the degree of inhibition was decreased to 78% at 24 h.

A single treatment of F2 liposomes inhibited *H. pylori* growth (cfu/ml) in a concentration dependent manner with the greatest inhibition observed with 125 μg/ml liposomes at 24 h post-treatment (**Figure 4.11**). Approximately 96% inhibition of growth (cfu/ml) was observed at 12 h post-treatment, which was sustained over 24 h to reach 98% inhibition relative to untreated *H.*

pylori (**Figure 4.12**). Similar inhibitions were observed when growth was assessed as a change in OD_{600} (**Figures 4.13**)

Two treatments of F2 liposomes were more effective than F1 liposomes, when OD_{600} (**Figure 4.14**) or cfu/ml (**Figure 4.15**) was used to monitor *H. pylori* growth after 24 h incubation. Changes in OD_{600} did not accruately reflect the changes observed in cful/ml.

Two treatments of F2 were able to achieve 100% inhibition in *H. pylori* growth at concentrations 75, 100 and 125 µg/ml when cfu/ml was used to monitor *H. pylori* growth, and was more effective than the same concentration of F1 liposomes (**Figure 4.16**).

Two treatments of F1 liposomes (**Figure 4.14**) were less effective than one treatment in inhibiting *H. pylori* growth (OD_{600}) at 24 h (**Figure 4.13**). The growth of untreated *H. pylori* in the two F1 liposome treatment experiment (**Figure 4.14**) was approximately twice the OD_{600} value observed in the single treatment experiment (**Figure 4.13**).

4.3.3 Assessment of F1 liposomes loading with UBA

SDS disrupted liposomes, exposed UBA to SYBR® Gold and increased the intensity of the UBA band. However, the UBA + liposomes band was more intense than the free UBA band (**Figure 4.17**).

As the ratio of liposomes increased relative to UBA, the intensity of the UBA band also increased, which suggests the liposomes increased the accessibility of UBA for staining by SYBR® Gold (**Figure 4.18**).

4.3.4 Effect of 20 nM UBA + F1 on urease activity of A64

The urease activity (OD_{560}) of *H. pylori* treated with unloaded F1 liiposomes increased with time and reached a maximum activity by 50 min (**Figure 4.19**). Similar urease activity was observed when *H. pylori* was treated with scr UBA + F1 liposomes. However, when treated with UBA+F1 liposomes, the *H. pylori* urease activity was reduced relative to *H. pylori* treated with unloaded F1 liposomes or scr UBA+ F1 liposomes.

20 nM UBA + 2.5 µg/ml F1 liposomes inhibited the urease activity (Vmax) of *H. pylori* A64 by 30% relative to unloaded F1 liposomes or scr UBA + F1 liposomes (**Figure 4.19**). The growth of *H. pylori* was unaffected by liposome treatment.

4.4 Discussion

4.4.1 Antimicrobial activity of stearylamine-containing liposomes

Cationic liposomes have been previously reported to exert antimicrobial activity against bacteria, fungi and protozoan (3-5). In the present study, stearylamine-containing liposomes inhibited *H. pylori* growth in a concentration dependant manner. Higher antimicrobial activity was observed when stearylamine concentration was increased.

Similar to the present study, the bactericidal effect of dioctadecyldimethylammonium bromide (DODAB) containing cationic liposomes was evaluated against four different types of bacteria (*E. coli*, *Salmonella typhimurium*, *Pseudomonas aeruginosa*, *Staphylococcus aureus)*. DODAB liposomes had antimicrobial activity against both Gram positive and Gram negative bacteria but the highest susceptibility was observed against *E.coli* (3). Larger liposomes (~ 250 nm) exerted better antimicrobial activity against *E. coli* than smaller liposomes (~ 86 nm). DODAB concentrations required for the bactericidal effect were 0.01- 0.1 mM, whereas a 40–50% of death of mouse fibroblasts was measured by flow cytometry in the presence of 1 mM DODAB indicating a potential for selective toxicity and therapeutic application (3). In the present study, only one size (~ 130 nm) of liposomes was assessed. Larger liposomes may exert better antimicrobial activity at lower stearylamine concentrations due to better stability than smaller liposomes. This may explain why larger liposomes may have better antimicrobial activity than smaller liposomes. Stearylamine is a fatty amine with poor water solubility. The incorporation of stearylamine into cationic liposomes solves the poor water solubility problem, allowing for better delivery of stearylamine to *H. pylori*. Moreover, cationic liposomes themselves exert antimicrobial activity by reacting with phospholipids in cytoplasmic membranes, causing membrane distortion and protoplast lysis under osmotic stress. Cationic liposomes may be adsorbed on the surface of microbial cells causing a change in overall charge from negative to positive (6). The positive charge on microbial cells has been linked to the biocidal action as proteins might be aggregated and/or precipitated (4).

Dey *et al.,* reported the antimicrobial activity of stearylamine-containing liposomes. In the previous study, stearylamine-containing liposomes (PC/SA) in a molar ratio of 7:2 had *in vitro* and *in vivo* antileishmanial activity. A concentration of 88 µg/mL of the PC/SA liposomes killed 95% of amastigotes, a stage in the life cycle of leishmania, in 24 h. The toxicity of PC/SA liposomes

for normal murine macrophages was also investigated. High concentrations of SA-PC liposomes (396-1,188 μg/mL) imparted 14.4-16.6 % toxicity to normal macrophages while 132 μg/mL of PC/SA liposomes caused less than 1% toxicity (13). In the present study, 125 μg/mL of F2 liposomes (PC/CH/SA in a molar ratio 7:2:1) inhibited *H. pylori* growth by 98% following 24 h incubation. The concentration used in this study was lower than that investigated by Dey *et al.,* and exerted minimum toxicity against macrophages. However, toxicity to gastric cell line still needs to be addressed.

Free fatty acids have antibacterial activity against many pathogenic bacteria (8). It is proposed that the target site of free fatty acid is the cell membrane as free fatty acids may disrupt the electron transport chain and oxidative phosphorylation. In addition, free fatty may inhibit enzymatic activity and cause impairment of nutrient uptake. Moreover, free fatty acids may generate peroxidation and auto-oxidation degradation products or cause direct lysis of bacterial cells (8). Unsaturated fatty acids are more active than saturated fatty acids with the same number of carbon atoms (14). As the number of double bond increases, the antibacterial activity tends to increase (14). The double bonds in free fatty acids may be either of *cis* or *trans* orientation. It has been reported that free fatty acids with double bonds in *cis* orientation tend to have greater antibacterial activity than those with *trans* orientation as *trans* fatty acids have structures that resemble saturated fatty acids. *cis*-bonds in unsaturated fatty acids cause a kink in the structure of carbon chain so when these fatty acids are incorporated in cell membranes of bacterial cells, they are not tightly packed leading to greater membrane instability (15).

In literature, I found two studies that reported the antimicrobial activity of free fatty acids in liposomal formulations. Yang *et al.,* investigated the antimicrobial activity of lauric acid, oleic acid, and palmitic acid against *Propionibacterium acnes*, the bacterium that promotes inflammatory acne. Among the three fatty acids, the greatest antimicrobial activity was observed for lauric acid. The incorporation of lauric acid into liposomal formulation enhanced the antibacterial activity (16). A recent study has reported antibacterial activity for liposomal linolenic acids against antibiotic-resistant *H. pylori*. The prepared liposomes were able to disrupt membranes of both *H. pylori* spiral and coccoid forms. The antibacterial activity of liposomal linolenic acid was compared to that of liposomal stearic acid and oleic acid. Liposomal linolenic showed the most potent bactericidal effect and completely killed *H. pylori* within 5 min (7). A recent publication by Thamphiwantana *et al.,* demonstrated *in vivo* activity of liposomal linolenic

acid against *H. pylori.* The treatment killed *H. pylori* and reduced bacterial load in mouse stomach (17). Linolenic acid-containing liposomes caused structural changes in bacterial cell membrane leading to increased cell permeability and leakage of cytoplasmic contents (18). Both lauric acid and linolenic acid-containing liposomes were anionic liposomes which do not exert antimicrobial activity without the free fatty acid. In the present study, the combined effect of cationic liposomes and antimicrobial activity of the fatty amine was investigated. Addition of linolenic acid to the stearylamine-containing liposomes may be investigated if additional activity and/or selective toxicity are needed. Stearylamine lacks double bonds while linolenic acid is a *cis* unsaturated fatty acid which may enhance the antibacterial activity of stearylamine-containing cationic liposomes.

In this study, stearylamine-containing liposomes had antibacterial activity against *H. pylori*. The antibacterial activity increased as the stearylamine concentration in liposomes increased. The antibacterial activity of stearylamine alone was not investigated due to the poor water solubility of stearylamine. In addition, it has been previously reported that liposomal free fatty acids had enhanced antibacterial activity when compared to free fatty acids alone **(16)**. Determination of the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) are under investigation. The toxicity of stearylamine-containing liposomes, at MIC and MBC concentrations, to gastric cell lines will be also evaluated. Coating these liposomes with mucoadhesive thiomer might be needed if the acid stability needed to be further enhanced (19).

The use of stearylamine-containing liposomes may offer a novel treatment approach that can be used to treat multidrug resistant *H. pylori.* Novel treatments are needed due to the wide spread resistance of *H. pylori* to commonly used antibiotics. The incorporation of targeting ligand into the liposomal composition may selectively target *H. pylori* without affecting commensal microflora or affecting gastric cells.

4.4.2 Urease inhibition by UBA + stearylamine-containing liposomes

Stearylamine-containing liposomes (F1 liposomes) were inferior to DOTAP® liposomes in inhibiting urease activity of *H. pylori* A64. Although F1 liposomes did not affect the growth of *H. pylori* A64 following 24 h incubation period, the viability of *H. pylori* might be partially affected throughout the incubation period which in turn affected the response of cells to the interfering treatment. Antisense DNA interferes with urease activity by interfering with transcription and/or

translation, which requires cells to be actively growing. It has been proposed that cationic liposomes that are toxic to cells, might not be ideal for the delivery of interfering molecules. Anionic liposomes, which lack such toxicity, may provide better delivery resulting in better interference with the enzyme activity (20). DOTAP® liposomes may be less toxic than F1 liposomes, that is why better interference of urease activity was observed with DOTAP® liposomes. The rationale behind using stearylamine instead of DOTAP was that stearylamine-containing liposomes were previously studied as a drug delivery system to optimize the delivery of antimicrobials to *H. pylori*. Jain *et al.,* were able to prepare acid stable polyelectrolyte coated liposomes to be used for *H. pylori* treatment. The prepared liposomes were cationic as they had stearylamine in their composition (21). Since stearylamine-containing liposomes were previously used to deliver interfering molecules to eukaryotic cells (22), they can be loaded with UBA to enhance its delivery in the acidic condition of the stomach. However, experiments showed less urease inhibition than that obtained by DOTAP® liposomes. The incorporation of DOTAP lipid into liposomes having PC and CH may enhance the stability of liposomes and offers better inhibition of urease activity. Another strategy is to prepare anionic liposomes loaded with UBA to protect antisense DNA from the effect of DNases without being toxic to *H. pylori* and so provide better interference with urease activity.

One of the limitations of the present study is the inability to determine the loading efficiency of UBA-loaded F1 liposomes by the membrane impermeability assay. This was due to the presence of free UBA in the UBA-loaded liposome solution, which interfered with the assay. The molecular size of free UBA was too small to separate from the UBA-loaded liposomes by centrifugation; it was not possible to remove the free UBA, as both free UBA and UBA-loaded F1 liposomes tended to float in the aqueous suspension. Liposomes enhance the delivery and stability of antisense DNA when it is loaded into liposomes. UBA molecules that are bound to the surface of liposomes are not protected from the effect of DNases. Labelling of liposomes and UBA with fluorescent marker may be used to assess the loading efficiency of liposomes.

Another limitation is the lack of assessment of the overall charge of the loaded liposomes. It is possible to overcome the toxicity of cationic liposomes to *H. pylori* by incorporating antisense DNA in appropriate concentration to achieve a neutral net charge. Although the positive charge may be needed for better loading of UBA, it is not required for the delivery of UBA to *H. pylori* since delivery of antisense oligonucleotides was possible using anionic liposomes (20).

In this study, the strategy of using stearylamine-containing liposomes did not improve urease inhibition by antisense DNA treatment. Optimization of the lipid composition, size and charge of loaded liposomes may provide better stability and delivery of interfering molecules. In addition, the use of more stable interfering molecules such as antisense peptide nucleic acid or phosphorothioate oligonucleotides may offer better stability and activity. Understanding the exact mechanism by which liposomes deliver interfering molecules to *H. pylori* may provide better insight into developing the ideal liposomes that protect DNA from degradation but at the same time provide optimum delivery to *H. pylori*. However, the slow and irregular growth rate of *H. pylori* will be always challenging in terms of developing and optimizing novel alternative approaches that rely on shutting key enzymes required for *H. pylori* survival.

In conclusion, although antisense DNA is an attractive treatment strategy due to the high specificity of the treatment, urease inhibition was not improved with the use of stearylamine-containing liposomes. Unloaded stearylamine-containing liposomes themselves have antibacterial activity against *H. pylori* and may offer an alternative treatment approach for individuals in communities where the prevalence of *H. pylori* infection is high and where standard treatment therapies often fail, such as in Aklavik, NWT.

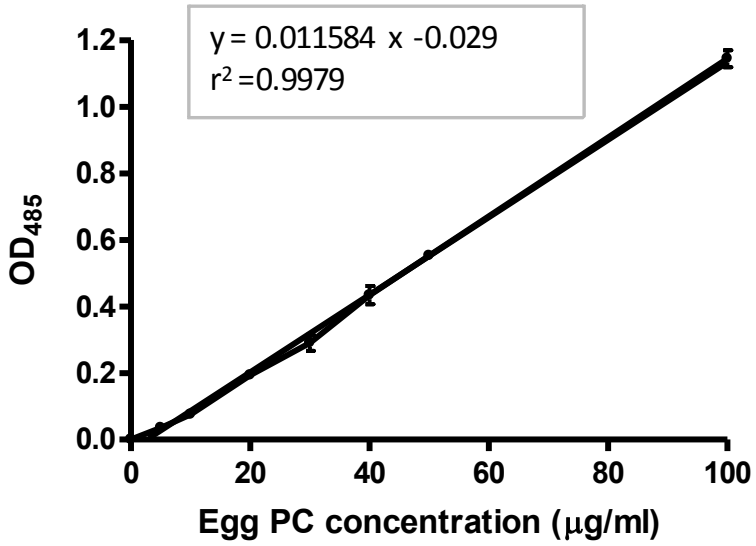


Figure 4.1 Standard curve for the Stewart assay.

The Stewart assay was used to assess the phosphatidylcholine (PC) content of prepared liposomes as described in 4.2.1.2.

Sample Name:	0.12 um latex size beads 1		
SOP Name:	latex beads.sop		
File Name:	liposome sizing.dts	**Dispersant Name:**	Water
Record Number:	15	**Dispersant RI:**	1.330
Material RI:	1.59	**Viscosity (cP):**	0.8872
Material Absorbtion:	0.010	**Measurement Date and Time:**	Thursday, November 07, 2013

Temperature (°C):	25.0	**Duration Used (s):**	60
Count Rate (kcps):	421.4	**Measurement Position (mm):**	4.65
Cell Description:	Disposable sizing cuvette	**Attenuator:**	5

			Size (d.nm):	**% Intensity:**	**St Dev (d.nm):**
Z-Average (d.nm):	119.1	**Peak 1:**	124.0	100.0	27.60
PdI:	0.009	**Peak 2:**	0.000	0.0	0.000
Intercept:	0.944	**Peak 3:**	0.000	0.0	0.000
Result quality :	Good				

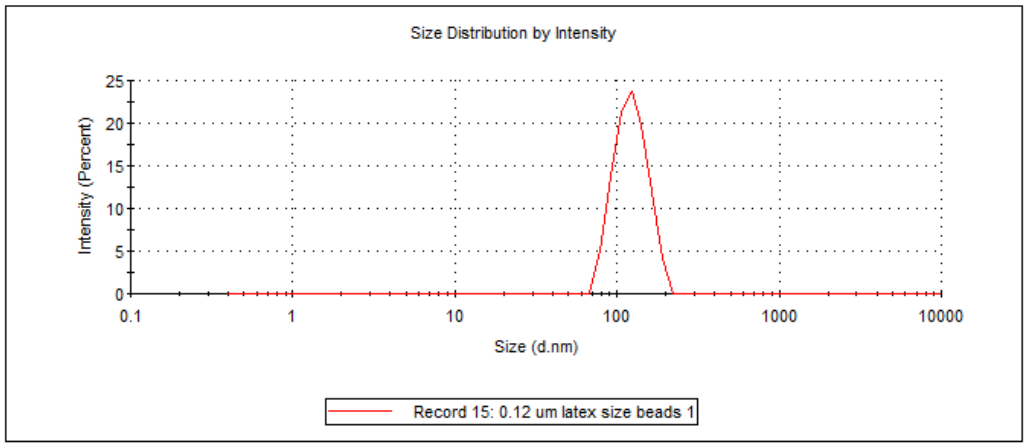


Figure 4.2 Sizing result for 120 nm standard latex beads

Standard latex beads served to calibrate the Malvern Zetasizer Nano S as described in 4.2.1.3. The average size of the120 nm standard latex beads was 119 ± 27.6 nm.

n = 2

Sample Name:	0.20 um latex size beads 1		
SOP Name:	latex beads.sop		
File Name:	liposome sizing.dts	**Dispersant Name:**	Water
Record Number:	16	**Dispersant RI:**	1.330
Material RI:	1.59	**Viscosity (cP):**	0.8872
Material Absorbtion:	0.010	**Measurement Date and Time:**	Thursday, November 07, 2013

Temperature (°C):	25.1	**Duration Used (s):**	60
Count Rate (kcps):	304.8	**Measurement Position (mm):**	4.65
Cell Description:	Disposable sizing cuvette	**Attenuator:**	5

			Size (d.nm):	**% Intensity:**	**St Dev (d.nm):**
Z-Average (d.nm):	201.0	**Peak 1:**	210.4	100.0	48.35
PdI:	0.021	**Peak 2:**	0.000	0.0	0.000
Intercept:	0.953	**Peak 3:**	0.000	0.0	0.000
Result quality :	Good				

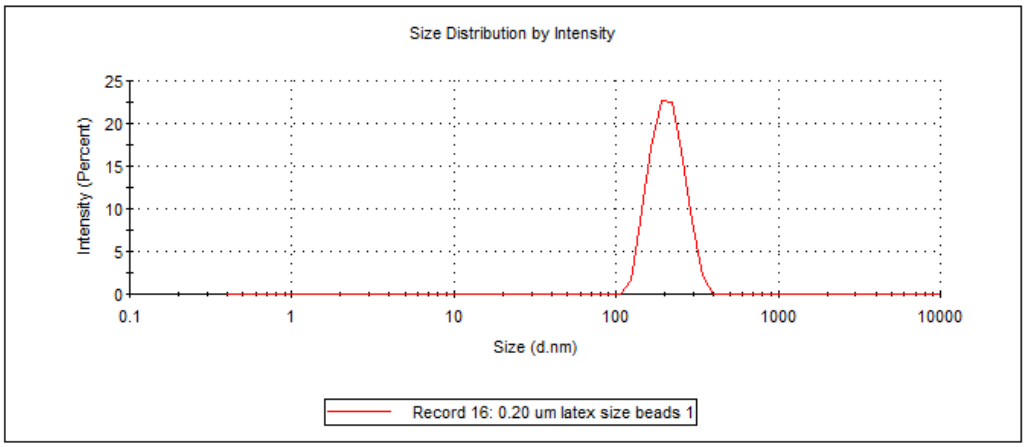


Figure 4.3 Sizing result for 200 nm standard latex beads

Standard latex beads served to calibrate the Malvern Zetasizer Nano S as described in 4.2.1.3. The average size of the 200 nm standard latex beads 201 ± 48.4 nm.

n = 2

Sample Name:	Liposomes PC:Ch:SA 1		
SOP Name:	liposomes.sop		
File Name:	liposome sizing.dts	**Dispersant Name:**	Water
Record Number:	17	**Dispersant RI:**	1.330
Material RI:	1.43	**Viscosity (cP):**	0.8872
Material Absorbtion:	0.010	**Measurement Date and Time:**	Thursday, November 07, 2013

Temperature (°C):	25.0	**Duration Used (s):**	60
Count Rate (kcps):	251.6	**Measurement Position (mm):**	4.65
Cell Description:	Disposable sizing cuvette	**Attenuator:**	6

			Size (d.nm):	**% Intensity:**	**St Dev (d.nm):**
Z-Average (d.nm):	130.7	**Peak 1:**	149.4	100.0	55.70
PdI:	0.128	**Peak 2:**	0.000	0.0	0.000
Intercept:	0.958	**Peak 3:**	0.000	0.0	0.000

Result quality : Good

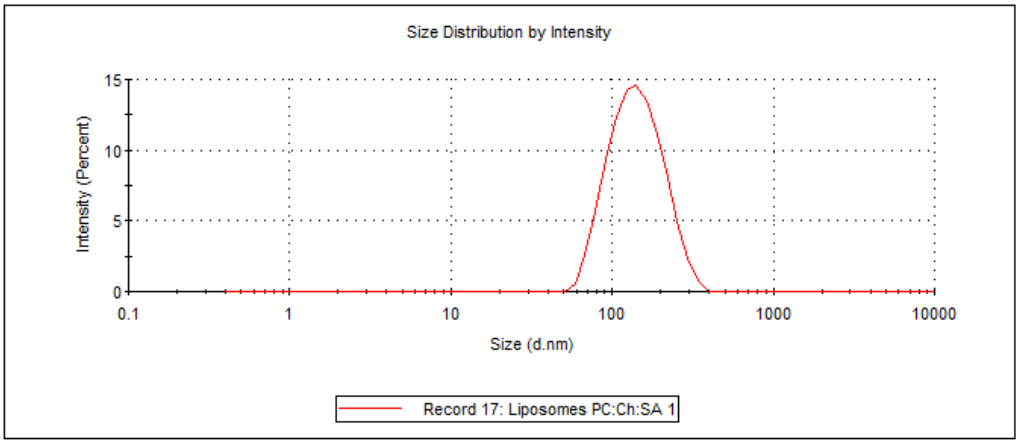


Figure 4.4 Sizing result for F1 liposomes (PC/CH/SA 7:3:1)

Liposome size (diameter) was determined in duplicate using the Malvern Zetasizer Nano S as described in 4.2.1.3. The average size of this one F1 liposome preparation was 131 ± 55.7 nm.

Sample Name:	Liposomes PC/CH/SA 7:3:2 1		
SOP Name:	mansettings.nano		
File Name:	liposome sizing.dts	**Dispersant Name:**	Water
Record Number:	40	**Dispersant RI:**	1.330
Material RI:	1.43	**Viscosity (cP):**	0.8872
Material Absorbtion:	0.010	**Measurement Date and Time:**	Tuesday, January 21, 2014

Temperature (°C):	25.0	**Duration Used (s):**	60
Count Rate (kcps):	436.3	**Measurement Position (mm):**	4.65
Cell Description:	Disposable sizing cuvette	**Attenuator:**	6

			Size (d.nm):	**% Intensity:**	**St Dev (d.nm):**
Z-Average (d.nm):	116.0	**Peak 1:**	138.7	100.0	59.07
PdI:	0.152	**Peak 2:**	0.000	0.0	0.000
Intercept:	0.939	**Peak 3:**	0.000	0.0	0.000
Result quality :	Good				

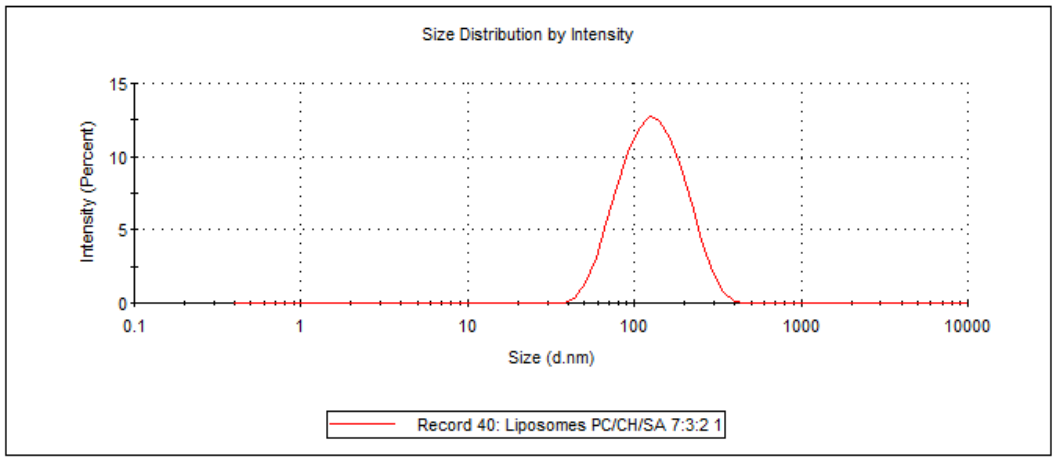


Figure 4.5 Sizing result for F2 liposomes (PC/CH/SA 7:3:2)

Liposome size (diameter) was determined in duplicate using the Malvern Zetasizer Nano S as described in 4.2.1.3. The average size of this one F2 liposome preparation was 116 ± 59.1 nm.

Sample Name:	Liposomes PC/CH/SA 7:3:1 + UBA 1		
SOP Name:	mansettings.nano		
File Name:	liposome sizing.dts	**Dispersant Name:**	Water
Record Number:	36	**Dispersant RI:**	1.330
Material RI:	1.43	**Viscosity (cP):**	0.8872
Material Absorbtion:	0.010	**Measurement Date and Time:**	Tuesday, January 21, 2014

Temperature (°C):	24.9	**Duration Used (s):**	60
Count Rate (kcps):	333.3	**Measurement Position (mm):**	4.65
Cell Description:	Disposable sizing cuvette	**Attenuator:**	6

			Size (d.nm):	**% Intensity:**	**St Dev (d.nm):**
Z-Average (d.nm):	117.7	**Peak 1:**	141.2	100.0	59.83
PdI:	0.159	**Peak 2:**	0.000	0.0	0.000
Intercept:	0.950	**Peak 3:**	0.000	0.0	0.000
Result quality :	Good				

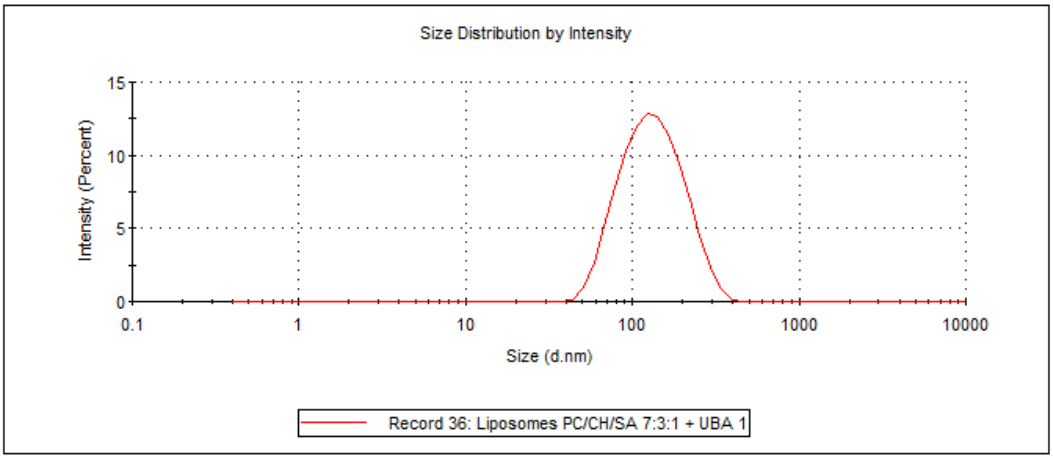


Figure 4.6 Sizing result for UBA loaded F1 liposomes

Liposome size (diameter) was determined in duplicate using the Malvern Zetasizer Nano S as described in 4.2.1.3. The average size of this one UBA loaded F1 liposome preparation was 118 ± 59.8 nm.

Sample Name:	Liposomes PC/CH/SA 7:3:1 + Scr UBA 1		
SOP Name:	mansettings.nano		
File Name:	liposome sizing.dts	**Dispersant Name:**	Water
Record Number:	38	**Dispersant RI:**	1.330
Material RI:	1.43	**Viscosity (cP):**	0.8872
Material Absorbtion:	0.010	**Measurement Date and Time:**	Tuesday, January 21, 2014

Temperature (°C):	24.9			**Duration Used (s):**	60
Count Rate (kcps):	382.0			**Measurement Position (mm):**	4.65
Cell Description:	Disposable sizing cuvette			**Attenuator:**	6

			Size (d.nm):	**% Intensity:**	**St Dev (d.nm):**
Z-Average (d.nm):	131.3	**Peak 1:**	151.2	100.0	58.77
PdI:	0.160	**Peak 2:**	0.000	0.0	0.000
Intercept:	0.944	**Peak 3:**	0.000	0.0	0.000
Result quality :	Good				

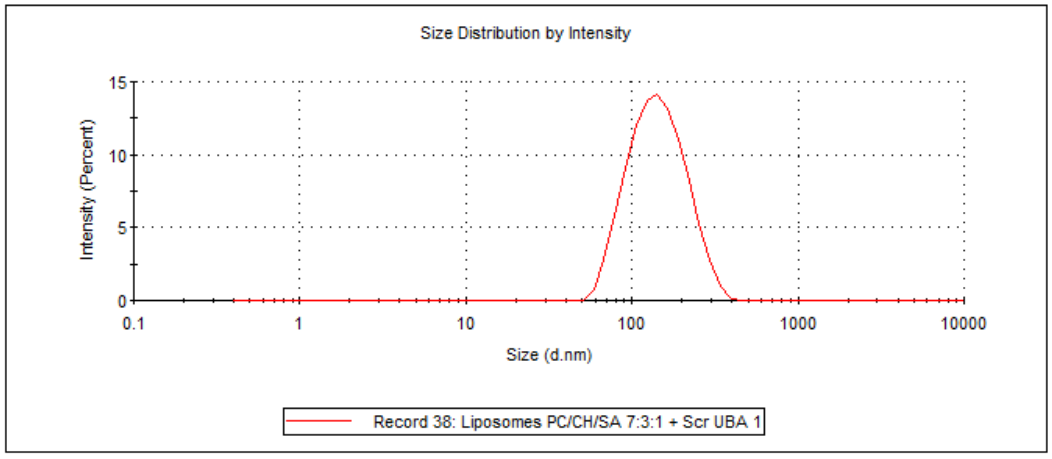


Figure 4.7 Sizing result for scr UBA loaded F1 liposomes

Liposome size (diameter) was determined in duplicate using the Malvern Zetasizer Nano S as described in 4.2.1.3. The average size of this one scr UBA loaded F1 liposome preparation was 131 ± 58.8 nm.

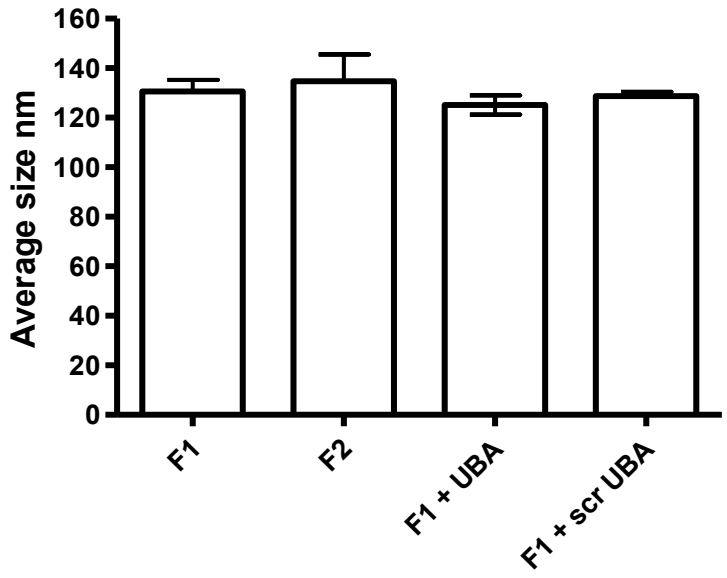


Figure 4.8 Average liposome size

Each liposome formulation was prepared on 3 different days as described in 4.2.1.1 and sized as described in 4.2.1.3.

n = 3 (each measurement was done in duplicate)

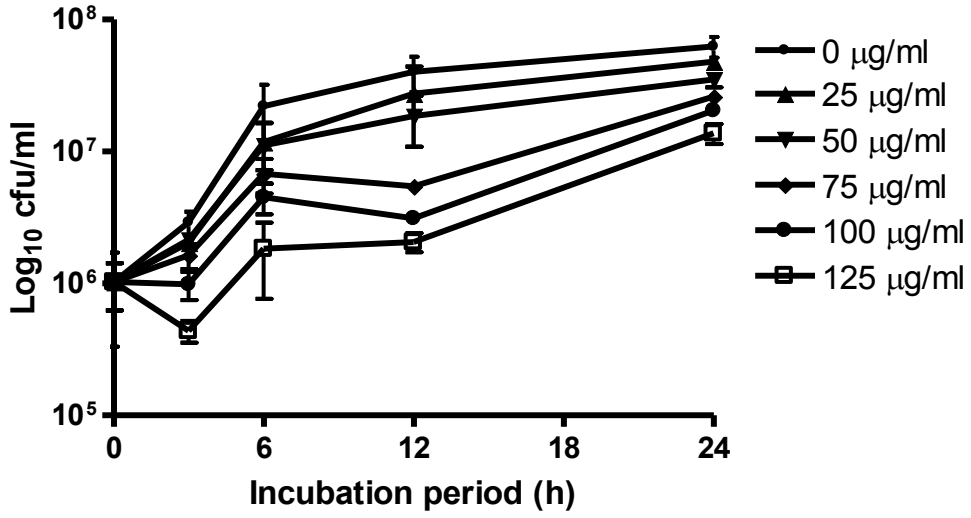


Figure 4.9 Effect of increasing concentrations of F1 liposomes on the growth (cfu/ml) of *H. pylori* A64 (one treatment at 0 h). The growth of *H. pylori* A64 (cfu/ml) was assessed at 3, 6, 12 and 24 h incubation after a single treatment at 0 h with different concentrations of F1 liposomes as described in 4.2.2.

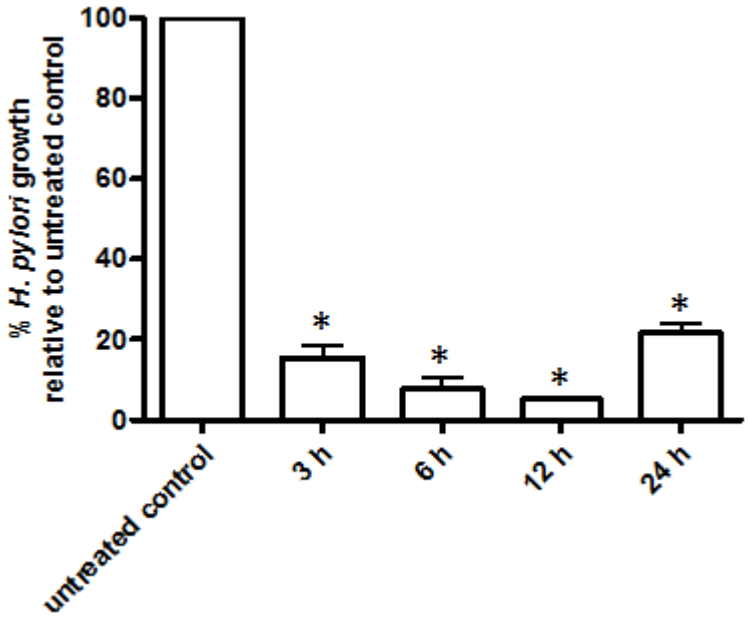


Figure 4.10 Effect of a single treatment of 125 μg/ml of F1 liposomes on the growth of *H. pylori* A64. *H. pylori* A64 growth from Figure 4.9 expressed relative to untreated control. *$p < 0.0001$, significant difference in *H. pylori* growth when compared to untreated control at time 0 h. n = 3 per incubation time

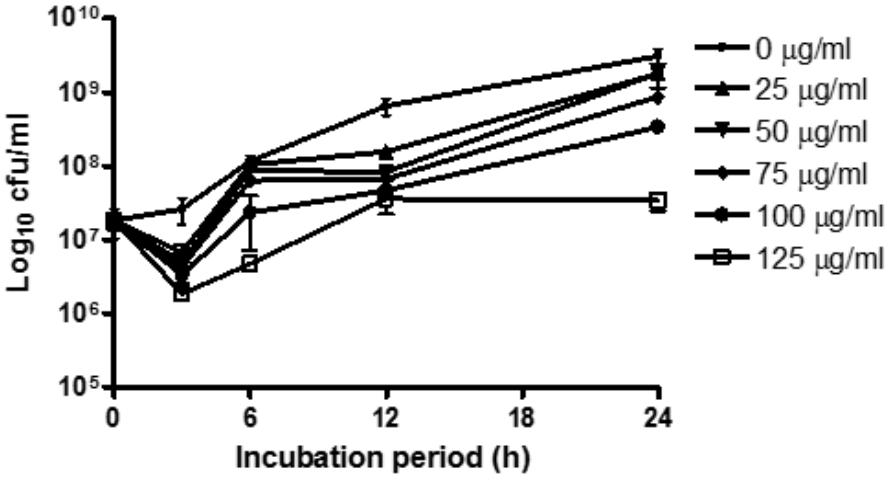


Figure 4.11 Effect of increasing concentrations of F2 liposomes on the growth (cfu/ml) of *H. pylori* A64 (one treatment at 0 h). The growth of *H. pylori* A64 (cfu/ml) was assessed at 3, 6, 12 and 24 h incubation after a single treatment at 0 h with different concentrations of F2 liposomes as described in 4.2.2.

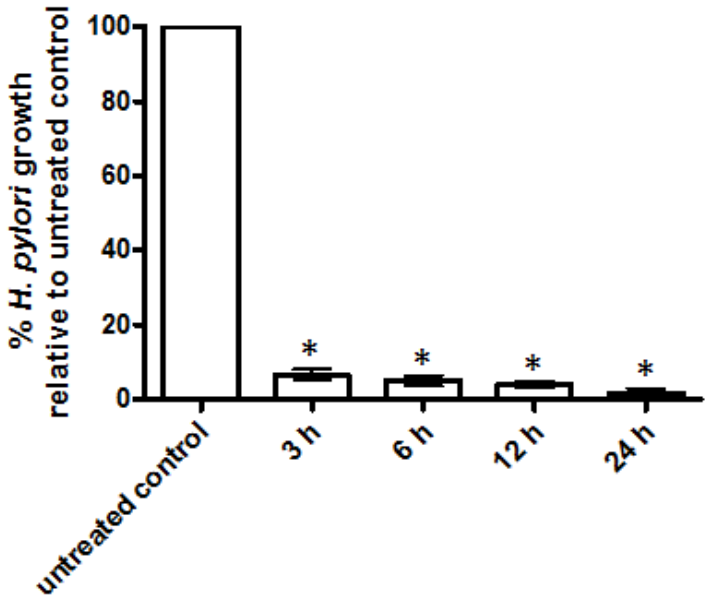


Figure 4.12 Effect of a single treatment of 125 µg/ml of F2 liposomes on the growth of *H. pylori* A64. *H. pylori* A64 growth from Figure 4.11 expressed relative to untreated control. *$p < 0.0001$, significant difference in *H. pylori* growth when compared to untreated control at time 0 h . n = 3 per incubation time

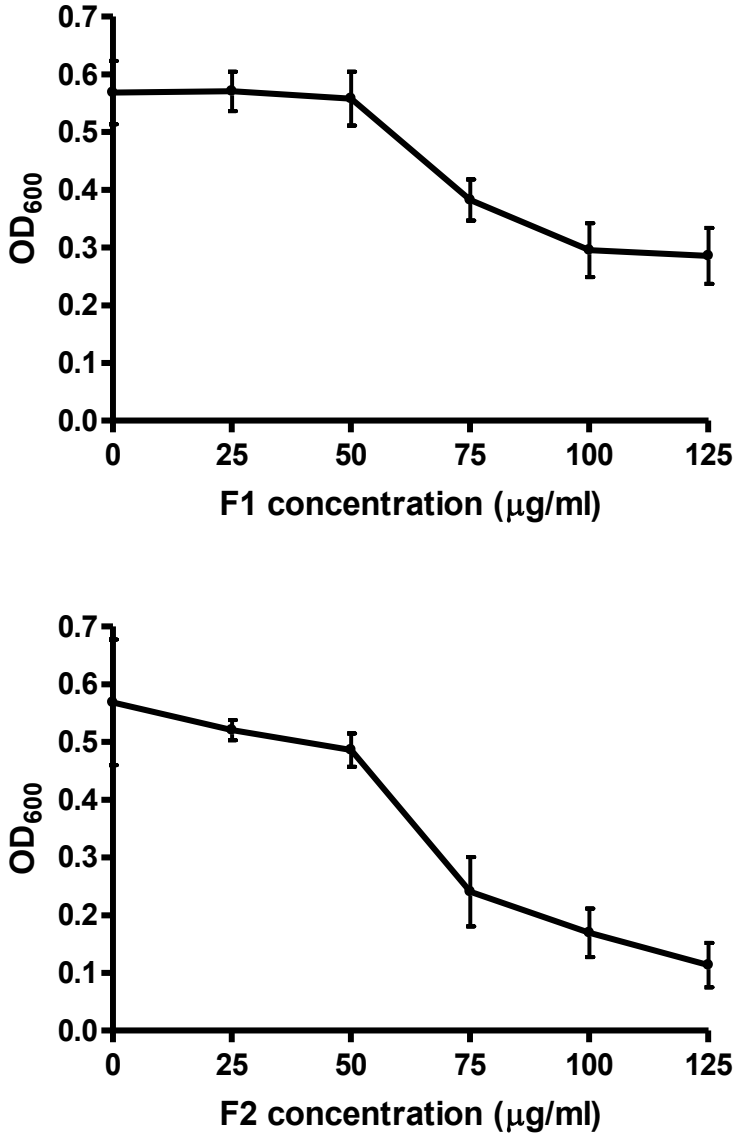


Figure 4.13 Effect of one treatment (at 0 h) of F1 or F2 liposomes on the growth of *H. pylori* A64 (OD_{600}). The growth of *H. pylori* A64 (OD_{600}) was assessed at 24 h incubation after one treatment at time zero with different concentrations of F1 or F2 liposomes as described in 4.2.2.

n = 3 per concentration

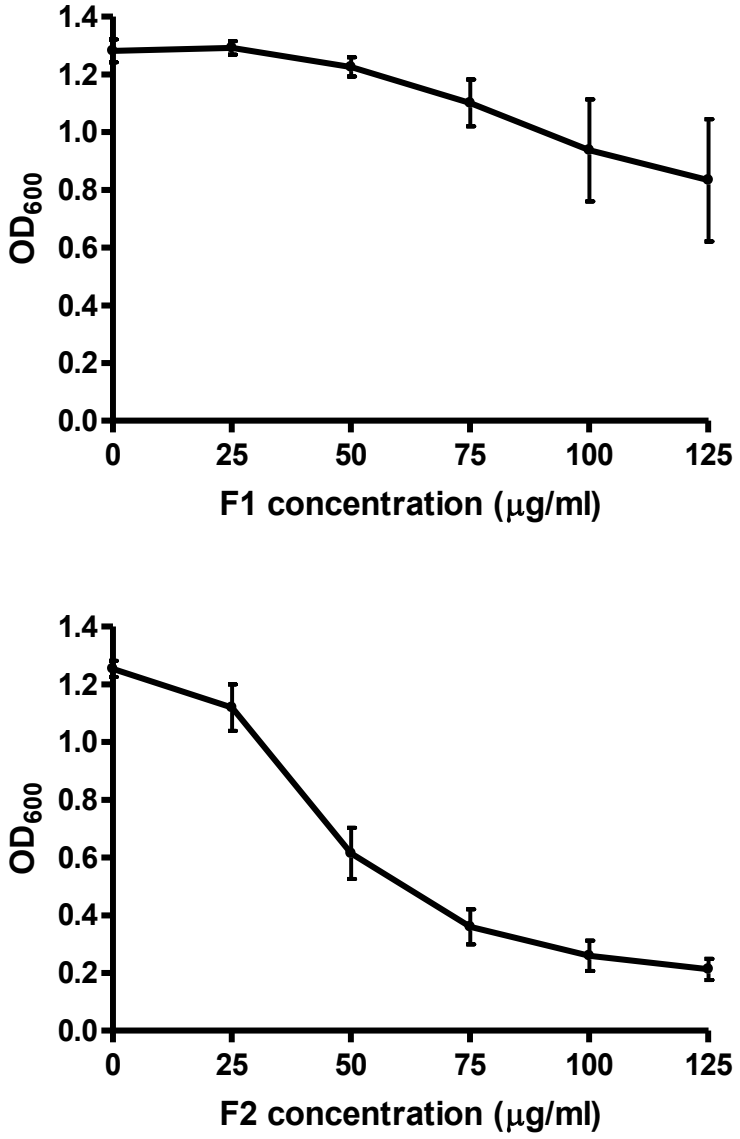
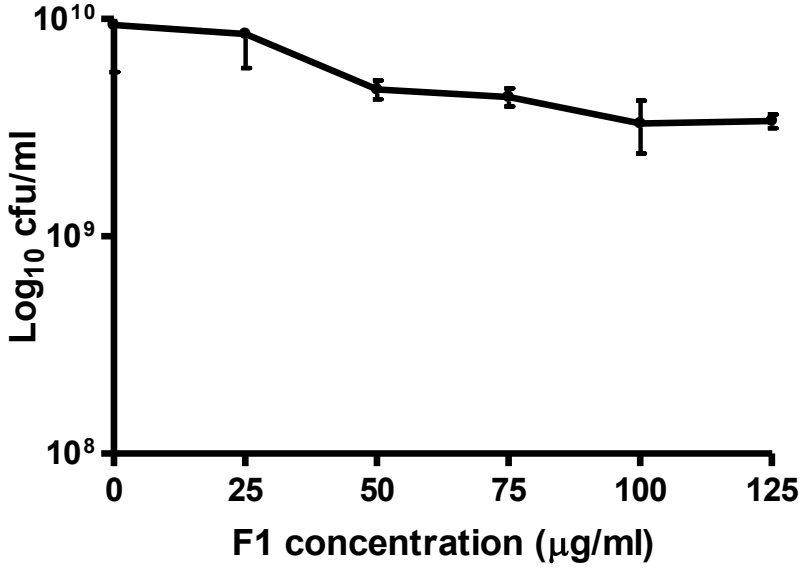


Figure 4.14 Effect of two treatments (at 0 h and 12 h) of F1 or F2 liposomes on the growth of *H. pylori* A64 (OD_{600})

The growth of *H. pylori* A64 (OD_{600}) was assessed at 24 h incubation after two treatments at time 0 h and 12 h with different concentrations of F1 or F2 liposomes as described in 4.2.2. n = 3 per concentration



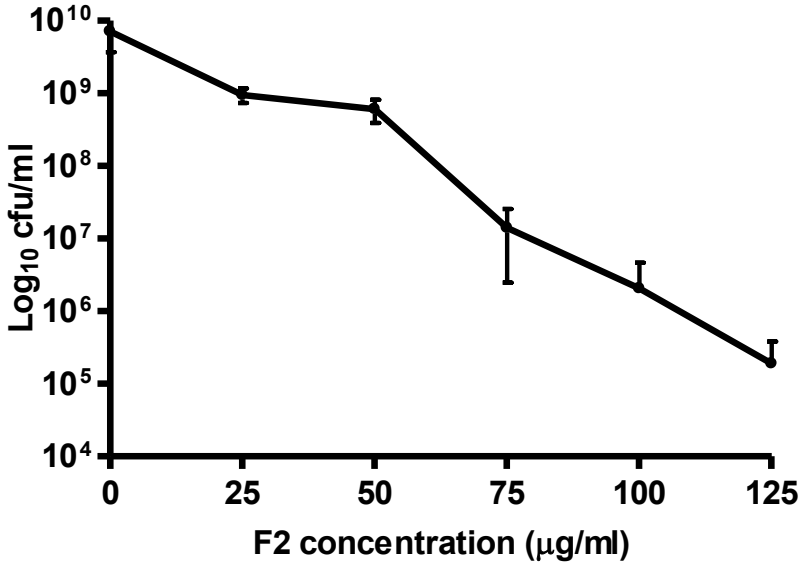


Figure 4.15 Effect of two treatments (at 0 h and 12h) of F1 or F2 liposomes on the growth of *H. pylori* A64 (cfu/ml)

The growth of *H. pylori* A64 (cfu/ml) was assessed at 24 h incubation after two treatments at time 0 h and 12 h with different concentrations of F1 or F2 liposomes as described in 4.2.2. n = 3 per concentration

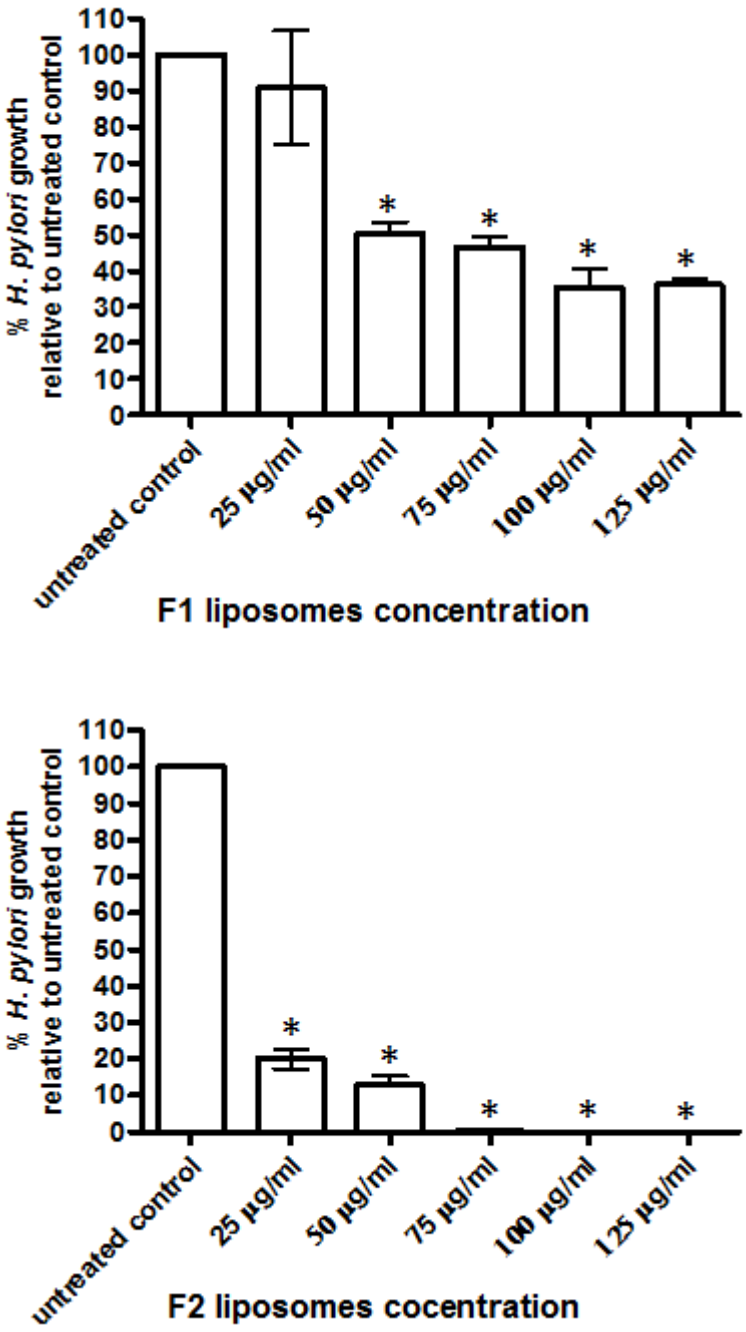


Figure 3.16 Effect of two treatments (at 0 h and 12h) of F1 or F2 liposomes on the growth of *H. pylori* A64 (cfu/ml)

H. pylori A64 growth (cfu/ml) from Figure 4.15 expressed relative to untreated control.

*$p < 0.0001$, significant difference in *H. pylori* growth when compared to untreated control at time 0 h. n = 3 per incubation time

Figure 4.17 Membrane impermeability assay

UBA loading efficiency into liposomes was assessed by comparing the intensity of UBA+liposomes with SDS-treated UBA+ liposomes, each stained with SYBR® Gold as described in 4.2.3.1. SDS disrupts liposomes, exposing UBA to SYBR® Gold stain. Free UBA served as a positive control. HEPES-buffered saline and unloaded liposomes served as negative controls.

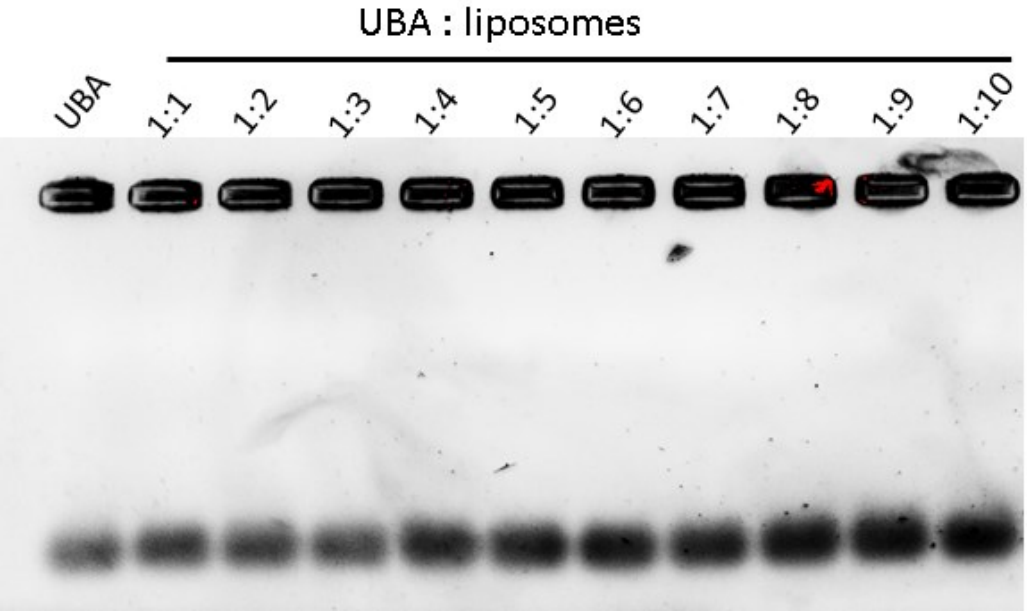


Figure 4.18 Effect of increasing concentrations of liposomes on the intensity of SYBR® Gold stained UBA

A constant amount of UBA was mixed with liposomes in an increasing weight ratio from 1:1 to 1:10, run through a 2% agarose gel, and then strained using SYBR® Gold as described 4.2.3.1.

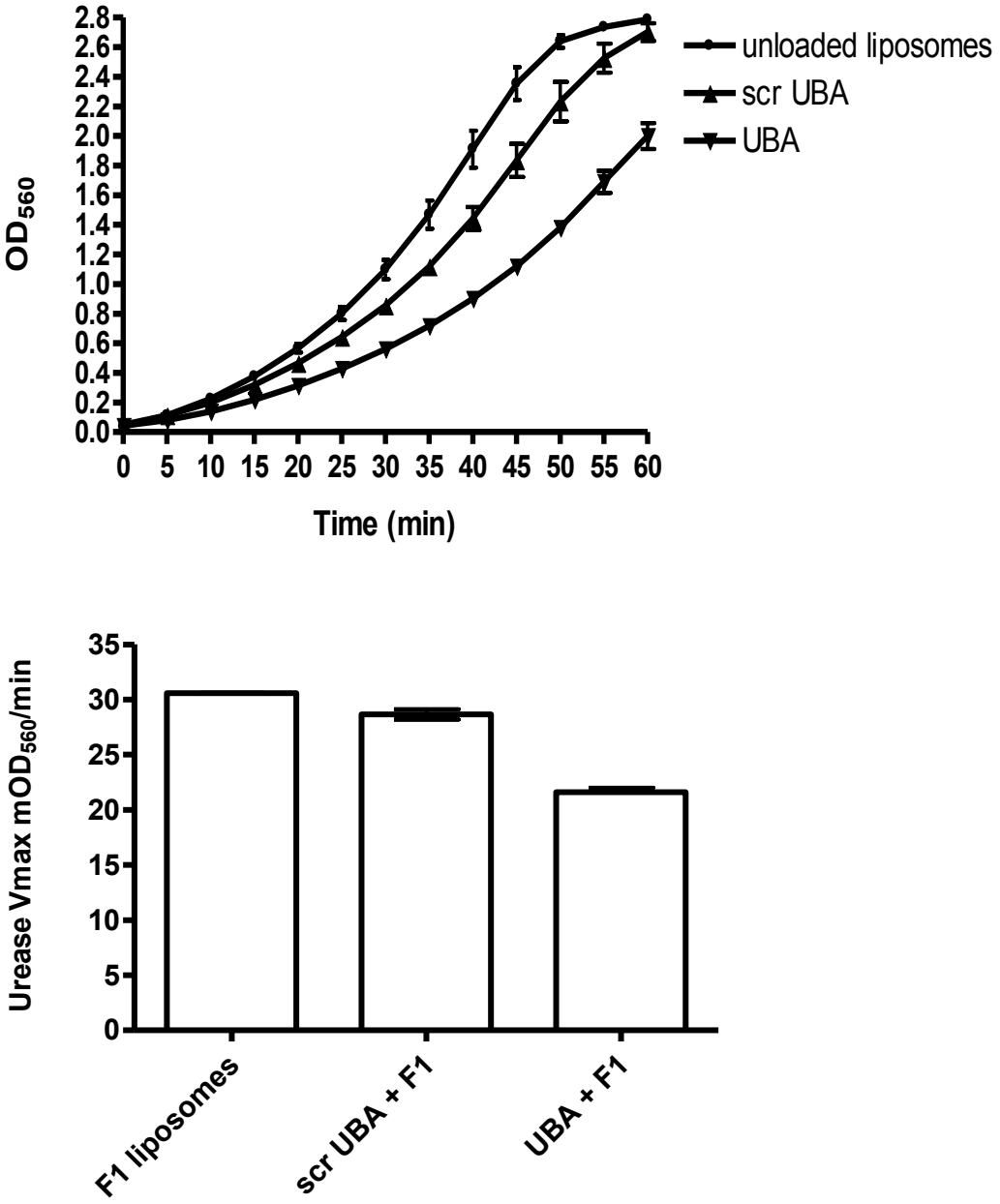


Figure 4.19 Effect of 20 nM UBA loaded F1 liposomes on the urease activity of *H. pylori* A64 . Urease activity of *H. pylori* A64 is measured as OD_{560}/min every 5 min up to 60 min as described in 3.2.2.2 (chapter 3) for unloaded F1 liposomes, 20 nM scr UBA + F1 liposomes, or 20 nM UBA + F1 liposomes as described in 3.2.3.3 (chapter 3). scr UBA refers to scrambled urease antisense treatment that serves as a negative antisense DNA control.

$p > 0.05$, 20 nM scr UBA + F1 liposomes, or 20 nM UBA + F1 liposomes vs unloaded F1 liposomes, n = 3 per treatment group

4.5 References

(1) Bangham AD, Standish MM, Watkins JC. Diffusion of univalent ions across the lamellae of swollen phospholipids. J Mol Biol 1965 Aug;13(1):238-252.

(2) Drulis-Kawa Z, Dorotkiewicz-Jach A. Liposomes as delivery systems for antibiotics. Int J Pharm 2010 Mar 15;387(1-2):187-198.

(3) Campanha MT, Mamizuka EM, Carmona-Ribeiro AM. Interactions between cationic liposomes and bacteria: the physical-chemistry of the bactericidal action. J Lipid Res 1999 Aug;40(8):1495-1500.

(4) Vieira DB, Carmona-Ribeiro AM. Cationic lipids and surfactants as antifungal agents: mode of action. J Antimicrob Chemother 2006 Oct;58(4):760-767.

(5) Banerjee A, Roychoudhury J, Ali N. Stearylamine-bearing cationic liposomes kill Leishmania parasites through surface exposed negatively charged phosphatidylserine. J Antimicrob Chemother 2008 Jan;61(1):103-110.

(6) Kanazawa A, Ikeda T, Endo T. A novel approach to mode of action of cationic biocides: morphological effect on antibacterial activity. J Appl Bacteriol 1995 Jan;78(1):55-60.

(7) Obonyo M, Zhang L, Thamphiwatana S, Pornpattananangkul D, Fu V, Zhang L. Antibacterial activities of liposomal linolenic acids against antibiotic-resistant *Helicobacter pylori*. Mol Pharm 2012 Sep 4;9(9):2677-2685.

(8) Desbois AP, Smith VJ. Antibacterial free fatty acids: activities, mechanisms of action and biotechnological potential. Appl Microbiol Biotechnol 2010 Feb;85(6):1629-1642.

(9) J. L. Holovati. Liposomal delivery of intracellular trehalose; 2008.

(10) Holovati JL, Acker JP. Spectrophotometric measurement of intraliposomal trehalose. Cryobiology 2007 Oct;55(2):98-107.

(11) Stewart JC. Colorimetric determination of phospholipids with ammonium ferrothiocyanate. Anal Biochem 1980 May 1;104(1):10-14.

(12) Zhang C, Tang N, Liu X, Liang W, Xu W, Torchilin VP. siRNA-containing liposomes modified with polyarginine effectively silence the targeted gene. J Control Release 2006 May 15;112(2):229-239.

(13) Dey T, Anam K, Afrin F, Ali N. Antileishmanial activities of stearylamine-bearing liposomes. Antimicrob Agents Chemother 2000 Jun;44(6):1739-1742.

(14) Knapp HR, Melly MA. Bactericidal effects of polyunsaturated fatty acids. J Infect Dis 1986 Jul;154(1):84-94.

(15) Chamberlain NR, Mehrtens BG, Xiong Z, Kapral FA, Boardman JL, Rearick JI. Correlation of carotenoid production, decreased membrane fluidity, and resistance to oleic acid killing in *Staphylococcus aureus* 18Z. Infect Immun 1991 Dec;59(12):4332-4337.

(16) Yang D, Pornpattananangkul D, Nakatsuji T, Chan M, Carson D, Huang CM, et al. The antimicrobial activity of liposomal lauric acids against *Propionibacterium acnes*. Biomaterials 2009 Oct;30(30):6035-6040.

(17) Thamphiwatana S, Gao W, Obonyo M, Zhang L. In vivo treatment of *Helicobacter pylori* infection with liposomal linolenic acid reduces colonization and ameliorates inflammation. Proc Natl Acad Sci U S A 2014 Dec 9;111(49):17600-17605.

(18) Jung SW, Thamphiwatana S, Zhang L, Obonyo M. Mechanism of antibacterial activity of liposomal linolenic acid against *Helicobacter pylori*. PLoS One 2015 Mar 20;10(3):e0116519.

(19) Hyun Sup Lee, Kwangmeyung Kim, Bong Hyun Jeong, Hyun Tae Moon, Youngro Byun. Stability of poly(Acrylic Acid)-grafted phospholipid liposomes in gastrointestinal conditions. Drug Development Research 2004;61(1):13-18.

(20) Fillion P, Desjardins A, Sayasith K, Lagace J. Encapsulation of DNA in negatively charged liposomes and inhibition of bacterial gene expression with fluid liposome-encapsulated antisense oligonucleotides. Biochim Biophys Acta 2001 Nov 1;1515(1):44-54.

(21) Jain P, Jain S, Prasad KN, Jain SK, Vyas SP. Polyelectrolyte coated multilayered liposomes (nanocapsules) for the treatment of *Helicobacter pylori* infection. Mol Pharm 2009 Mar-Apr;6(2):593-603.

(22) Silva AL, Alexandrino F, Verissimo LM, Agnez-Lima LF, Egito LC, de Oliveira AG, et al. Physical factors affecting plasmid DNA compaction in stearylamine-containing nanoemulsions intended for gene delivery. Pharmaceuticals (Basel) 2012 Jun 18;5(6):643-654.

Chapter 5

Summarizing Discussion and Future Directions

5.1 Summarizing Discussion

In this thesis I focus on two themes. First, the investigation of the genotypic features of *H. pylori* and gastric histopathology of infected research participants from Aklavik, NWT. Second, alternative approaches for the treatment of *H. pylori* infections.

Aklavik is a remote Aboriginal community in northern Canada where the prevalence of *H. pylori* infection is 2-3 times higher than the estimated prevalence in other urban communities in Canada (1-3). The work presented in Chapter 2 is a collaborative work of the CAN*Help* Working Group; a group that was established to link University of Alberta researchers with northern community leaders and health officials (4). The main goal of the group is to address community concerns regarding health risks of *H. pylori* infections among northern communities. The Aklavik *H. pylori* Project is the initial project of the CAN*Help* Working Group to address community concerns regarding a perceived high incidence of gastric cancer in Aklavik (4). The project is a community driven research project that actively engages the community through knowledge translation.

The clinical outcomes of *H. pylori* infections are hypothesized to be dependent on several virulence genes, as well as specified environmental and host factors (5). The major virulence genes (*cagA, vacA, babA2*) encode two toxins (CagA and VacA) and one adhesin (BabA). The CagA toxin disturbs cellular functions by interacting with certain cellular protein that has an important role in mitogenic cellular transduction, which may lead to the development of gastric cancer (6). The VacA toxin, sometimes but not always, induces cell vacuolation, disrupts mitochondrial functions and stimulates apoptosis (7). The BabA protein is involved in *H. pylori* adhesion to gastric mucosa, which is required for gastric colonization (8).

Chapter 2 of this thesis is the first description of the frequency of major *H. pylori* virulence genotypes and estimation of their association with different gastric histopathology outcomes for 121 research participants of Aklavik, NWT (population ~600) with recoverable *H. pylori* from gastric biopsies. Similar to other published studies, the vac*A* and *babA2* genes were detected in virtually all (up to 97%) *H. pylori* from Aklavik research participants. However, the *cagA* gene

was detected in only one third of *H. pylori* from Aklavik participants. Unlike the *vacA* gene and *babA2* gene, the *cagA* gene is present in an insertion region in the chromosome that was acquired from other bacteria (9). This region is known as the *cag* pathogenicity island, which encodes for the machinery required for CagA protein injection into the host cells (10). The *cagA* gene has been detected in about 70% of *H. pylori* strains worldwide, but this rate varies by geographical location from 90–95% of *H. pylori* in East Asian countries such as South Korea, China and Japan, versus only about 40% in Western countries such as Australia, United States of America and England (11). The low prevalence of *cagA* in *H. pylori* from Aklavik (34%) was similar to that observed in Western countries. As expected, the presence of *cagA* in Aklavik isolates was more likely to be associated with individuals diagnosed with severe chronic gastritis, gastric atrophy and/or intestinal metaplasia than those without, as determined by histopathology assessment of their gastric biopsies.

BabA adherence is mediated through the fucosylated Lewis b blood-group antigen (8). Fucosylation results in molecular mimicry between *H. pylori* lipopolysaccharide and host glycoconjugates, which helps *H. pylori* to evade host immune responses (12). Virtually all Aklavik research participants were infected with *H. pylori* that carry the *babA2* gene. Two different amplicon sizes were obtained by PCR among *H. pylori* isolates from Aklavik. The unexpected amplicon size (248 bp) was more prevalent in *H. pylori* from research participants with moderate to severe chronic gastritis than those with absent or mild chronic gastritis. This suggested that the 248 bp amplicon may be a marker for a *babA2* variant associated with more severe gastric histopathology as compared with histopathology associated with the 271 bp amplicon. Examination of *babA2* gene expression of the Aklavik *H. pylori* by mRNA detection revealed that *babA2* amplicon size could not predict whether the gene was expressed and therefore cannot predict the presence of BabA protein. Expression of the *babA2* gene is regulated by phase variation (13) as are some other *H. pylori* genes. Phase variation refers to the expression of heterogeneic phenotypes in a clonal bacterial population (14). It is not known whether there are differences in BabA binding in *H. pylori* associated with more severe gastric pathology. Neil and coworkers recently reported that although BabA-mediated adherence to Lewis b antigen was stable in most *H. pylori* positive individuals studied, up to 22% had reduced or lost ability to bind Lewis b antigen (15). They also identified amino acids sequences that affected the strength of BabA binding to Lewis b antigen. It is unknown whether the Aklavik isolates have a variable ability to bind to the

stomach epithelium of infected individuals, or whether variable binding of BabA could contribute to different disease outcomes. The greater prevalence of the 248 bp *babA2* amplicon with severe or moderate chronic gastritis versus mild/absent chronic gastritis, may be related to the strength of BabA binding to the gastric mucosa, but this requires further investigation. The lack of association of the *babA2* gene with gastric atrophy or intestinal metaplasia supports the importance of the role of BabA for the establishment of *H. pylori* infection.

Almost all of the *H. pylori* from Aklavik research participants were positive for the *vacA* gene. Polymorphism within *vacA* is reported to to induce cell changes such as vacuolation (16). Two major polymorphic regions have been identified within *vacA*: the signal region (*s1* or *s2*) and the midregion (*m1* or *m2*) (17). The *vacA s1/m1* strains being classified as the most virulent, while those with *s1/m2* were classified as having intermediate virulence and those with *s2/m2* as the least virulent (18). Another *vacA* polymorphic site, designated as the intermediate (*i*) region, was identified as having two sequence types (*i1* and *i2*) (19). In *H. pylori* isolated from Aklavik research participants, the *vacA s1, i1* and *m1* types were associated with the presence of gastric atrophy than without atrophy, whereas *vacA s1* was associated with the presence of intestinal metaplasia than the absence of intestinal metaplasia. Winter *et al,* recently reported a strong association between *vacA i1* and precancerous intestinal metaplasia in mice (20). The same study also examined human gastric biopsy specimens and reported a strong association of the *vacA i1* type with intestinal metaplasia (20). The *vacA* i2 type was associated with the absence of intestinal metaplasia, even if the *H. pylori* was positive for *vacA s1* and *cagA* genes (20). Rhead reported an association between the *i1* type and gastric adenocarcinoma but not with *i2* among 73 Iranian patients (19). Gastric cancer was not detected in the Aklavik research participants.

Investigation of the association of three major virulence factors (*cagA*, *vacA* and *babA2*) with the presence of gastric diseases using crude odds ratios, revealed that Aklavik individuals infected with the *babA2* 248 amplicon variant were most likely to have severe acute or severe chronic gastritis than moderate/mild/absent gastritis. This supports the importance of the BabA adhesin in the colonization of the gastric mucosa. Aklavik research participants infected with *cagA*-positive, *vacA i1*-positive *or vacA m1*-positive *H. pylori* were more likely to have moderate/severe acute gastritis than mild/absent acute gastritis. This was not observed for individuals with moderate/severe chronic gastritis versus mild/absent chronic gastritis. Individuals with gastric atrophy were more likely to be infected with *cagA*-positive, *vacA s1*-positive *or vacA*

m1 positive *H. pylori,* while those with intestinal metaplasia tended to be infected with *cagA*-positive or *vacA s1* positive *H. pylori.* These findings suggest the presence of particular polymorphisms or variants the *babA2* and *vacA* genes promote the progression from gastritis, to atrophy, metaplasia and ultimately the development of gastric cancer. However, this study did not identify unique *H. pylori* genotypes that could predict different clinical outcomes among residents of Aklavik as proposed in the first hypothesis of this thesis.

Crude odds ratios do not account for the presence of confounding variables, and therefore limit the interpretation of the findings. Future studies will use more detailed multivariate analyses on a larger genotype profile that will account for confounding variables. Since the CAN*Help* Working Group has extended their activities to other communities in the Northwest Territories and Yukon, the greater sample size will allow a more reliable assessment of the influence of virulence factors on the pathogenesis of *H. pylori* infections. The resulting odds ratio calculations with narrower confidence intervals would provide more strength of association of genotype with histopathology. Understanding the associations of other virulence factors with histopathology will provide better insight into the role of different virulence factors in developing gastric diseases.

H. pylori is genetically one of the most diverse bacterial species due to the highest rate of genetic recombination. Infection with more than one strain is not uncommon due to such high level of genetic recombination (21). More than one type of *vacA* gene (*m1/m2* and/or *i1/i2*) was detected in 11% of *H. pylori* cultures from Aklavik research participants, indicating infection with more than one strain. Didelot *et al* investigated genomic evolution within two *H. pylori*-infected families from South Africa and observed little genetic recombination for many individuals while in some, substitutions were introduced 100 times more often than mutation (22). Comparison of the genomes revealed more frequent transmission events between some close family members and those living within the same home, but this was not the case most for most individuals (22). Krebes *et al*., recently reported extensive bidirectional exchange among *H. pylori* infected family members (23). Kersulyte *et al*., reported evidence of extensive genomic rearrangement in an Aklavik strain (24). One Aklavik *H. pylori* strain contains extra iron-cofactored urease genes and about 140 rearrangements in its chromosome relative to previously described strains although strains typically differ from one another by less than 10 rearrangements (24). In a community with high prevalence of *H. pylori* infection, such as Aklavik, infection with more than one *H. pylori* strain as reported in this thesis, may be underestimated.

The high prevalence of acute and chronic gastritis (more than 80%) observed in gastric biopsies collected from research participants justifies concerns about *H. pylori* infection raised by members of the Aklavik community. In addition, the detection of precancerous lesions (atrophy or intestinal metaplasia) supports their concerns regarding increased risk of gastric cancer. The association of certain *H. pylori* genotypes with gastric diseases supports the concerns raised by community members regarding the perceived risk of gastric cancer. Accordingly, the community is concerned with treatment strategies to effectively treat *H. pylori* infections.

Standard therapies may fail to treat *H. pylori* infections due to antimicrobial resistance (25), re-infection (26), patient compliance, and the high cost of currently used drugs (27). *H. pylori* can be treated by different treatment regimens of which triple, sequential and quadruple therapies are the most common. In triple therapy, a proton pump inhibitor (PPI) with amoxicillin and clarithromycin or metronidazole are taken for 7 to 10 days (28). Sequential therapy is a 10-day course that includes a 5-day treatment with PPI + amoxicillin, followed by another 5-day treatment with PPI + clarithromycin + tinidazole or metronidazole (29). Quadruple therapy is composed of a combination of bismuth subcitrate potassium, metronidazole, tetracycline, and omeprazole (30). The effectiveness of standard triple therapy and sequential therapy was investigated for the community of Aklavik. A total number of 89 research participants had treatment results; 49 had standard triple therapy (PPI-CA) and 40 had sequential therapy (ST). The intention to treat effectiveness was 55% (95% CI 41% - 69%) for PPI-CA and 57% (95% CI 43% - 71%) for ST (31). Because of the relatively low eradication rate of Aklavik *H. pylori* infections, alternative treatment approaches are needed.

Gene silencing is an attractive field of research due to the specificity of the treatment. The first-in-humans trial of gene silencing therapy through RNA interference in endometrial cancer patient with liver involvement was published in 2013. The interfering RNA was loaded in lipid nanoparticles and was administered intravenously biweekly. The treatment resulted in target downregulation, and antitumor activity, including complete regression of liver metastases in endometrial cancer patient (32). However, no clinical trials have been done to treat bacterial infections using gene therapy.

Chapter 3 discusses the possibility of interfering with the production of urease, an enzyme that is essential for *H. pylori* colonization, using gene therapy. Urease catalyzes the hydrolysis of urea to form carbon dioxide (CO_2) and ammonia (NH_3), which neutralizes the gastric acid, thereby

allowing *H. pylori* to grow (33). In Chapter 3, I discussed three novel strategies that can be used to inhibit *H. pylori* urease activity. The first strategy used a plasmid carrying a mutant urease gene; however, the plasmid did not knockdown urease expression. The second strategy loaded antisense DNA against the urease gene into DOTAP® cationic liposomes. The antisense DNA inhibited urease activity by 40% when incorporated into DOTAP® cationic liposomes. In the absence of DOTAP®, fifty times more concentration of the antisense DNA was required to achieve the same inhibition. This suggests that DOTAP® protects the antisense DNA from degradation and enhances its delivery to *H. pylori*. However, 100% inhibition in urease activity could not be achieved and so the effect of the treatment on *H. pylori* survival in an acidic medium was not assessed. Further modification of liposome content and interfering treatment may be needed to achieve 100% inhibition in urease activity. The third strategy used siRNA against the urease gene but no inhibition of urease expression was obtained. This suggests that the siRNA may be ineffective in *H. pylori* due to the absence of RNase III, which is required for the endonucleolytic cleavage of the complementary target mRNA (34). The findings of the second approach provided evidence to support part of the second hypothesis of this thesis in that liposome-mediated delivery of antisense DNA against the urease gene of *H. pylori* will specifically disrupt the ability of *H. pylori* isolates to produce urease, but did not provide evidence that it could impair *H. pylori* survival in the stomach.

Bucker and colleagues simulated prandial and post-prandial stomach conditions in a Mongolian gerbil model of *H. pylori* infection (35). They observed that adult gerbils were able to reacidify the gastric mucosa more quickly after a meal than young babies, which coincided with an impaired ability for *H. pylori* to colonize the gastric mucosa of adults vs babies (35). This suggests that an urease antisense DNA treatment may have the potential to be more effective in babies than adults if similar response is observed in humans.

The antisense treatment did not affect *H. pylori* growth as all experiments were done in neutral culture media where urease in not required for survival. Although the antimicrobial activity of cationic liposomes were previously reported, DOTAP® cationic liposomes did not have any effect on *H. pylori* growth rate at concentrations used in the experiments performed in Chapter 3. Creation of *in vitro* acidic culture medium that simulates gastric juice faces number of challenges. In the literature, a number of studies demonstrated survival of *H. pylori* when incubated at pH 4.0. However, no study has demonstrated *H. pylori* growth in an acidic medium (36,37). An

undergraduate student in our lab tried designing acidic culture media that would support *H. pylori* growth under various acidic pH conditions. However, *H. pylori* demonstrated survival but not growth in pH more than 4. Since the antisense DNA inhibits urease activity by interfering with transcription and/or translation (38), the treatment is only effective when added to actively growing *H. pylori*. It is not expected for antisense DNA to have a significant effect on urease production by *H. pylori* when cells are in the stationary phase. During the exponential phase, cell division proceeds at a constant rate allowing for production of mRNA, which is the target site for antisense DNA treatment. An acidic medium that supports *H. pylori* growth is needed to test the effect of antisense DNA on *H. pylori* growth in conditions that are similar to the physiological condition, however, more research is needed to design this medium.

The oral delivery of antisense oligonucleotide-loaded liposomes will face number of challenges as liposomes may be rapidly degraded by the high acidity of the stomach and the action of digestive enzymes. Coating loaded liposomes with polymers such as chitosan and pectin may enhance their acid stability and mucoadhesive properties (39); however, it is unknown whether or not coating may affect the delivery of interfering molecules to *H. pylori.* Oral liposomal delivery of antimicrobials and other drugs were previously reported but the oral delivery of liposomes loaded with antisense oligonucleotides has not been investigated.

The Aklavik *H. pylori* isolates demonstrated variation in their urease activity. The same concentration of antisense DNA + DOTAP® exerted variation in urease inhibition when the treatment was applied to different Aklavik *H. pylori* isolates. The variation was not only due to differences in urease activity but also due to differences in growth rate among Aklavik *H. pylori* isolates. Such variation may also affect the response to the antisense DNA *in vivo*. In addition, the urease activity differs *in vivo* depending upon the pH of the surrounding environment. Activation begins at a pH of 6.5 and reaches its maximum at pH 5.5. Constant urease activity was observed at pH between 2.5 and 3.0. Urease activity is maintained over a broad range of acidic pH but is lost at pH levels where ammonia production might be toxic to *H. pylori* (40). The *in vivo* variation in urease activity at different pH values may limit the effectiveness of the any treatment that interfere with urease production especially if the treatment was given at constant rate regardless the pH values. Other *H. pylori* genes that are essential for gastric colonization, such as motility genes should be investigated as possible alternative to the urease gene.

Cationic liposomes have been previously reported to exert antimicrobial activity against bacteria, fungi and protozoan (41-43). The antibacterial activity of two formulations (F1 and F2) of stearylamine-containing liposomes was reported for the first time in Chapter 4 of this thesis, and demonstrated antibacterial activity against *H. pylori*. The prepared liposomes had egg phosphatidylcholine (PC), cholesterol (CH) and stearylamine (SA) in a molar ratio of 7:3:1 (F1 liposomes) and 7:3:2 (F2 liposomes). When two treatments of at least 75 µg/uL F2 liposomes were given at 0 and 12 h, *H. pylori* growth was inhibited by 100%. Preliminary data from the Keelan lab revealed a minimal toxicity to gastric epithelial cell lines for F2 liposomes when used at the minimal inhibitory concentration (200 µg/mL). Stearylamine-containing liposomes may be alternative treatment approach for *H. pylori* infections resistant to commonly used antibiotics. These findings provide evidence to support the third hypothesis of this thesis that stearylamine-containing cationic liposomes will inhibit the growth of *H. pylori*.

Liposomal free fatty acids were previously reported to have antimicrobial activity against *H. pylori*. A recent study has reported antibacterial activity for liposomal linolenic acids against antibiotic-resistant *H. pylori*. The liposomal linolenic acid was compared to that of liposomal stearic acid and oleic acid. Liposomal linolenic had the most potent bactericidal effect and completely killed *H. pylori* within 5 min with minimal toxicity (44). The design of liposomes containing both stearylamine and linolenic acid may further enhance antimicrobial activity without being toxic to gastric cells. Coating of the designed liposomes with chitosan or pectin will be needed to achieve acid stability. Polymer coating may enhance the antimicrobial activity of the cationic liposomes due to changes in the overall charge as these polymers are cationic (39). However, coating might be challenging due to the similarity in charge between liposomes and the coating polymers so further modification in liposomes charge may be needed to achieve maximum coating without affecting the antimicrobial activity. Another strategy is the use of synthetic polymers such as Eudragit® which combines both mucoadhesive and pH dependent content release. Although Eudragit® was specifically designed to achieve drug release in the small intestine, it can be used to achieve acid stability without releasing stearylamine content. The antimicrobial activity of stearylamine-containing liposomes depends on the overall liposome charge rather than stearylamine delivery to *H. pylori*. Eudragit®-coated liposomes showed high stability at pH = 1.4 and 6.3 but rapidly degraded by bile (in the duodenum) which will minimize stearylamine delivery to the small intestine and colon (45).

The antisense DNA loaded F1 liposomes were inferior to antisense DNA loaded DOTAP® in inhibiting urease activity. Further modification of the lipid content and/or interfering molecules may be needed to maximize urease inhibition. The incorporation of DOTAP® lipid into liposomes having PC and CH may enhance the stability of liposomes and offers better inhibition of urease activity. Another strategy is to prepare anionic liposomes loaded with UBA to protect antisense DNA from the effect of DNases without being toxic to *H. pylori* and so provide better interference with urease activity.

In summary, the work presented in this thesis is the first report on the genotypic features of *H. pylori* isolates from Aklavik, a remote community in the north where the prevalence of *H. pylori* infections is high. Novel alternative approaches to treat *H. pylori* infections were investigated. Interfering with urease expression may be a specific treatment approach if more research is performed to modify the lipid composition of liposomes and/or interfering molecules to completely abolish urease expression. Stearylamine-containing liposomes have antibacterial activity against *H. pylori* and may offer a valuable treatment approach.

Concluding remarks

There is a gap in our understanding of the characteristic genotypic features of *H. pylori* isolated from northern Canadian communities where the prevalence of *H. pylori* is high and there is high level of treatment failure. The Aklavik project is a community driven research project that was initiated by community members due to their concerns regarding a perceived increased risk of gastric cancer in their community. The work presented in this study is the first report of the characteristic genotypic features of *H. pylori* isolates from Aklavik and their association with clinical outcomes. The association of genotypes such as *cagA* and *vacA* with more severe gastritis or intestinal metaplasia may identify *H. pylori* isolates that must be eradicated to avoid the development of more severe gastric disease. The identification of about 140 rearrangements in the chromosome of one Aklavik *H. pylori* strain relative to previously described strains from other populations illustrates the unique characteristics of Aklavik isolates (7). Understanding the characteristics of these isolates may give insight into their role in causing gastric diseases and the best methods for effective treatment.

Antimicrobial resistance and treatment failure are global health concerns not only in *H. pylori* infections but also in other infectious diseases. There is a great demand for alternative

treatment approaches that would specifically target infectious agents. The work presented in this thesis is the first report of the possibility of inhibiting *H. pylori* urease by using antisense DNA. Interfering with the production of enzymes that are essential for pathogen survival in its host is considered to be a specific and effective treatment. The use of cationic liposomes enhances the delivery and stability of antisense DNA. Complete inhibition of urease activity was not possible in this study; however, changing liposome composition and/or interfering agent may further suppress urease activity. This strategy, if well optimized, may be valuable alternative approach to treat bacterial infections that are resistant to conventional treatment approaches.

Cationic liposomes have antimicrobial activity against bacteria, fungi and protozoa. This study is the first report of the antimicrobial activity of stearylamine-containing liposomes against *H. pylori*. Preliminary data from the Keelan lab showed minimum toxicity to gastric cell lines at the minimum inhibitory and minimum bactericidal concentrations. The novel treatment approach may offer alternative treatment to *H. pylori* infections that are resistant to conventional treatment strategies.

5.2 Future Directions

5.2.1 Genotypic features of *H. pylori* isolates from Aklavik NWT

5.2.1.1 To sequence *vacA i*-region and subtype *vacA s1* region

The correlation of genotypic features to histopathological outcomes revealed the importance of the *vacA* intermediate region as a predictor for acute gastritis. Polymorphism in the *vacA* i-region at amino acid position 196 was previously found to impact *H. pylori* induced disease development (46). Sequence analysis of *vacA* intermediate regions of Aklavik isolates associated with moderate/severe acute gastritis may give insight into the virulence characteristics of those isolates and the reason for their increased virulence. Among Aklavik isolates, *vacAs1* type was not significantly associated with more severe clinical outcomes. Further typing of *vacA s1* into *s1a, s1b* or *s1c* may identify the actual role of *vacA s1* in *H. pylori* pathogenesis.

5.2.1.2 To combine genotypic data from other northern communities

Much more genotype information has been determined for the *H. pylori* isolates collected from the northern communities. Since the Aklavik *H. pylori* project has been extended to other communities in Northern Canada such as Old Crow, Tuktoyaktuk and Fort McPherson. Combining the data from several communities may overcome the problem of small sample size and provide more power in the data analysis to assess the prevalence of severe disease outcome for residents of Northern Canada and the contribution of virulence factors in the pathogenesis of *H. pylori* infection.

5.2.1.3 To study immune gene polymorphisms of Aklavik participants

Host immune gene polymorphisms such as the interleukin-1 gene cluster and gastric acid secretion were previously reported to influence *H. pylori*'s ability to colonize the stomach and establish a clinical condition (47,48). Understanding the immune gene characteristics of Aklavik research participants with more severe clinical outcomes relative to participants with mild or absent clinical presentation may recognize patients with increased risk for disease progression. Furthermore, assessing the roles of both host and pathogen risk factor in disease development will strengthen our knowledge regarding risk factors for residents within the northern communities.

5.2.2 Inhibition of urease activity by antisense DNA

5.2.2.1 To modify antisense DNA structure for better stability

DOTAP mediated delivery of unmodified antisense DNA against the *ureB* gene suppressed the urease activity of *H. pylori* by 40%. Modification of antisense DNA structure may enhance its stability and maximize its activity. Phosphorothioates modified antisense DNA are more stable in cells and tissues. Modification is performed by substituting non-bridging oxygen at each phosphorus in the oligonucleotide chain by sulfur. Substitution retains the charge and solubility of the unmodified phosphodiester oligomer, as well as its ability to hybridize with target mRNAs (49). Another strategy is using peptide nucleic acids which are oligonucleotide analogues in which the deoxyribose or ribose-phosphate backbone has been replaced by a pseudopeptide skeleton. They bind DNA and RNA with high specificity and selectivity, leading to PNA–RNA and PNA–DNA hybrids that are more stable than the corresponding nucleic acid complexes. The binding affinity and selectivity of PNAs for nucleic acids can be modified by the introduction of stereogenic centers (such as D-Lys-based units) into the PNA backbone (50).

5.2.2.2 To modify the lipid composition of liposomes for better delivery

Since the stearylamine-containing liposomes was inferior to DOTAP® in enhancing UBA stability and delivery, further modification of the lipid content may be needed. Liposomes containing egg phosphatidylcholine, cholesterol and DOTAP may provide better delivery for UBA. Coating of the liposomes with a polymer may be needed to ensure the acid stability as the acid stability of DOTAP® is uncertain.

5.2.3 Antimicrobial activity of stearylamine-containing liposomes

5.2.3.1 To investigate the effect of increasing stearylamine concentration

The F2 liposomes containing egg phosphatidylcholine, cholesterol and stearylamine in a molar ratio 7:3:2 showed much higher antimicrobial activity than F1 liposomes containing the same ingredients in a molar ratio of 7:3:1. Further increase in stearylamine concentration may enhance the potency of the liposomes.

5.2.3.2 To determine the MIC and MBC of stearylamine-containing liposomes

The lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism following overnight incubation is known as the minimum inhibitory concentration (MIC), while the lowest concentration of an antimicrobial that will kill an organism following overnight incubation is known as minimum bactericidal concentration (MBC) (51). MICs and MBCs are used to determine the *in vitro* activity of new antimicrobials and so determine the minimum concentration at which they could be used *in vivo*.

5.2.3.3 To assess the toxicity of liposomes to gastric cells

Stearylamine-containing liposomes should be used in a concentration that is non-toxic to gastric cell lines. Liposomes bearing phosphatidylcholine and stearylamine in a molar ratio of 7:2 killed Leishmania *in vitro* and *in vivo*, without having any adverse effect to human macrophages but no investigation was performed on gastric cells (43). Although increasing the stearylamine concentration enhances the antimicrobial activity of the liposomes, higher concentrations might lack the selective toxicity and may prevent their use as antimicrobial agents. The ideal formulation will have maximum inhibitory effect on *H. pylori* without harming gastric cells.

5.2.3.4 To target liposomes to *H. pylori*

The use of liposomes targeted to a molecule specific to the surface of *H. pylori* would have the advantage of affecting only *H. pylori* without harming other bacteria living as commensals in the gastro-intestinal tract, and would also prevent the toxicity of these liposomes to eukaryotic cells. Targeting can be achieved by incorporating a neoglycolipid with a structure similar to Le^b that can bind the BabA protein. The same strategy was previously used to enhance the specific delivery of antimicrobials to *H. pylori* (52).

5.2.3.5 To coat the liposomes with polymers to enhance acid stability

Coating of liposomes with mucoadhesive thiomer might be needed if the acid stability needed to be further enhanced (53). This mucoadhesive thiomer may also be essential for prolonged contact time with gastric mucosa for optimum therapeutic effect of the new therapy.

5.3 References

(1) Naja F, Kreiger N, Sullivan T. *Helicobacter pylori* infection in Ontario: prevalence and risk factors. Can J Gastroenterol 2007 Aug;21(8):501-506.

(2) Veldhuyzen van Zanten SJ, Pollak PT, Best LM, Bezanson GS, Marrie T. Increasing prevalence of *Helicobacter pylori* infection with age: continuous risk of infection in adults rather than cohort effect. J Infect Dis 1994 Feb;169(2):434-437.

(3) Segal I, Otley A, Issenman R, Armstrong D, Espinosa V, Cawdron R, et al. Low prevalence of *Helicobacter pylori* infection in Canadian children: a cross-sectional analysis. Can J Gastroenterol 2008 May;22(5):485-489.

(4) Cheung J, Goodman K, Munday R, Heavner K, Huntington J, Morse J, et al. *Helicobacter pylori* infection in Canada's arctic: searching for the solutions. Can J Gastroenterol 2008 Nov;22(11):912-916.

(5) Sgouros SN, Bergele C. Clinical outcome of patients with *Helicobacter pylori* infection: the bug, the host, or the environment? Postgrad Med J 2006 May;82(967):338-342.

(6) Higashi H, Tsutsumi R, Fujita A, Yamazaki S, Asaka M, Azuma T, et al. Biological activity of the *Helicobacter pylori* virulence factor CagA is determined by variation in the tyrosine phosphorylation sites. Proc Natl Acad Sci U S A 2002 Oct 29;99(22):14428-14433.

(7) Wada A, Yamasaki E, Hirayama T. *Helicobacter pylori* vacuolating cytotoxin, VacA, is responsible for gastric ulceration. J Biochem 2004 Dec;136(6):741-746.

(8) Ilver D, Arnqvist A, Ogren J, Frick IM, Kersulyte D, Incecik ET, et al. *Helicobacter pylori* adhesin binding fucosylated histo-blood group antigens revealed by retagging. Science 1998 Jan 16;279(5349):373-377.

(9) Kusters JG, van Vliet AH, Kuipers EJ. Pathogenesis of *Helicobacter pylori* infection. Clin Microbiol Rev 2006 Jul;19(3):449-490.

(10) Censini S, Lange C, Xiang Z, Crabtree JE, Ghiara P, Borodovsky M, et al. *cag*, a pathogenicity island of *Helicobacter pylori*, encodes type I-specific and disease-associated virulence factors. Proc Natl Acad Sci U S A 1996 Dec 10;93(25):14648-14653.

(11) Jones KR, Whitmire JM, Merrell DS. A Tale of Two Toxins: *Helicobacter pylori* CagA and VacA Modulate Host Pathways that Impact Disease. Front Microbiol 2010 Nov 23;1:115.

(12) Appelmelk BJ, Simoons-Smit I, Negrini R, Moran AP, Aspinall GO, Forte JG, et al. Potential role of molecular mimicry between *Helicobacter pylori* lipopolysaccharide and host Lewis blood group antigens in autoimmunity. Infect Immun 1996 Jun;64(6):2031-2040.

(13) Styer CM, Hansen LM, Cooke CL, Gundersen AM, Choi SS, Berg DE, et al. Expression of the BabA adhesin during experimental infection with *Helicobacter pylori*. Infect Immun 2010 Apr;78(4):1593-1600.

(14) van der Woude MW, Baumler AJ. Phase and antigenic variation in bacteria. Clin Microbiol Rev 2004 Jul;17(3):581-611, table of contents.

(15) Nell S, Kennemann L, Schwarz S, Josenhans C, Suerbaum S. Dynamics of Lewis b binding and sequence variation of the babA adhesin gene during chronic *Helicobacter pylori* infection in humans. MBio 2014 Dec 16;5(6):10.1128/mBio.02281-14.

(16) Jafari F, Shokrzadeh L, Dabiri H, Baghaei K, Yamaoka Y, Zojaji H, et al. *vacA* genotypes of *Helicobacter pylori* in relation to *cagA* status and clinical outcomes in Iranian populations. Jpn J Infect Dis 2008 Jul;61(4):290-293.

(17) Atherton JC, Cover TL, Twells RJ, Morales MR, Hawkey CJ, Blaser MJ. Simple and accurate PCR-based system for typing vacuolating cytotoxin alleles of *Helicobacter pylori*. J Clin Microbiol 1999 Sep;37(9):2979-2982.

(18) Atherton JC, Cao P, Peek RM,Jr, Tummuru MK, Blaser MJ, Cover TL. Mosaicism in vacuolating cytotoxin alleles of *Helicobacter pylori*. Association of specific *vacA* types with cytotoxin production and peptic ulceration. J Biol Chem 1995 Jul 28;270(30):17771-17777.

(19) Rhead JL, Letley DP, Mohammadi M, Hussein N, Mohagheghi MA, Eshagh Hosseini M, et al. A new *Helicobacter pylori* vacuolating cytotoxin determinant, the intermediate region, is associated with gastric cancer. Gastroenterology 2007 Sep;133(3):926-936.

(20) Winter JA, Letley DP, Cook KW, Rhead JL, Zaitoun AA, Ingram RJ, et al. A role for the vacuolating cytotoxin, *VacA*, in colonization and *Helicobacter pylori*-induced metaplasia in the stomach. J Infect Dis 2014 Sep 15;210(6):954-963.

(21) Suerbaum S, Smith JM, Bapumia K, Morelli G, Smith NH, Kunstmann E, et al. Free recombination within *Helicobacter pylori*. Proc Natl Acad Sci U S A 1998 Oct 13;95(21):12619-12624.

(22) Didelot X, Nell S, Yang I, Woltemate S, van der Merwe S, Suerbaum S. Genomic evolution and transmission of *Helicobacter pylori* in two South African families. Proc Natl Acad Sci U S A 2013 Aug 20;110(34):13880-13885.

(23) Krebes J, Didelot X, Kennemann L, Suerbaum S. Bidirectional genomic exchange between *Helicobacter pylori* strains from a family in Coventry, United Kingdom. Int J Med Microbiol 2014 Nov;304(8):1135-1146.

(24) Kersulyte D, Bertoli MT, Tamma S, Keelan M, Munday R, Geary J, et al. Complete Genome Sequences of Two *Helicobacter pylori* Strains from a Canadian Arctic Aboriginal Community. Genome Announc 2015 Apr 16;3(2):10.1128/genomeA.00209-15.

(25) Meyer JM, Silliman NP, Wang W, Siepman NY, Sugg JE, Morris D, et al. Risk factors for *Helicobacter pylori* resistance in the United States: the surveillance of *H. pylori* antimicrobial resistance partnership (SHARP) study, 1993-1999. Ann Intern Med 2002 Jan 1;136(1):13-24.

(26) Cameron EA, Bell GD, Baldwin L, Powell KU, Williams SG. Long-term study of re-infection following successful eradication of *Helicobacter pylori* infection. Aliment Pharmacol Ther 2006 May 1;23(9):1355-1358.

(27) Al-Eidan FA, McElnay JC, Scott MG, McConnell JB. Management of *Helicobacter pylori* eradication--the influence of structured counselling and follow-up. Br J Clin Pharmacol 2002 Feb;53(2):163-171.

(28) Garza-Gonzalez E, Perez-Perez GI, Maldonado-Garza HJ, Bosques-Padilla FJ. A review of *Helicobacter pylori* diagnosis, treatment, and methods to detect eradication. World J Gastroenterol 2014 Feb 14;20(6):1438-1449.

(29) Berrutti M, Pellicano R, Astegiano M, Smedile A, Saracco G, Morgando A, et al. *Helicobacter pylori* eradication: metronidazole or tinidazole? Data from Turin, Italy. Minerva Gastroenterol Dietol 2008 Dec;54(4):355-358.

(30) Lahaie R, Farley A, Dallaire C, Archambault A, Fallone CA, Ponich T, et al. Bismuth-based quadruple therapy with bismuth subcitrate, metronidazole, tetracycline and omeprazole in the eradication of *Helicobacter pylori*. Can J Gastroenterol 2001 Sep;15(9):581-585.

(31) Morse AL, Goodman KJ, Munday R, Chang HJ, Morse JW, Keelan M, et al. A randomized controlled trial comparing sequential with triple therapy for *Helicobacter pylori* in an Aboriginal community in the Canadian North. Can J Gastroenterol 2013 Dec;27(12):701-706.

(32) Tabernero J, Shapiro GI, LoRusso PM, Cervantes A, Schwartz GK, Weiss GJ, et al. First-in-humans trial of an RNA interference therapeutic targeting VEGF and KSP in cancer patients with liver involvement. Cancer Discov 2013 Apr;3(4):406-417.

(33) Tsujii M, Kawano S, Tsuji S, Fusamoto H, Kamada T, Sato N. Mechanism of gastric mucosal damage induced by ammonia. Gastroenterology 1992 Jun;102(6):1881-1888.

(34) Elbashir SM, Harborth J, Lendeckel W, Yalcin A, Weber K, Tuschl T. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. Nature 2001 May 24;411(6836):494-498.

(35) Bucker R, Azevedo-Vethacke M, Groll C, Garten D, Josenhans C, Suerbaum S, et al. *Helicobacter pylori* colonization critically depends on postprandial gastric conditions. Sci Rep 2012;2:994.

(36) Marcus EA, Inatomi N, Nagami GT, Sachs G, Scott DR. The effects of varying acidity on *Helicobacter pylori* growth and the bactericidal efficacy of ampicillin. Aliment Pharmacol Ther 2012 Nov;36(10):972-979.

(37) West AP, Millar MR, Tompkins DS. Effect of physical environment on survival of *Helicobacter pylori*. J Clin Pathol 1992 Mar;45(3):228-231.

(38) Matzke M, Matzke AJ, Kooter JM. RNA: guiding gene silencing. Science 2001 Aug 10;293(5532):1080-1083.

(39) Hua S. Orally administered liposomal formulations for colon targeted drug delivery. Front Pharmacol 2014 Jun 10;5:138.

(40) Sachs G, Scott DR, Weeks DL, Rektorscheck M, Melchers K. Regulation of Urease for Acid Habitation. In: Mobley HLT, Mendz GL, Hazell SL, editors. *Helicobacter pylori*: Physiology and Genetics Washington (DC): ASM Press; 2001.

(41) Campanha MT, Mamizuka EM, Carmona-Ribeiro AM. Interactions between cationic liposomes and bacteria: the physical-chemistry of the bactericidal action. J Lipid Res 1999 Aug;40(8):1495-1500.

(42) Vieira DB, Carmona-Ribeiro AM. Cationic lipids and surfactants as antifungal agents: mode of action. J Antimicrob Chemother 2006 Oct;58(4):760-767.

(43) Banerjee A, Roychoudhury J, Ali N. Stearylamine-bearing cationic liposomes kill Leishmania parasites through surface exposed negatively charged phosphatidylserine. J Antimicrob Chemother 2008 Jan;61(1):103-110.

(44) Obonyo M, Zhang L, Thamphiwatana S, Pornpattananangkul D, Fu V, Zhang L. Antibacterial activities of liposomal linolenic acids against antibiotic-resistant *Helicobacter pylori*. Mol Pharm 2012 Sep 4;9(9):2677-2685.

(45) Barea MJ, Jenkins MJ, Gaber MH, Bridson RH. Evaluation of liposomes coated with a pH responsive polymer. Int J Pharm 2010 Dec 15;402(1-2):89-94.

(46) Jones KR, Jang S, Chang JY, Kim J, Chung IS, Olsen CH, et al. Polymorphisms in the intermediate region of VacA impact *Helicobacter pylori*-induced disease development. J Clin Microbiol 2011 Jan;49(1):101-110.

(47) El-Omar EM, Carrington M, Chow WH, McColl KE, Bream JH, Young HA, et al. Interleukin-1 polymorphisms associated with increased risk of gastric cancer. Nature 2000 Mar 23;404(6776):398-402.

(48) Kusters JG, van Vliet AH, Kuipers EJ. Pathogenesis of *Helicobacter pylori* infection. Clin Microbiol Rev 2006 Jul;19(3):449-490.

(49) Stein CA, Subasinghe C, Shinozuka K, Cohen JS. Physicochemical properties of phosphorothioate oligodeoxynucleotides. Nucleic Acids Res 1988 Apr 25;16(8):3209-3221.

(50) Menchise V, De Simone G, Tedeschi T, Corradini R, Sforza S, Marchelli R, et al. Insights into peptide nucleic acid (PNA) structural features: the crystal structure of a D-lysine-based chiral PNA-DNA duplex. Proc Natl Acad Sci U S A 2003 Oct 14;100(21):12021-12026.

(51) Andrews JM. Determination of minimum inhibitory concentrations. J Antimicrob Chemother 2001 Jul;48 Suppl 1:5-16.

(52) Bardonnet PL, Faivre V, Boullanger P, Piffaretti JC, Falson F. Pre-formulation of liposomes against *Helicobacter pylori*: characterization and interaction with the bacteria. Eur J Pharm Biopharm 2008 Aug;69(3):908-922.

(53) Hyun Sup Lee, Kwangmeyung Kim, Bong Hyun Jeong, Hyun Tae Moon, Youngro Byun. Stability of poly(Acrylic Acid)-grafted phospholipid liposomes in gastrointestinal conditions. Drug Development Research 2004;61(1):13-18.

Bibliography

Abdullah SM, Hussein NR, Salih AM, Merza MA, Goreal AA, Odeesh OY, et al. Infection with *Helicobacter pylori* strains carrying *babA2* and *cagA* is associated with an increased risk of peptic ulcer disease development in Iraq. Arab J Gastroenterol 2012 Dec;13(4):166-169.

Adams BL, Bates TC, Oliver JD. Survival of *Helicobacter pylori* in a natural freshwater environment. Appl Environ Microbiol 2003 Dec;69(12):7462-7466.

Aebischer T, Bumann D, Epple HJ, Metzger W, Schneider T, Cherepnev G, et al. Correlation of T cell response and bacterial clearance in human volunteers challenged with *Helicobacter pylori* revealed by randomised controlled vaccination with Ty21a-based *Salmonella* vaccines. Gut 2008 Aug;57(8):1065-1072.

Agudo S, Perez-Perez G, Alarcon T, Lopez-Brea M. High prevalence of clarithromycin-resistant *Helicobacter pylori* strains and risk factors associated with resistance in Madrid, Spain. J Clin Microbiol 2010 Oct;48(10):3703-3707.

Ahmad MM, Rahman M, Rumi AK, Islam S, Huq F, Chowdhury MF, et al. Prevalence of *Helicobacter pylori* in asymptomatic population--a pilot serological study in Bangladesh. J Epidemiol 1997 Dec;7(4):251-254.

Ahmed N. 23 years of the discovery of *Helicobacter pylori*: is the debate over? Ann Clin Microbiol Antimicrob 2005 Oct 31;4:17.

Akbarzadeh A, Rezaei-Sadabady R, Davaran S, Joo SW, Zarghami N, Hanifehpour Y, et al. Liposome: classification, preparation, and applications. Nanoscale Res Lett 2013 Feb 22;8(1):102-276X-8-102.

Akhiani AA, Stensson A, Schon K, Lycke NY. IgA antibodies impair resistance against *Helicobacter pylori* infection: studies on immune evasion in IL-10-deficient mice. J Immunol 2005 Jun 15;174(12):8144-8153.

Al-Eidan FA, McElnay JC, Scott MG, McConnell JB. Management of *Helicobacter pylori* eradication--the influence of structured counselling and follow-up. Br J Clin Pharmacol 2002 Feb;53(2):163-171.

Allen TM, Hansen C. Pharmacokinetics of stealth versus conventional liposomes: effect of dose. Biochim Biophys Acta 1991 Sep 30;1068(2):133-141.

Allmon A, Stevermer JJ. Sequential therapy boosts H pylori eradication rates. J Fam Pract 2008 Oct;57(10):651-654.

Alm RA, Bina J, Andrews BM, Doig P, Hancock RE, Trust TJ. Comparative genomics of *Helicobacter pylori*: analysis of the outer membrane protein families. Infect Immun 2000 Jul;68(7):4155-4168.

Al-Sulami AA, Al-Edani TA, Al-Abdula AA. Culture Method and PCR for the Detection of *Helicobacter pylori* in Drinking Water in Basrah Governorate Iraq. Gastroenterol Res Pract 2012;2012:245167.

Altman E, Chandan V, Harrison B. The potential of dextran-based glycoconjugates for development of *Helicobacter pylori* vaccine. Glycoconj J 2014 Jan;31(1):13-24.

Alvarado-Esquivel C. Seroepidemiology of *Helicobacter pylori* Infection in Tepehuanos Aged 15 Years and Older in Durango, Mexico. J Pathog 2013;2013:243246.

Andersen, P. and Wadström T. Basic Bacteriology and Culture. In: Mobley HLT, Mendz GL, Hazell SL, editor. *Helicobacter pylori*: Physiology and Genetics. Washington (DC): ASM; 2001.

Andrews JM. Determination of minimum inhibitory concentrations. J Antimicrob Chemother 2001 Jul;48 Suppl 1:5-16.

Appelmelk BJ, Simoons-Smit I, Negrini R, Moran AP, Aspinall GO, Forte JG, et al. Potential role of molecular mimicry between *Helicobacter pylori* lipopolysaccharide and host Lewis blood group antigens in autoimmunity. Infect Immun 1996 Jun;64(6):2031-2040.

Argent RH, Kidd M, Owen RJ, Thomas RJ, Limb MC, Atherton JC. Determinants and consequences of different levels of CagA phosphorylation for clinical isolates of *Helicobacter pylori*. Gastroenterology 2004 Aug;127(2):514-523.

Argent RH, Zhang Y, Atherton JC. Simple method for determination of the number of *Helicobacter pylori* CagA variable-region EPIYA tyrosine phosphorylation motifs by PCR. J Clin Microbiol 2005 Feb;43(2):791-795.

Asfeldt AM, Lochen ML, Straume B, Steigen SE, Florholmen J, Goll R, et al. Accuracy of a monoclonal antibody-based stool antigen test in the diagnosis of *Helicobacter pylori* infection. Scand J Gastroenterol 2004 Nov;39(11):1073-1077.

Aspholm-Hurtig M, Dailide G, Lahmann M, Kalia A, Ilver D, Roche N, et al. Functional adaptation of BabA, the *H. pylori* ABO blood group antigen binding adhesin. Science 2004 Jul 23;305(5683):519-522.

Atherton JC, Cao P, Peek RM,Jr, Tummuru MK, Blaser MJ, Cover TL. Mosaicism in vacuolating cytotoxin alleles of *Helicobacter pylori*. Association of specific *vacA* types with cytotoxin production and peptic ulceration. J Biol Chem 1995 Jul 28;270(30):17771-17777.

Atherton JC, Cover TL, Twells RJ, Morales MR, Hawkey CJ, Blaser MJ. Simple and accurate PCR-based system for typing vacuolating cytotoxin alleles of *Helicobacter pylori*. J Clin Microbiol 1999 Sep;37(9):2979-2982.

Atherton JC, Cover TL, Twells RJ, Morales MR, Hawkey CJ, Blaser MJ. Simple and accurate PCR-based system for typing vacuolating cytotoxin alleles of *Helicobacter pylori*. J Clin Microbiol 1999 Sep;37(9):2979-2982.

Ayoola AE, Ageely HM, Gadour MO, Pathak VP. Prevalence of *Helicobacter pylori* infection among patients with dyspepsia in South-Western Saudi Arabia. Saudi Med J 2004 Oct;25(10):1433-1438.

Azagarsamy MA, Gomez-Escudero A, Yesilyurt V, Vachet RW, Thayumanavan S. Amphiphilic nanoassemblies for the detection of peptides and proteins using fluorescence and mass spectrometry. Analyst 2009 Apr;134(4):635-649.

Aziz F, Chen X, Yang X, Yan Q. Prevalence and correlation with clinical diseases of *Helicobacter pylori cagA* and *vacA* genotype among gastric patients from Northeast China. Biomed Res Int 2014;2014:142980.

Azuma T, Yamakawa A, Yamazaki S, Fukuta K, Ohtani M, Ito Y, et al. Correlation between variation of the 3' region of the *cagA* gene in *Helicobacter pylori* and disease outcome in Japan. J Infect Dis 2002 Dec 1;186(11):1621-1630.

Azuma T. *Helicobacter pylori* CagA protein variation associated with gastric cancer in Asia. J Gastroenterol 2004;39(2):97-103.

Backert S, Clyne M, Tegtmeyer N. Molecular mechanisms of gastric epithelial cell adhesion and injection of CagA by *Helicobacter pylori*. Cell Commun Signal 2011 Nov 1;9:28-811X-9-28.

Backert S, Kwok T, Konig W. Conjugative plasmid DNA transfer in *Helicobacter pylori* mediated by chromosomally encoded relaxase and TraG-like proteins. Microbiology 2005 Nov;151(Pt 11):3493-3503.

Banerjee A, Roychoudhury J, Ali N. Stearylamine-bearing cationic liposomes kill Leishmania parasites through surface exposed negatively charged phosphatidylserine. J Antimicrob Chemother 2008 Jan;61(1):103-110.

Bangham AD, Standish MM, Watkins JC. Diffusion of univalent ions across the lamellae of swollen phospholipids. J Mol Biol 1965 Aug;13(1):238-252.

Barbas III CF, Burton, D.R. Scott, J.K. and Silverman, G.J. Quantification of DNA and RNA. Cold Spring Harbor Protocol 2007.

Bardonnet PL, Faivre V, Boullanger P, Piffaretti JC, Falson F. Pre-formulation of liposomes against *Helicobacter pylori*: characterization and interaction with the bacteria. Eur J Pharm Biopharm 2008 Aug;69(3):908-922.

Barea MJ, Jenkins MJ, Gaber MH, Bridson RH. Evaluation of liposomes coated with a pH responsive polymer. Int J Pharm 2010 Dec 15;402(1-2):89-94.

Barenholz, Y. and Lasic, D.D editor. Handbook of Nonmedical Applications of Liposomes from design to microreactors. United States of America: CRC Press Inc.; 1996.

Bartlett JG, Madariaga-Vignudo L, O'Neil JD, Kuhnlein HV: Identifying indigenous peoples for health research in a global context: a review of perspectives and challenges. Int J Circumpolar Health 2007, 66(4)**:**287-307.

Basso D, Zambon CF, Letley DP, Stranges A, Marchet A, Rhead JL, et al. Clinical relevance of *Helicobacter pylori cagA* and *vacA* gene polymorphisms. Gastroenterology 2008 Jul;135(1):91-99.

Bauerfeind P, Garner R, Dunn BE, Mobley HL. Synthesis and activity of *Helicobacter pylori* urease and catalase at low pH. Gut 1997 Jan;40(1):25-30.

Belda S, Saez J, Santibanez M, Rodriguez JC, Sola-Vera J, Ruiz-Garcia M, et al. Relationship between bacterial load, morbidity and *cagA* gene in patients infected by *Helicobacter pylori*. Clin Microbiol Infect 2012 Jul;18(7):E251-3.

Benaissa M, Babin P, Quellard N, Pezennec L, Cenatiempo Y, Fauchere JL. Changes in *Helicobacter pylori* ultrastructure and antigens during conversion from the bacillary to the coccoid form. Infect Immun 1996 Jun;64(6):2331-2335.

Benktander J, Angstrom J, Breimer ME, Teneberg S. Redefinition of the carbohydrate binding specificity of *Helicobacter pylori* BabA adhesin. J Biol Chem 2012 Sep 14;287(38):31712-31724.

Bentires-Alj M, Paez JG, David FS, Keilhack H, Halmos B, Naoki K, et al. Activating mutations of the noonan syndrome-associated SHP2/PTPN11 gene in human solid tumors and adult acute myelogenous leukemia. Cancer Res 2004 Dec 15;64(24):8816-8820.

Bermejo F, Boixeda D, Gisbert JP, Defarges V, Sanz JM, Redondo C, et al. Rapid urease test utility for *Helicobacter pylori* infection diagnosis in gastric ulcer disease. Hepatogastroenterology 2002 Mar-Apr;49(44):572-575.

Bernstein CN, McKeown I, Embil JM, Blanchard JF, Dawood M, Kabani A, et al. Seroprevalence of *Helicobacter pylori*, incidence of gastric cancer, and peptic ulcer-associated hospitalizations in a Canadian Indian population. Dig Dis Sci 1999 Apr;44(4):668-674.

Berrutti M, Pellicano R, Astegiano M, Smedile A, Saracco G, Morgando A, et al. *Helicobacter pylori* eradication: metronidazole or tinidazole? Data from Turin, Italy. Minerva Gastroenterol Dietol 2008 Dec;54(4):355-358.

Beswick EJ, Pinchuk IV, Minch K, Suarez G, Sierra JC, Yamaoka Y, et al. The *Helicobacter pylori* urease B subunit binds to CD74 on gastric epithelial cells and induces NF-kappaB activation and interleukin-8 production. Infect Immun 2006 Feb;74(2):1148-1155.

Bhai S. A., Yadav V.,Mamatha Y. and Prasanth V.V. Liposomes: an overview. Journal of pharmaceutical and scientific innovation 2012;1(1).

Bhuiyan TR, Qadri F, Saha A, Svennerholm AM. Infection by *Helicobacter pylori* in Bangladeshi children from birth to two years: relation to blood group, nutritional status, and seasonality. Pediatr Infect Dis J 2009 Feb;28(2):79-85.

Biernat MM, Gosciniak G, Iwanczak B. Prevalence of *Helicobacter pylori cagA*, *vacA*, *iceA*, *babA2* genotypes in Polish children and adolescents with gastroduodenal disease. Postepy Hig Med Dosw (Online) 2014 Aug 22;68:1015-1021.

Bogstedt AK, Nava S, Wadstrom T, Hammarstrom L. *Helicobacter pylori* infections in IgA deficiency: lack of role for the secretory immune system. Clin Exp Immunol 1996 Aug;105(2):202-204.

Boren T, Falk P, Roth KA, Larson G, Normark S. Attachment of *Helicobacter pylori* to human gastric epithelium mediated by blood group antigens. Science 1993 Dec 17;262(5141):1892-1895.

Bourke B, Ceponis P, Chiba N, Czinn S, Ferraro R, Fischbach L, et al. Canadian Helicobacter Study Group Consensus Conference: Update on the approach to *Helicobacter pylori* infection in children and adolescents--an evidence-based evaluation. Can J Gastroenterol 2005 Jul;19(7):399-408.

Brantl S, Bruckner R. Small regulatory RNAs from low-GC Gram-positive bacteria. RNA Biol 2014 May;11(5):443-456.

Brantl S. Antisense-RNA regulation and RNA interference. Biochim Biophys Acta 2002 May 3;1575(1-3):15-25.

Brantl S. Regulatory mechanisms employed by cis-encoded antisense RNAs. Curr Opin Microbiol 2007 Apr;10(2):102-109.

Brown LM. *Helicobacter pylori*: epidemiology and routes of transmission. Epidemiol Rev 2000;22(2):283-297.

Bucker R, Azevedo-Vethacke M, Groll C, Garten D, Josenhans C, Suerbaum S, et al. *Helicobacter pylori* colonization critically depends on postprandial gastric conditions. Sci Rep 2012;2:994.

Bunn JE, MacKay WG, Thomas JE, Reid DC, Weaver LT. Detection of *Helicobacter pylori* DNA in drinking water biofilms: implications for transmission in early life. Lett Appl Microbiol 2002;34(6):450-454.

Bures J, Kopacova M, Koupil I, Seifert B, Skodova Fendrichova M, Spirkova J, et al. Significant decrease in prevalence of *Helicobacter pylori* in the Czech Republic. World J Gastroenterol 2012 Aug 28;18(32):4412-4418.

Burgers R, Schneider-Brachert W, Reischl U, Behr A, Hiller KA, Lehn N, et al. *Helicobacter pylori* in human oral cavity and stomach. Eur J Oral Sci 2008 Aug;116(4):297-304.

Burucoa C, Lhomme V, Fauchere JL. Performance criteria of DNA fingerprinting methods for typing of *Helicobacter pylori* isolates: experimental results and meta-analysis. J Clin Microbiol 1999 Dec;37(12):4071-4080.

Calvet X, Sanchez-Delgado J, Montserrat A, Lario S, Ramirez-Lazaro MJ, Quesada M, et al. Accuracy of diagnostic tests for *Helicobacter pylori*: a reappraisal. Clin Infect Dis 2009 May 15;48(10):1385-1391.

Cameron EA, Bell GD, Baldwin L, Powell KU, Williams SG. Long-term study of re-infection following successful eradication of *Helicobacter pylori* infection. Aliment Pharmacol Ther 2006 May 1;23(9):1355-1358.

Campanha MT, Mamizuka EM, Carmona-Ribeiro AM. Interactions between cationic liposomes and bacteria: the physical-chemistry of the bactericidal action. J Lipid Res 1999 Aug;40(8):1495-1500.

Capelle LG, de Vries AC, Haringsma J, Ter Borg F, de Vries RA, Bruno MJ, et al. The staging of gastritis with the OLGA system by using intestinal metaplasia as an accurate alternative for atrophic gastritis. Gastrointest Endosc 2010 Jun;71(7):1150-1158.

Caracciolo G, Amenitsch H. Cationic liposome/DNA complexes: from structure to interactions with cellular membranes. Eur Biophys J 2012 Oct;41(10):815-829.

Carraher S, Chang HJ, Munday R, Goodman KJ, CANHelp Working Group. *Helicobacter pylori* incidence and re-infection in the Aklavik *H. pylori* Project. Int J Circumpolar Health 2013 Aug 5;72:10.3402/ijch.v72i0.21594. eCollection 2013.

Cendron L, Zanotti G. Structural and functional aspects of unique type IV secretory components in the *Helicobacter pylori* cag-pathogenicity island. FEBS J 2011 Apr;278(8):1223-1231.

Censini S, Lange C, Xiang Z, Crabtree JE, Ghiara P, Borodovsky M, et al. cag, a pathogenicity island of *Helicobacter pylori*, encodes type I-specific and disease-associated virulence factors. Proc Natl Acad Sci U S A 1996 Dec 10;93(25):14648-14653.

Cevc G editor. Phospholipids Handbook. New York, USA: Marcel Dekker Inc.; 1993.

Chehter EZ, Bacci MR, Fonseca FL, Goncalves JA, Buchalla G, Shiraichi SA, et al. Diagnosis of the infection by the *Helicobacter pylori* through stool examination: Method standardization in adults. Clin Biochem 2013 Oct;46(15):1622-1624.

Chen J, Lin L, Li N, She F. Enhancement of *Helicobacter pylori* outer inflammatory protein DNA vaccine efficacy by co-delivery of interleukin-2 and B subunit heat-labile toxin gene encoded plasmids. Microbiol Immunol 2012 Feb;56(2):85-92.

Chen MY, He CY, Meng X, Yuan Y. Association of *Helicobacter pylori babA2* with peptic ulcer disease and gastric cancer. World J Gastroenterol 2013 Jul 14;19(26):4242-4251.

Cheung J, Goodman K, Munday R, Heavner K, Huntington J, Morse J, et al. *Helicobacter pylori* infection in Canada's arctic: searching for the solutions. Can J Gastroenterol 2008 Nov;22(11):912-916.

Cheung J, Goodman KJ, Girgis S, Bailey R, Morse J, Fedorak RN, et al. Disease manifestations of *Helicobacter pylori* infection in Arctic Canada: using epidemiology to address community concerns. BMJ Open 2014 Jan 8;4(1):e003689-2013-003689.

Chi H, Bair MJ, Wu MS, Chiu NC, Hsiao YC, Chang KY. Prevalence of *Helicobacter pylori* infection in high-school students on Lanyu Island, Taiwan: risk factor analysis and effect on growth. J Formos Med Assoc 2009 Dec;108(12):929-936.

Chomvarin C, Kulsantiwong P, Chantarasuk Y, Chantrakooptungool S, Kanjanahareutai S. Comparison of media and antibiotic supplements for isolation of *Helicobacter pylori* from gastric biopsies. Southeast Asian J Trop Med Public Health 2006 Nov;37(6):1163-1169.

Chomvarin C, Namwat W, Chaicumpar K, Mairiang P, Sangchan A, Sripa B, et al. Prevalence of *Helicobacter pylori vacA*, *cagA*, *cagE, iceA* and *babA2* genotypes in Thai dyspeptic patients. Int J Infect Dis 2008 Jan;12(1):30-36.

Chung C, Olivares A, Torres E, Yilmaz O, Cohen H, Perez-Perez G. Diversity of VacA intermediate region among *Helicobacter pylori* strains from several regions of the world. J Clin Microbiol 2010 Mar;48(3):690-696.

Chung JW, Jung YK, Kim YJ, Kwon KA, Kim JH, Lee JJ, et al. Ten-day sequential versus triple therapy for *Helicobacter pylori* eradication: a prospective, open-label, randomized trial. J Gastroenterol Hepatol 2012 Nov;27(11):1675-1680.

Clemens J, Albert MJ, Rao M, Huda S, Qadri F, Van Loon FP, et al. Sociodemographic, hygienic and nutritional correlates of *Helicobacter pylori* infection of young Bangladeshi children. Pediatr Infect Dis J 1996 Dec;15(12):1113-1118.

Clyne M, Dolan B, Reeves EP. Bacterial factors that mediate colonization of the stomach and virulence of *Helicobacter pylori*. FEMS Microbiol Lett 2007 Mar;268(2):135-143.

Coderch L, Fonollosa J, De Pera M, Estelrich J, De La Maza A, Parra JL. Influence of cholesterol on liposome fluidity by EPR. Relationship with percutaneous absorption. J Control Release 2000 Jul 31;68(1):85-95.

Colquhoun A, Arnold M, Ferlay J, Goodman KJ, Forman D, Soerjomataram I. Global patterns of cardia and non-cardia gastric cancer incidence in 2012. Gut 2015 Mar 6.

Colquhoun A, Geary J, Goodman KJ. Challenges in conducting community-driven research created by differing ways of talking and thinking about science: a researcher's perspective. Int J Circumpolar Health 2013 Aug 5;72:10.3402/ijch.v72i0.21232. eCollection 2013.

Corthesy-Theulaz I, Porta N, Glauser M, Saraga E, Vaney AC, Haas R, et al. Oral immunization with *Helicobacter pylori* urease B subunit as a treatment against Helicobacter infection in mice. Gastroenterology 1995 Jul;109(1):115-121.

Covacci A, Censini S, Bugnoli M, Petracca R, Burroni D, Macchia G, et al. Molecular characterization of the 128-kDa immunodominant antigen of *Helicobacter pylori* associated with cytotoxicity and duodenal ulcer. Proc Natl Acad Sci U S A 1993 Jun 15;90(12):5791-5795.

Croxen MA, Ernst PB, Hoffman PS. Antisense RNA modulation of alkyl hydroperoxide reductase levels in *Helicobacter pylori* correlates with organic peroxide toxicity but not infectivity. J Bacteriol 2007 May;189(9):3359-3368.

Daniels, R. (April 2001). Galenic principles of modern skin care products. Skin care forum Issue 25. http://www.skin-care-forum.basf.com. Accessed 21/02/2014.

de la Cruz F, Frost LS, Meyer RJ, Zechner EL. Conjugative DNA metabolism in Gram-negative bacteria. FEMS Microbiol Rev 2010 Jan;34(1):18-40.

Dehghani SM, Karamifar H, Raeesi T, Haghighat M. Growth parameters in children with dyspepsia symptoms and *Helicobacter pylori* infection. Indian Pediatr 2013 Mar;50(3):324-326.

Del Valle J. Peptic Ulcer Disease. In: Longo D, Fauci A, Kasper D, Hauser S, Jameson, JL and Loscalzo, J, editors. Harrison's Principles of Internal Medicine. 18th ed. USA: The McGraw-Hill Companies, Inc.; 2012.

Delihas N. Regulation of gene expression by trans-encoded antisense RNAs. Mol Microbiol 1995 Feb;15(3):411-414.

Department of Health and Social Services, Government of the Northwest Territories. The NWT Health Status Report 2005. 2005.

Department of Health and Social Services, Government of the Northwest Territories Cancer in the Northwest Territories, 1990-2000. A descriptive report. 2003.

Desbois AP, Smith VJ. Antibacterial free fatty acids: activities, mechanisms of action and biotechnological potential. Appl Microbiol Biotechnol 2010 Feb;85(6):1629-1642.

Dey T, Anam K, Afrin F, Ali N. Antileishmanial activities of stearylamine-bearing liposomes. Antimicrob Agents Chemother 2000 Jun;44(6):1739-1742.

Didelot X, Nell S, Yang I, Woltemate S, van der Merwe S, Suerbaum S. Genomic evolution and transmission of *Helicobacter pylori* in two South African families. Proc Natl Acad Sci U S A 2013 Aug 20;110(34):13880-13885.

Doh KO, Kim BK, Lee TJ, Park JW, Seu YB. Synthesis of NBD-labeled DOTAP analog to track intracellular delivery of liposome. J Microbiol Biotechnol 2013 Jan;23(1):131-135.

Dore MP, Graham DY, Sepulveda AR. Different penicillin-binding protein profiles in amoxicillin-resistant *Helicobacter pylori*. Helicobacter 1999 Sep;4(3):154-161.

Dore MP, Osato MS, Malaty HM, Graham DY. Characterization of a culture method to recover *Helicobacter pylori* from the feces of infected patients. Helicobacter 2000 Sep;5(3):165-168.

Dore MP, Sepulveda AR, El-Zimaity H, Yamaoka Y, Osato MS, Mototsugu K, et al. Isolation of *Helicobacter pylori* from sheep-implications for transmission to humans. Am J Gastroenterol 2001 May;96(5):1396-1401.

Drulis-Kawa Z, Dorotkiewicz-Jach A. Liposomes as delivery systems for antibiotics. Int J Pharm 2010 Mar 15;387(1-2):187-198.

Dua J.S., Rana A. C. and Bhandari A. K. LIPOSOME: METHODS OF PREPARATION AND APPLICATIONS . International Journal of Pharmaceutical Studies and Research 2012;3(2).

Dufourc EJ. Sterols and membrane dynamics. J Chem Biol 2008 Nov;1(1-4):63-77.

Dunn BE, Cohen H, Blaser MJ. *Helicobacter pylori.* Clin Microbiol Rev 1997 Oct;10(4):720-741.

Eaton KA, Brooks CL, Morgan DR, Krakowka S. Essential role of urease in pathogenesis of gastritis induced by *Helicobacter pylori* in gnotobiotic piglets. Infect Immun 1991 Jul;59(7):2470-2475.

El Dine SS, Mubarak M, Salama R, El Raziky M, El Sherbiny, E. and others. Low Seroprevalence of Anti-CagA Antibodies Inspite of High Seroprevalence of Anti-H pylori Antibodies in Rural Egyptian Community. . Res J Med Med Sci. 2008;3:118.

Elbashir SM, Harborth J, Lendeckel W, Yalcin A, Weber K, Tuschl T. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. Nature 2001 May 24;411(6836):494-498.

El-Omar EM, Carrington M, Chow WH, McColl KE, Bream JH, Young HA, et al. Interleukin-1 polymorphisms associated with increased risk of gastric cancer. Nature 2000 Mar 23;404(6776):398-402.

Epidemiology of, and risk factors for, *Helicobacter pylori* infection among 3194 asymptomatic subjects in 17 populations. The EUROGAST Study Group. Gut 1993 Dec;34(12):1672-1676.

Everhart JE, Kruszon-Moran D, Perez-Perez GI, Tralka TS, McQuillan G. Seroprevalence and ethnic differences in *Helicobacter pylori* infection among adults in the United States. J Infect Dis 2000 Apr;181(4):1359-1363.

Fajardo CA, Quiroga AJ, Coronado A, Labrador K, Acosta N, Delgado P, et al. CagA EPIYA polymorphisms in Colombian *Helicobacter pylori* strains and their influence on disease-associated cellular responses. World J Gastrointest Oncol 2013 Mar 15;5(3):50-59.

Falsafi T, Valizadeh N, Najafi M, Ehsani A, Khani A, Landarani Z, et al. Culture of *Helicobacter pylori* from stool samples in children. Can J Microbiol 2007 Mar;53(3):411-416.

Fan XG, Chua A, Li TG, Zeng QS. Survival of *Helicobacter pylori* in milk and tap water. J Gastroenterol Hepatol 1998 Nov;13(11):1096-1098.

Faundez G, Troncoso M, Figueroa G. *cagA* and *vacA* in strains of *Helicobacter pylori* from ulcer and non-ulcerative dyspepsia patients. BMC Gastroenterol 2002 Sep 10;2:20.

Fernandez-Gonzalez E, Backert S. DNA transfer in the gastric pathogen *Helicobacter pylori*. J Gastroenterol 2014 Apr;49(4):594-604.

Filipe MI, Munoz N, Matko I, Kato I, Pompe-Kirn V, Jutersek A, et al. Intestinal metaplasia types and the risk of gastric cancer: a cohort study in Slovenia. Int J Cancer 1994 May 1;57(3):324-329.

Fillion P, Desjardins A, Sayasith K, Lagace J. Encapsulation of DNA in negatively charged liposomes and inhibition of bacterial gene expression with fluid liposome-encapsulated antisense oligonucleotides. Biochim Biophys Acta 2001 Nov 1;1515(1):44-54.

Fischer W, Hofreuter D, Haas R. Natural Transformation, Recombination, and Repair. In: Mobley HLT, Mendz GL, Hazell SL, editors. *Helicobacter pylori*: Physiology and Genetics Washington (DC): ASM Press; 2001.

Francesco VD, Zullo A, Hassan C, Giorgio F, Rosania R, Ierardi E. Mechanisms of *Helicobacter pylori* antibiotic resistance: An updated appraisal. World J Gastrointest Pathophysiol 2011 Jun 15;2(3):35-41.

Gao W, Cheng H, Hu F, Li J, Wang L, Yang G, et al. The evolution of *Helicobacter pylori* antibiotics resistance over 10 years in Beijing, China. Helicobacter 2010 Oct;15(5):460-466.

Garza-Gonzalez E, Perez-Perez GI, Maldonado-Garza HJ, Bosques-Padilla FJ. A review of *Helicobacter pylori* diagnosis, treatment, and methods to detect eradication. World J Gastroenterol 2014 Feb 14;20(6):1438-1449.

Gatti LL, Fagundes e Souza EK, Leite KR, Bastos EL, Vicentini LR, Silva LC, et al. *cagA, vacA* alelles and *babA2* genotypes of *Helicobacter pylori* associated with gastric disease in Brazilian adult patients. Diagn Microbiol Infect Dis 2005 Apr;51(4):231-235.

Georgopoulos SD, Papastergiou V, Karatapanis S. Current options for the treatment of *Helicobacter pylori*. Expert Opin Pharmacother 2013 Feb;14(2):211-223.

Gerhard M, Lehn N, Neumayer N, Boren T, Rad R, Schepp W, et al. Clinical relevance of the *Helicobacter pylori* gene for blood-group antigen-binding adhesin. Proc Natl Acad Sci U S A 1999 Oct 26;96(22):12778-12783.

Ghotaslou R, Milani M, Akhi MT, Nahaei MR, Hasani A, Hejazi MS, et al. Diversity of *Helicobacter pylori cagA* and *vacA* Genes and Its Relationship with Clinical Outcomes in Azerbaijan, Iran. Adv Pharm Bull 2013;3(1):57-62.

Goodman KJ, Correa P, Tengana Aux HJ, Ramirez H, DeLany JP, Guerrero Pepinosa O, et al. *Helicobacter pylori* infection in the Colombian Andes: a population-based study of transmission pathways. Am J Epidemiol 1996 Aug 1;144(3):290-299.

Goodman KJ, Jacobson K, Veldhuyzen van Zanten S. *Helicobacter pylori* infection in Canadian and related Arctic Aboriginal populations. Can J Gastroenterol 2008 Mar;22(3):289-295.

Gordon, J. and McLeod, J.W. The practical application of the direct oxidase reaction in bacteriology. The Journal of Pathology and Bacteriology 1928;31(2):185.

Graham DY, Fischbach L. *Helicobacter pylori* treatment in the era of increasing antibiotic resistance. Gut 2010 Aug;59(8):1143-1153.

Graham DY, Opekun AR, Osato MS, El-Zimaity HM, Lee CK, Yamaoka Y, et al. Challenge model for *Helicobacter pylori* infection in human volunteers. Gut 2004 Sep;53(9):1235-1243.

Graham DY. History of *Helicobacter pylori*, duodenal ulcer, gastric ulcer and gastric cancer. World J Gastroenterol 2014 May 14;20(18):5191-5204.

Gramley WA, Asghar A, Frierson HF,Jr, Powell SM. Detection of *Helicobacter pylori* DNA in fecal samples from infected individuals. J Clin Microbiol 1999 Jul;37(7):2236-2240.

Guo L, Li X, Tang F, He Y, Xing Y, Deng X, et al. Immunological features and the ability of inhibitory effects on enzymatic activity of an epitope vaccine composed of cholera toxin B subunit and B cell epitope from *Helicobacter pylori* urease A subunit. Appl Microbiol Biotechnol 2012 Mar;93(5):1937-1945.

Han J, Kim D, Morris KV. Promoter-associated RNA is required for RNA-directed transcriptional gene silencing in human cells. Proc Natl Acad Sci U S A 2007 Jul 24;104(30):12422-12427.

Haque M, Hirai Y, Yokota K, Mori N, Jahan I, Ito H, et al. Lipid profile of Helicobacter spp.: presence of cholesteryl glucoside as a characteristic feature. J Bacteriol 1996 Apr;178(7):2065-2070.

Harris P, Perez-Perez G, Zylberberg A, Rollan A, Serrano C, Riera F, et al. Relevance of adjusted cut-off values in commercial serological immunoassays for *Helicobacter pylori* infection in children. Dig Dis Sci 2005 Nov;50(11):2103-2109.

Hatakeyama M. Deregulation of SHP-2 tyrosine phosphatase by the *Helicobacter pylori* virulence factor CagA. Keio J Med 2002 Dec;51 Suppl 2:26-32.

Hatakeyama M. Oncogenic mechanisms of the *Helicobacter pylori* CagA protein. Nat Rev Cancer 2004 Sep;4(9):688-694.

Hatakeyama M. The role of *Helicobacter pylori* CagA in gastric carcinogenesis. Int J Hematol 2006 Nov;84(4):301-308.

Hatakeyama M. The role of *Helicobacter pylori* CagA in gastric carcinogenesis. Int J Hematol 2006 Nov;84(4):301-308.

Hayashi T, Morohashi H, Hatakeyama M. Bacterial EPIYA effectors--where do they come from? What are they? Where are they going? Cell Microbiol 2013 Mar;15(3):377-385.

Heiner CR, Hunkapiller KL, Chen SM, Glass JI, Chen EY. Sequencing multimegabase-template DNA with BigDye terminator chemistry. Genome Res 1998 May;8(5):557-561.

Higashi H, Tsutsumi R, Fujita A, Yamazaki S, Asaka M, Azuma T, et al. Biological activity of the *Helicobacter pylori* virulence factor CagA is determined by variation in the tyrosine phosphorylation sites. Proc Natl Acad Sci U S A 2002 Oct 29;99(22):14428-14433.

Higashi H, Yokoyama K, Fujii Y, Ren S, Yuasa H, Saadat I, et al. EPIYA motif is a membrane-targeting signal of *Helicobacter pylori* virulence factor CagA in mammalian cells. J Biol Chem 2005 Jun 17;280(24):23130-23137.

Hirschl AM, Makristathis A. Methods to detect *Helicobacter pylori*: from culture to molecular biology. Helicobacter 2007 Nov;12 Suppl 2:6-11.

Hofreuter D, Odenbreit S, Henke G, Haas R. Natural competence for DNA transformation in *Helicobacter pylori*: identification and genetic characterization of the comB locus. Mol Microbiol 1998 Jun;28(5):1027-1038.

Holovati JL, Acker JP. Spectrophotometric measurement of intraliposomal trehalose. Cryobiology 2007 Oct;55(2):98-107.

Holovati, J. L. Liposomal delivery of intracellular trehalose; 2008.

Homan M, Luzar B, Kocjan BJ, Orel R, Mocilnik T, Shrestha M, et al. Prevalence and clinical relevance of *cagA*, *vacA*, and *iceA* genotypes of *Helicobacter pylori* isolated from Slovenian children. J Pediatr Gastroenterol Nutr 2009 Sep;49(3):289-296.

Homan M, Sterbenc A, Kocjan BJ, Luzar B, Zidar N, Orel R, et al. Prevalence of the *Helicobacter pylori babA2* gene and correlation with the degree of gastritis in infected Slovenian children. Antonie Van Leeuwenhoek 2014 Oct;106(4):637-645.

Horiki N, Omata F, Uemura M, Suzuki S, Ishii N, Iizuka Y, et al. Annual change of primary resistance to clarithromycin among *Helicobacter pylori* isolates from 1996 through 2008 in Japan. Helicobacter 2009 Oct;14(5):86-90.

Horiuchi T, Ohkusa T, Watanabe M, Kobayashi D, Miwa H, Eishi Y. *Helicobacter pylori* DNA in drinking water in Japan. Microbiol Immunol 2001;45(7):515-519.

Hu D, Shao J, Wang L, Zheng H, Xu Y, Song G, et al. Prevalence and risk factors of *Helicobacter pylori* infection in Chinese maritime workers. Ann Hum Biol 2013 Nov-Dec;40(6):472-476.

Hu LT, Foxall PA, Russell R, Mobley HL. Purification of recombinant *Helicobacter pylori* urease apoenzyme encoded by ureA and ureB. Infect Immun 1992 Jul;60(7):2657-2666.

Hu LT, Mobley HL. Expression of catalytically active recombinant *Helicobacter pylori* urease at wild-type levels in *Escherichia coli*. Infect Immun 1993 Jun;61(6):2563-2569.

Hua S. Orally administered liposomal formulations for colon targeted drug delivery. Front Pharmacol 2014 Jun 10;5:138.

Huang JQ, Zheng GF, Sumanac K, Irvine EJ, Hunt RH. Meta-analysis of the relationship between *cagA* seropositivity and gastric cancer. Gastroenterology 2003 Dec;125(6):1636-1644.

Hulten K, Han SW, Enroth H, Klein PD, Opekun AR, Gilman RH, et al. *Helicobacter pylori* in the drinking water in Peru. Gastroenterology 1996 Apr;110(4):1031-1035.

Hyun Sup Lee, Kwangmeyung Kim, Bong Hyun Jeong, Hyun Tae Moon, Youngro Byun. Stability of poly(Acrylic Acid)-grafted phospholipid liposomes in gastrointestinal conditions. Drug Development Research 2004;61(1):13-18.

Iankov ID, Haralambieva IH, Galanis E. Immunogenicity of attenuated measles virus engineered to express *Helicobacter pylori* neutrophil-activating protein. Vaccine 2011 Feb 11;29(8):1710-1720.

Ilver D, Arnqvist A, Ogren J, Frick IM, Kersulyte D, Incecik ET, et al. *Helicobacter pylori* adhesin binding fucosylated histo-blood group antigens revealed by retagging. Science 1998 Jan 16;279(5349):373-377.

Integrated DNA Technology. IDT SciTools® Web Tools. 2012; Available at: https://www.idtdna.com/pages/scitools. Accessed 09, 2011.

Iranikhah A, Ghadir MR, Sarkeshikian S, Saneian H, Heiari A, Mahvari M. Stool antigen tests for the detection of *Helicobacter pylori* in children. Iran J Pediatr 2013 Apr;23(2):138-142.

Isaka Y, Imai E, Takahara S, Rakugi H. Oligonucleotidic therapeutics. Expert Opin Drug Discov 2008 Sep;3(9):991-996.

Ito S, Kohli Y, Kato T, Murakita H, Ohotaki Y, Hirai M, et al. Differences in urease activity in live *Helicobacter pylori* cultured from patients with gastroduodenal diseases. Eur J Gastroenterol Hepatol 1995 Aug;7 Suppl 1:S83-8.

Jackman RP, Schlichting C, Carr W, Dubois A. Prevalence of *Helicobacter pylori* in United States Navy submarine crews. Epidemiol Infect 2006 Jun;134(3):460-464.

Jacobson K. The changing prevalence of *Helicobacter pylori* infection in Canadian children: should screening be performed in high-risk children? Can J Gastroenterol 2005 Jul;19(7):412-414.

Jafari F, Shokrzadeh L, Dabiri H, Baghaei K, Yamaoka Y, Zojaji H, et al. *vacA* genotypes of *Helicobacter pylori* in relation to *cagA* status and clinical outcomes in Iranian populations. Jpn J Infect Dis 2008 Jul;61(4):290-293.

Jain AK, Jain SK. Development and characterization of nanolipobeads-based dual drug delivery system for *H. pylori* targeting. J Drug Target 2013 Jul;21(6):593-603.

Jain P, Jain S, Prasad KN, Jain SK, Vyas SP. Polyelectrolyte coated multilayered liposomes (nanocapsules) for the treatment of *Helicobacter pylori* infection. Mol Pharm 2009 Mar-Apr;6(2):593-603.

Jeon B, Zhang Q. Sensitization of *Campylobacter jejuni* to fluoroquinolone and macrolide antibiotics by antisense inhibition of the CmeABC multidrug efflux transporter. J Antimicrob Chemother 2009 May;63(5):946-948.

Jones KR, Jang S, Chang JY, Kim J, Chung IS, Olsen CH, et al. Polymorphisms in the intermediate region of VacA impact *Helicobacter pylori*-induced disease development. J Clin Microbiol 2011 Jan;49(1):101-110.

Jones KR, Joo YM, Jang S, Yoo YJ, Lee HS, Chung IS, et al. Polymorphism in the CagA EPIYA motif impacts development of gastric cancer. J Clin Microbiol 2009 Apr;47(4):959-968.

Jones KR, Whitmire JM, Merrell DS. A Tale of Two Toxins: *Helicobacter pylori* CagA and VacA Modulate Host Pathways that Impact Disease. Front Microbiol 2010 Nov 23;1:115.

Judd LM, Alderman BM, Howlett M, Shulkes A, Dow C, Moverley J, et al. Gastric cancer development in mice lacking the SHP2 binding site on the IL-6 family co-receptor gp130. Gastroenterology 2004 Jan;126(1):196-207.

Jung SW, Thamphiwatana S, Zhang L, Obonyo M. Mechanism of antibacterial activity of liposomal linolenic acid against *Helicobacter pylori*. PLoS One 2015 Mar 20;10(3):e0116519.

Kadayifci A, Buyukhatipoglu H, Cemil Savas M, Simsek I. Eradication of *Helicobacter pylori* with triple therapy: an epidemiologic analysis of trends in Turkey over 10 years. Clin Ther 2006 Nov;28(11):1960-1966.

Kaijser B, Falen EMarshall BJ, Barrett LJ, Prakash C, et al. Protection of *Campylobacter pyloridis* (CP) but not *Campylobacter jejuni* (CJ) against acid susceptibility by urea. In: Kaijser B, Falen E, eds. Campylobacter IV. Proceedings of the 4th International Workshop on Campylobacter Infections. Goteborg: University of Goteborg, 1988:402–3.

Kanazawa A, Ikeda T, Endo T. A novel approach to mode of action of cationic biocides: morphological effect on antibacterial activity. J Appl Bacteriol 1995 Jan;78(1):55-60.

Karmali PP, Chaudhuri A. Cationic liposomes as non-viral carriers of gene medicines: resolved issues, open questions, and future promises. Med Res Rev 2007 Sep;27(5):696-722.

Kawata Y, Yano S, Kojima H. *Escherichia coli* can be transformed by a liposome-mediated lipofection method. Biosci Biotechnol Biochem 2003 May;67(5):1179-1181.

Kelly C, Jefferies C, Cryan SA. Targeted liposomal drug delivery to monocytes and macrophages. J Drug Deliv 2011;2011:727241.

Kent C. Eukaryotic phospholipid biosynthesis. Annu Rev Biochem 1995;64:315-343.

Kersulyte D, Bertoli MT, Tamma S, Keelan M, Munday R, Geary J, et al. Complete Genome Sequences of Two *Helicobacter pylori* Strains from a Canadian Arctic Aboriginal Community. Genome Announc 2015 Apr 16;3(2):10.1128/genomeA.00209-15.

Kidd M, Lastovica AJ, Atherton JC, Louw JA. Heterogeneity in the *Helicobacter pylori vacA* and *cagA* genes: association with gastroduodenal disease in South Africa? Gut 1999 Oct;45(4):499-502.

Kim JS, Ji JS, Choi H, Kim JH. Sequential therapy or triple therapy for *Helicobacter pylori* infection in Asians: systematic review and meta-analysis. Clin Res Hepatol Gastroenterol 2014 Feb;38(1):118-125.

Koch M, Meyer TF, Moss SF. Inflammation, immunity, vaccines for *Helicobacter pylori* infection. Helicobacter 2013 Sep;18 Suppl 1:18-23.

Koletzko S, Richy F, Bontems P, Crone J, Kalach N, Monteiro ML, et al. Prospective multicentre study on antibiotic resistance of *Helicobacter pylori* strains obtained from children living in Europe. Gut 2006 Dec;55(12):1711-1716.

Konturek JW. Discovery by Jaworski of *Helicobacter pylori* and its pathogenetic role in peptic ulcer, gastritis and gastric cancer. J Physiol Pharmacol 2003 Dec;54 Suppl 3:23-41.

Korkmaz H, Kesli R, Karabagli P, Terzi Y. Comparison of the diagnostic accuracy of five different stool antigen tests for the diagnosis of *Helicobacter pylori* infection. Helicobacter 2013 Oct;18(5):384-391.

Kosikowska P, Berlicki L. Urease inhibitors as potential drugs for gastric and urinary tract infections: a patent review. Expert Opin Ther Pat 2011 Apr 4.

Krebes J, Didelot X, Kennemann L, Suerbaum S. Bidirectional genomic exchange between *Helicobacter pylori* strains from a family in Coventry, United Kingdom. Int J Med Microbiol 2014 Nov;304(8):1135-1146.

Kuipers EJ, Israel DA, Kusters JG, Blaser MJ. Evidence for a conjugation-like mechanism of DNA transfer in *Helicobacter pylori*. J Bacteriol 1998 Jun;180(11):2901-2905.

Kuipers EJ, Thijs JC, Festen HP. The prevalence of *Helicobacter pylori* in peptic ulcer disease. Aliment Pharmacol Ther 1995;9 Suppl 2:59-69.

Kuipers EJ, Uyterlinde AM, Pena AS, Hazenberg HJ, Bloemena E, Lindeman J, et al. Increase of *Helicobacter pylori*-associated corpus gastritis during acid suppressive therapy: implications for long-term safety. Am J Gastroenterol 1995 Sep;90(9):1401-1406.

Kuipers EJ, Uyterlinde AM, Pena AS, Roosendaal R, Pals G, Nelis GF, et al. Long-term sequelae of *Helicobacter pylori* gastritis. Lancet 1995 Jun 17;345(8964):1525-1528.

Kuipers EJ. *Helicobacter pylori* and the risk and management of associated diseases: gastritis, ulcer disease, atrophic gastritis and gastric cancer. Aliment Pharmacol Ther 1997 Apr;11 Suppl 1:71-88.

Kusters JG, van Vliet AH, Kuipers EJ. Pathogenesis of *Helicobacter pylori* infection. Clin Microbiol Rev 2006 Jul;19(3):449-490.

Kuwahara H, Miyamoto Y, Akaike T, Kubota T, Sawa T, Okamoto S, et al. *Helicobacter pylori* urease suppresses bactericidal activity of peroxynitrite via carbon dioxide production. Infect Immun 2000 Aug;68(8):4378-4383.

Ladewig K, Niebert M, Xu ZP, Gray PP, Lu GQ. Efficient siRNA delivery to mammalian cells using layered double hydroxide nanoparticles. Biomaterials 2010 Mar;31(7):1821-1829.

Lahaie R, Farley A, Dallaire C, Archambault A, Fallone CA, Ponich T, et al. Bismuth-based quadruple therapy with bismuth subcitrate, metronidazole, tetracycline and omeprazole in the eradication of *Helicobacter pylori*. Can J Gastroenterol 2001 Sep;15(9):581-585.

Laheij RJ, Rossum LG, Jansen JB, Straatman H, Verbeek AL. Evaluation of treatment regimens to cure *Helicobacter pylori* infection--a meta-analysis. Aliment Pharmacol Ther 1999 Jul;13(7):857-864.

Lasch J., Weissig V. and Brandle M. Preparation of liposomes. In: Torchilin V. P., Weissig V., editor. Liposomes. second ed. UK: Oxford University Press; 2003. p. 3.

Lawrence BP, Brown WJ. Autophagic vacuoles rapidly fuse with pre-existing lysosomes in cultured hepatocytes. J Cell Sci 1992 Jul;102 (Pt 3)(Pt 3):515-526.

Layek B, Mukherjee B. Tamoxifen citrate encapsulated sustained release liposomes: preparation and evaluation of physicochemical properties. Sci Pharm 2010;78(3):507-515.

Leal YA, Flores LL, Fuentes-Panana EM, Cedillo-Rivera R, Torres J. 13C-urea breath test for the diagnosis of *Helicobacter pylori* infection in children: a systematic review and meta-analysis. Helicobacter 2011 Aug;16(4):327-337.

Life technologies. http://www.appliedbiosystems.com/absite/us/en/home.html. Accessed 9/2011.

Linke S, Lenz J, Gemein S, Exner M, Gebel J. Detection of *Helicobacter pylori* in biofilms by real-time PCR. Int J Hyg Environ Health 2010 Jun;213(3):176-182.

Lu Y, Redlinger TE, Avitia R, Galindo A, Goodman K. Isolation and genotyping of *Helicobacter pylori* from untreated municipal wastewater. Appl Environ Microbiol 2002 Mar;68(3):1436-1439.

Luzza F, Mancuso M, Imeneo M, Contaldo A, Giancotti L, Pensabene L, et al. Evidence favouring the gastro-oral route in the transmission of *Helicobacter pylori* infection in children. Eur J Gastroenterol Hepatol 2000 Jun;12(6):623-627.

Ma B, Simala-Grant JL, Taylor DE. Fucosylation in prokaryotes and eukaryotes. Glycobiology 2006 Dec;16(12):158R-184R.

MacFaddin JF. Biochemical tests for identification of medical bacteria. 3rd ed. ed. Philadelphia, PA: Lippincott Williams & Wilkins; 2000.

Machida-Montani A, Sasazuki S, Inoue M, Natsukawa S, Shaura K, Koizumi Y, et al. Association of *Helicobacter pylori* infection and environmental factors in non-cardia gastric cancer in Japan. Gastric Cancer 2004;7(1):46-53.

Maeda S, Ogura K, Yoshida H, Kanai F, Ikenoue T, Kato N, et al. Major virulence factors, VacA and CagA, are commonly positive in *Helicobacter pylori* isolates in Japan. Gut 1998 Mar;42(3):338-343.

Mahdavi J, Sonden B, Hurtig M, Olfat FO, Forsberg L, Roche N, et al. *Helicobacter pylori* SabA adhesin in persistent infection and chronic inflammation. Science 2002 Jul 26;297(5581):573-578.

Malaty HM, Paykov V, Bykova O, Ross A, Graham DP, Anneger JF, et al. *Helicobacter pylori* and socioeconomic factors in Russia. Helicobacter 1996 Jun;1(2):82-87.

Malfertheiner P, Bazzoli F, Delchier JC, Celinski K, Giguere M, Riviere M, et al. *Helicobacter pylori* eradication with a capsule containing bismuth subcitrate potassium, metronidazole, and tetracycline given with omeprazole versus clarithromycin-based triple therapy: a randomised, open-label, non-inferiority, phase 3 trial. Lancet 2011 Mar 12;377(9769):905-913.

Malfertheiner P, Megraud F, O'Morain CA, Atherton J, Axon AT, Bazzoli F, et al. Management of *Helicobacter pylori* infection--the Maastricht IV/ Florence Consensus Report. Gut 2012 May;61(5):646-664.

Malfertheiner P, Schultze V, Rosenkranz B, Kaufmann SH, Ulrichs T, Novicki D, et al. Safety and immunogenicity of an intramuscular *Helicobacter pylori* vaccine in noninfected volunteers: a phase I study. Gastroenterology 2008 Sep;135(3):787-795.

Manconi M, Isola R, Falchi AM, Sinico C, Fadda AM. Intracellular distribution of fluorescent probes delivered by vesicles of different lipidic composition. Colloids Surf B Biointerfaces 2007 Jun 15;57(2):143-151.

Mapstone NP, Lynch DA, Lewis FA, Axon AT, Tompkins DS, Dixon MF, et al. PCR identification of *Helicobacter pylori* in faeces from gastritis patients. Lancet 1993 Feb 13;341(8842):447.

Marcus EA, Inatomi N, Nagami GT, Sachs G, Scott DR. The effects of varying acidity on *Helicobacter pylori* growth and the bactericidal efficacy of ampicillin. Aliment Pharmacol Ther 2012 Nov;36(10):972-979.

Marshall BJ, Armstrong JA, McGechie DB, Glancy RJ. Attempt to fulfil Koch's postulates for pyloric *Campylobacter*. Med J Aust 1985 Apr 15;142(8):436-439.

Marshall BJ, Barrett LJ, Prakash C, McCallum RW, Guerrant RL. Urea protects Helicobacter (Campylobacter) pylori from the bactericidal effect of acid. Gastroenterology 1990 Sep;99(3):697-702.

Marshall BJ, Warren JR. Unidentified curved bacilli in the stomach of patients with gastritis and peptic ulceration. Lancet 1984 Jun 16;1(8390):1311-1315.

Marshall BJ. One Hundred Years of Discovery and Rediscovery of *Helicobacter pylori* and Its Association with Peptic Ulcer Disease. In: Mobley HLT, Mendz GL, Hazell SL, editors. *Helicobacter pylori*: Physiology and Genetics Washington (DC): ASM Press; 2001.

Maruyama K. PEG-immunoliposome. Biosci Rep 2002 Apr;22(2):251-266.

Masek T, Vopalensky V, Suchomelova P, Pospisek M. Denaturing RNA electrophoresis in TAE agarose gels. Anal Biochem 2005 Jan 1;336(1):46-50.

Matos JI, de Sousa HA, Marcos-Pinto R, Dinis-Ribeiro M. *Helicobacter pylori* CagA and VacA genotypes and gastric phenotype: a meta-analysis. Eur J Gastroenterol Hepatol 2013 Dec;25(12):1431-1441.

Mattar R, dos Santos AF, Eisig JN, Rodrigues TN, Silva FM, Lupinacci RM, et al. No correlation of *babA2* with *vacA* and *cagA* genotypes of *Helicobacter pylori* and grading of gastritis from peptic ulcer disease patients in Brazil. Helicobacter 2005 Dec;10(6):601-608.

Matzke M, Matzke AJ, Kooter JM. RNA: guiding gene silencing. Science 2001 Aug 10;293(5532):1080-1083.

McClain MS, Cao P, Iwamoto H, Vinion-Dubiel AD, Szabo G, Shao Z, et al. A 12-amino-acid segment, present in type s2 but not type s1 *Helicobacter pylori* VacA proteins, abolishes cytotoxin activity and alters membrane channel formation. J Bacteriol 2001 Nov;183(22):6499-6508.

McColl KE, el-Omar E, Gillen D. Interactions between *H. pylori* infection, gastric acid secretion and anti-secretory therapy. Br Med Bull 1998;54(1):121-138.

McKeown I, Orr P, Macdonald S, Kabani A, Brown R, Coghlan G, et al. *Helicobacter pylori* in the Canadian arctic: seroprevalence and detection in community water samples. Am J Gastroenterol 1999 Jul;94(7):1823-1829.

Megraud F, Lehours P. *Helicobacter pylori* detection and antimicrobial susceptibility testing. Clin Microbiol Rev 2007 Apr;20(2):280-322.

Menchise V, De Simone G, Tedeschi T, Corradini R, Sforza S, Marchelli R, et al. Insights into peptide nucleic acid (PNA) structural features: the crystal structure of a D-lysine-based chiral PNA-DNA duplex. Proc Natl Acad Sci U S A 2003 Oct 14;100(21):12021-12026.

Meyer JM, Silliman NP, Wang W, Siepman NY, Sugg JE, Morris D, et al. Risk factors for *Helicobacter pylori* resistance in the United States: the surveillance of *H. pylori* antimicrobial resistance partnership (SHARP) study, 1993-1999. Ann Intern Med 2002 Jan 1;136(1):13-24.

Mhaskar RS, Ricardo I, Azliyati A, Laxminarayan R, Amol B, Santosh W, et al. Assessment of risk factors of *Helicobacter pylori* infection and peptic ulcer disease. J Glob Infect Dis 2013 Apr;5(2):60-67.

Michetti P, Corthesy-Theulaz I, Davin C, Haas R, Vaney AC, Heitz M, et al. Immunization of BALB/c mice against Helicobacter felis infection with *Helicobacter pylori* urease. Gastroenterology 1994 Oct;107(4):1002-1011.

Miehlke S, Kirsch C, Agha-Amiri K, Gunther T, Lehn N, Malfertheiner P, et al. The *Helicobacter pylori vacA s1, m1* genotype and *cagA* is associated with gastric carcinoma in Germany. Int J Cancer 2000 Aug 1;87(3):322-327.

Miernyk K, Morris J, Bruden D, McMahon B, Hurlburt D, Sacco F, et al. Characterization of *Helicobacter pylori cagA* and *vacA* genotypes among Alaskans and their correlation with clinical disease. J Clin Microbiol 2011 Sep;49(9):3114-3121.

Miller JF, Dower WJ, Tompkins LS. High-voltage electroporation of bacteria: genetic transformation of *Campylobacter jejuni* with plasmid DNA. Proc Natl Acad Sci U S A 1988 Feb;85(3):856-860.

Miyaji H, Azuma T, Ito S, Abe Y, Gejyo F, Hashimoto N, et al. *Helicobacter pylori* infection occurs via close contact with infected individuals in early childhood. J Gastroenterol Hepatol 2000 Mar;15(3):257-262.

Mizushima T, Sugiyama T, Komatsu Y, Ishizuka J, Kato M, Asaka M. Clinical relevance of the *babA2* genotype of *Helicobacter pylori* in Japanese clinical isolates. J Clin Microbiol 2001 Jul;39(7):2463-2465.

Mobley HL, Cortesia MJ, Rosenthal LE, Jones BD. Characterization of urease from Campylobacter pylori. J Clin Microbiol 1988 May;26(5):831-836.

Mobley HL. The role of *Helicobacter pylori* urease in the pathogenesis of gastritis and peptic ulceration. Aliment Pharmacol Ther 1996 Apr;10 Suppl 1:57-64.

Mobley HLT. Urease. In: Mobley HLT, Mendz GL, Hazell SL, editors. *Helicobacter pylori*: Physiology and Genetics Washington (DC): ASM Press; 2001.

Mohammad MA, Hussein L, Coward A, Jackson SJ. Prevalence of *Helicobacter pylori* infection among Egyptian children: impact of social background and effect on growth. Public Health Nutr 2008 Mar;11(3):230-236.

Mori J, Vranac T, Smrekar B, Cernilec M, Serbec VC, Horvat S, et al. Chimeric flagellin as the self-adjuvanting antigen for the activation of immune response against *Helicobacter pylori*. Vaccine 2012 Aug 31;30(40):5856-5863.

Morris A, Ali MR, Brown P, Lane M, Patton K. Campylobacter pylori infection in biopsy specimens of gastric antrum: laboratory diagnosis and estimation of sampling error. J Clin Pathol 1989 Jul;42(7):727-732.

Morris A, Nicholson G. Ingestion of Campylobacter pyloridis causes gastritis and raised fasting gastric pH. Am J Gastroenterol 1987 Mar;82(3):192-199.

Morse AL, Goodman KJ, Munday R, Chang HJ, Morse JW, Keelan M, et al. A randomized controlled trial comparing sequential with triple therapy for *Helicobacter pylori* in an Aboriginal community in the Canadian North. Can J Gastroenterol 2013 Dec;27(12):701-706.

Moss SF, Moise L, Lee DS, Kim W, Zhang S, Lee J, et al. HelicoVax: epitope-based therapeutic *Helicobacter pylori* vaccination in a mouse model. Vaccine 2011 Mar 3;29(11):2085-2091.

Mueller D, Tegtmeyer N, Brandt S, Yamaoka Y, De Poire E, Sgouras D, et al. c-Src and c-Abl kinases control hierarchic phosphorylation and function of the CagA effector protein in Western and East Asian *Helicobacter pylori* strains. J Clin Invest 2012 Apr 2;122(4):1553-1566.

Mufamadi MS, Pillay V, Choonara YE, Du Toit LC, Modi G, Naidoo D, et al. A review on composite liposomal technologies for specialized drug delivery. J Drug Deliv 2011;2011:939851.

Naja F, Kreiger N, Sullivan T. *Helicobacter pylori* infection in Ontario: prevalence and risk factors. Can J Gastroenterol 2007 Aug;21(8):501-506.

Nakao M, Malfertheiner P. Growth inhibitory and bactericidal activities of lansoprazole compared with those of omeprazole and pantoprazole against *Helicobacter pylori*. Helicobacter 1998 Mar;3(1):21-27.

Nakashima N, Tamura T. Gene silencing in *Escherichia coli* using antisense RNAs expressed from doxycycline-inducible vectors. Lett Appl Microbiol 2013 Mar 11.

Nasa M, Choksey A, Phadke A, Sawant P. Sequential therapy versus standard triple-drug therapy for *Helicobacter pylori* eradication: a randomized study. Indian J Gastroenterol 2013 Nov;32(6):392-396.

Nell S, Kennemann L, Schwarz S, Josenhans C, Suerbaum S. Dynamics of Lewis b binding and sequence variation of the babA adhesin gene during chronic *Helicobacter pylori* infection in humans. MBio 2014 Dec 16;5(6):10.1128/mBio.02281-14.

Nicolau C, Le Pape A, Soriano P, Fargette F, Muh JP, Juhel MF. In vivo transfer and expression of the liposome encapsulated rat insulin gene. Prog Clin Biol Res 1982;102 pt A:321-330.

Nii T, Ishii F. Encapsulation efficiency of water-soluble and insoluble drugs in liposomes prepared by the microencapsulation vesicle method. Int J Pharm 2005 Jul 14;298(1):198-205.

Nilsson CL, Larsson T, Gustafsson E, Karlsson KA, Davidsson P. Identification of protein vaccine candidates from *Helicobacter pylori* using a preparative two-dimensional electrophoretic procedure and mass spectrometry. Anal Chem 2000 May 1;72(9):2148-2153.

Northwest Territories Bureau of Statistics. 2014; Available at: http://www.statsnwt.ca/community-data/infrastructure/aklavik.html. Accessed 10/6, 2014.

Obonyo M, Zhang L, Thamphiwatana S, Pornpattananangkul D, Fu V, Zhang L. Antibacterial activities of liposomal linolenic acids against antibiotic-resistant *Helicobacter pylori*. Mol Pharm 2012 Sep 4;9(9):2677-2685.

O'connor A, Taneike I, Nami A, Fitzgerald N, Murphy P, Ryan B, et al. *Helicobacter pylori* resistance to metronidazole and clarithromycin in Ireland. Eur J Gastroenterol Hepatol 2010 Sep;22(9):1123-1127.

Odenbreit S, Till M, Hofreuter D, Faller G, Haas R. Genetic and functional characterization of the alpAB gene locus essential for the adhesion of *Helicobacter pylori* to human gastric tissue. Mol Microbiol 1999 Mar;31(5):1537-1548.

Ogiwara H, Graham DY, Yamaoka Y. *vacA* i-region subtyping. Gastroenterology 2008 Apr;134(4):1267; author reply 1268.

Ogura K, Maeda S, Nakao M, Watanabe T, Tada M, Kyutoku T, et al. Virulence factors of *Helicobacter pylori* responsible for gastric diseases in Mongolian gerbil. J Exp Med 2000 Dec 4;192(11):1601-1610.

Okuda M, Miyashiro E, Booka M, Tsuji T, Nakazawa T. *Helicobacter pylori* colonization in the first 3 years of life in Japanese children. Helicobacter 2007 Aug;12(4):324-327.

Oliveira AG, Santos A, Guerra JB, Rocha GA, Rocha AM, Oliveira CA, et al. *babA2*- and *cagA*-positive *Helicobacter pylori* strains are associated with duodenal ulcer and gastric carcinoma in Brazil. J Clin Microbiol 2003 Aug;41(8):3964-3966.

Oliveira JG, Ferreira CH, Camerin AC, Rota CA, Meurer L, Silveira TR. Prevalence of infection with *cagA*-positive *Helicobacter pylori* strains among children and adolescents in southern Brazil. Arq Gastroenterol 2014 Sep;51(3):180-185.

Olson F, Hunt CA, Szoka FC, Vail WJ, Papahadjopoulos D. Preparation of liposomes of defined size distribution by extrusion through polycarbonate membranes. Biochim Biophys Acta 1979 Oct 19;557(1):9-23.

Onder G, Aydin A, Akarca U, Tekin F, Ozutemiz O, Ilter T. High *Helicobacter pylori* resistance rate to clarithromycin in Turkey. J Clin Gastroenterol 2007 Sep;41(8):747-750.

Oriol R, Mollicone R, Cailleau A, Balanzino L, Breton C. Divergent evolution of fucosyltransferase genes from vertebrates, invertebrates, and bacteria. Glycobiology 1999 Apr;9(4):323-334.

O'Rourke J, Bode G. Morphology and Ultrastructure. In: Mobley HLT, Mendz GL, Hazell SL, editors. *Helicobacter pylori*: Physiology and Genetics Washington (DC): ASM Press; 2001.

Orozco A, Ogura T, Hirosawa T, Garduno R, Kubo I. In hydrolyzed cow's milk *Helicobacter pylori* becomes nonculturable and the growth of *Salmonella typhi* and *Escherichia coli* is inhibited. J Food Sci 2007 Oct;72(8):M306-9.

O'Toole PW, Clyne M. Cell Envelope. In: Mobley HLT, Mendz GL, Hazell SL, editors. *Helicobacter pylori*: Physiology and Genetics Washington (DC): ASM Press; 2001.

Ottiwet O, Chomvarin C, Chaicumpar K, Namwat W, Mairiang P. Nested polymerase chain reaction for detection of *Helicobacter pylori* in gastric biopsy specimens. Southeast Asian J Trop Med Public Health 2010 Nov;41(6):1423-1431.

Ouattara B, Simard RE, Holley RA, Piette GJ, Begin A. Antibacterial activity of selected fatty acids and essential oils against six meat spoilage organisms. Int J Food Microbiol 1997 Jul 22;37(2-3):155-162.

Oudbier JH, Langenberg W, Rauws EA, Bruin-Mosch C. Genotypical variation of Campylobacter pylori from gastric mucosa. J Clin Microbiol 1990 Mar;28(3):559-565.

Ozaydin N, Turkyilmaz SA, Cali S. Prevalence and risk factors of *Helicobacter pylori* in Turkey: a nationally-representative, cross-sectional, screening with the (1)(3)C-Urea breath test. BMC Public Health 2013 Dec 21;13:1215-2458-13-1215.

Pagliaccia C, de Bernard M, Lupetti P, Ji X, Burroni D, Cover TL, et al. The m2 form of the *Helicobacter pylori* cytotoxin has cell type-specific vacuolating activity. Proc Natl Acad Sci U S A 1998 Aug 18;95(17):10212-10217.

Palframan SL, Kwok T, Gabriel K. Vacuolating cytotoxin A (VacA), a key toxin for *Helicobacter pylori* pathogenesis. Front Cell Infect Microbiol 2012;2:92.

Panayotopoulou EG, Sgouras DN, Papadakos K, Kalliaropoulos A, Papatheodoridis G, Mentis AF, et al. Strategy to characterize the number and type of repeating EPIYA phosphorylation motifs in the carboxyl terminus of CagA protein in *Helicobacter pylori* clinical isolates. J Clin Microbiol 2007 Feb;45(2):488-495.

Paniagua GL, Monroy E, Rodriguez R, Arroniz S, Rodriguez C, Cortes JL, et al. Frequency of *vacA*, *cagA* and *babA2* virulence markers in *Helicobacter pylori* strains isolated from Mexican patients with chronic gastritis. Ann Clin Microbiol Antimicrob 2009 Apr 30;8:14-0711-8-14.

Papenfort K, Vogel J. Regulatory RNA in bacterial pathogens. Cell Host Microbe 2010 Jul 22;8(1):116-127.

Parkinson AJ, Gold BD, Bulkow L, Wainwright RB, Swaminathan B, Khanna B, et al. High prevalence of *Helicobacter pylori* in the Alaska native population and association with low serum ferritin levels in young adults. Clin Diagn Lab Immunol 2000 Nov;7(6):885-888.

Patil SD, Sharma R, Srivastava S, Navani NK, Pathania R. Downregulation of *yidC* in *Escherichia coli* by Antisense RNA Expression Results in Sensitization to Antibacterial Essential Oils Eugenol and Carvacrol. PLoS One 2013;8(3):e57370.

Peck B, Ortkamp M, Diehl KD, Hundt E, Knapp B. Conservation, localization and expression of HopZ, a protein involved in adhesion of *Helicobacter pylori*. Nucleic Acids Res 1999 Aug 15;27(16):3325-3333.

Peek RM,Jr, Crabtree JE. Helicobacter infection and gastric neoplasia. J Pathol 2006 Jan;208(2):233-248.

Pelicic V, Jackson M, Reyrat JM, Jacobs WR,Jr, Gicquel B, Guilhot C. Efficient allelic exchange and transposon mutagenesis in Mycobacterium tuberculosis. Proc Natl Acad Sci U S A 1997 Sep 30;94(20):10955-10960.

Percival SL, Thomas JG. Transmission of *Helicobacter pylori* and the role of water and biofilms. J Water Health 2009 Sep;7(3):469-477.

Pereira WN, Ferraz MA, Zabaglia LM, de Labio RW, Orcini WA, Bianchi Ximenez JP, et al. Association among *H. pylori* virulence markers *dupA*, *cagA* and *vacA* in Brazilian patients. J Venom Anim Toxins Incl Trop Dis 2014 Jan 23;20(1):1.

Peretz A, Paritsky M, Nasser O, Brodsky D, Glyatman T, Segal S, et al. Resistance of *Helicobacter pylori* to tetracycline, amoxicillin, clarithromycin and metronidazole in Israeli children and adults. J Antibiot (Tokyo) 2014 Apr 30.

Perez-Perez GI, Blaser MJ. *Campylobacter* and *Helicobacter*. In: Baron S, editor. Medical Microbiology. 4th ed. Galveston (TX): The University of Texas Medical Branch at Galveston; 1996.

Pohl MA, Romero-Gallo J, Guruge JL, Tse DB, Gordon JI, Blaser MJ. Host-dependent Lewis (Le) antigen expression in *Helicobacter pylori* cells recovered from Leb-transgenic mice. J Exp Med 2009 Dec 21;206(13):3061-3072.

Pomorski T, Hrafnsdottir S, Devaux PF, van Meer G. Lipid distribution and transport across cellular membranes. Semin Cell Dev Biol 2001 Apr;12(2):139-148.

Posselt G, Backert S, Wessler S. The functional interplay of *Helicobacter pylori* factors with gastric epithelial cells induces a multi-step process in pathogenesis. Cell Commun Signal 2013 Oct 7;11:77-811X-11-77.

Qiu J, Wei XH, Geng F, Liu R, Zhang JW, Xu YH. Multivesicular liposome formulations for the sustained delivery of interferon alpha-2b. Acta Pharmacol Sin 2005 Nov;26(11):1395-1401.

Quaglia NC, Dambrosio A, Normanno G, Parisi A, Patrono R, Ranieri G, et al. High occurrence of *Helicobacter pylori* in raw goat, sheep and cow milk inferred by glmM gene: a risk of food-borne infection? Int J Food Microbiol 2008 May 10;124(1):43-47.

Rad R, Gerhard M, Lang R, Schoniger M, Rosch T, Schepp W, et al. The *Helicobacter pylori* blood group antigen-binding adhesin facilitates bacterial colonization and augments a nonspecific immune response. J Immunol 2002 Mar 15;168(6):3033-3041.

Radosz-Komoniewska H, Bek T, Jozwiak J, Martirosian G. Pathogenicity of *Helicobacter pylori* infection. Clin Microbiol Infect 2005 Aug;11(8):602-610.

Rajinikanth PS, Mishra B. Preparation and in vitro characterization of gellan based floating beads of acetohydroxamic acid for eradication of *H. pylori*. Acta Pharm 2007 Dec;57(4):413-427.

Reoch J. The Decomposition of Urea. J Anat Physiol 1875 May;9(Pt 2):368-385.

Reva I, Takano T, Higuchi W, Iwao Y, Taneike I, Nakagawa S, et al. Virulence genotypes and drug resistance of *Helicobacter pylori* from Vladivostok, Russia: another feature in the Far East. Microbiol Immunol 2012 Mar;56(3):198-202.

Rhead JL, Letley DP, Mohammadi M, Hussein N, Mohagheghi MA, Eshagh Hosseini M, et al. A new *Helicobacter pylori* vacuolating cytotoxin determinant, the intermediate region, is associated with gastric cancer. Gastroenterology 2007 Sep;133(3):926-936.

Ribeiro ML, Godoy AP, Benvengo YH, Mendonca S, Pedrazzoli J,Jr. Clinical relevance of the *cagA*, *vacA* and *iceA* genotypes of *Helicobacter pylori* in Brazilian clinical isolates. FEMS Immunol Med Microbiol 2003 May 25;36(3):181-185.

Rimbara E, Sasatsu M, Graham DY. PCR detection of *Helicobacter pylori* in clinical samples. Methods Mol Biol 2013;943:279-287.

Rocha A, Ruiz S, Coll JM. Improvement of DNA transfection with cationic liposomes. J Physiol Biochem 2002 Mar;58(1):45-56.

Rohrer S, Holsten L, Weiss E, Benghezal M, Fischer W, Haas R. Multiple pathways of plasmid DNA transfer in *Helicobacter pylori*. PLoS One 2012;7(9):e45623.

Rossi G, Ruggiero P, Peppoloni S, Pancotto L, Fortuna D, Lauretti L, et al. Therapeutic vaccination against *Helicobacter pylori* in the beagle dog experimental model: safety, immunogenicity, and efficacy. Infect Immun 2004 Jun;72(6):3252-3259.

Rothenbacher D, Winkler M, Gonser T, Adler G, Brenner H. Role of infected parents in transmission of *Helicobacter pylori* to their children. Pediatr Infect Dis J 2002 Jul;21(7):674-679.

Rudel T, Kepp O, Kozjak-Pavlovic V. Interactions between bacterial pathogens and mitochondrial cell death pathways. Nat Rev Microbiol 2010 Oct;8(10):693-705.

Sachs G, Scott DR, Weeks DL, Rektorscheck M, Melchers K. Regulation of Urease for Acid Habitation. In: Mobley HLT, Mendz GL, Hazell SL, editors. *Helicobacter pylori*: Physiology and Genetics Washington (DC): ASM Press; 2001.

Safaei HG, Rahimi E, Zandi A, Rashidipour A. *Helicobacter pylori* as a zoonotic infection: the detection of *H. pylori* antigens in the milk and faeces of cows. J Res Med Sci 2011 Feb;16(2):184-187.

Sahara S, Sugimoto M, Vilaichone RK, Mahachai V, Miyajima H, Furuta T, et al. Role of *Helicobacter pylori cagA* EPIYA motif and *vacA* genotypes for the development of gastrointestinal diseases in Southeast Asian countries: a meta-analysis. BMC Infect Dis 2012 Sep 21;12:223-2334-12-223.

Salama NR, Hartung ML, Muller A. Life in the human stomach: persistence strategies of the bacterial pathogen *Helicobacter pylori*. Nat Rev Microbiol 2013 Jun;11(6):385-399.

Salokangas, R. and Parlee, B. The influence of family history on learning opportunities of Inuvialuit youth. Erudit 2009;33:191.

Sarker SA, Mahalanabis D, Hildebrand P, Rahaman MM, Bardhan PK, Fuchs G, et al. *Helicobacter pylori*: prevalence, transmission, and serum pepsinogen II concentrations in children of a poor periurban community in Bangladesh. Clin Infect Dis 1997 Nov;25(5):990-995.

Schmilovitz-Weiss H, Sehayek-Shabat V, Eliakim R, Skapa E, Avni Y, Shirin H. Applicability of a short/rapid 13C-urea breath test for *Helicobacter pylori*: retrospective multicenter chart review study. BMC Gastroenterol 2012 Jan 19;12:8-230X-12-8.

Secka O, Antonio M, Berg DE, Tapgun M, Bottomley C, Thomas V, et al. Mixed infection with *cagA* positive and *cagA* negative strains of *Helicobacter pylori* lowers disease burden in The Gambia. PLoS One 2011;6(11):e27954.

Segal ED, Cha J, Lo J, Falkow S, Tompkins LS. Altered states: involvement of phosphorylated CagA in the induction of host cellular growth changes by *Helicobacter pylori*. Proc Natl Acad Sci U S A 1999 Dec 7;96(25):14559-14564.

Segal ED, Lange C, Covacci A, Tompkins LS, Falkow S. Induction of host signal transduction pathways by *Helicobacter pylori*. Proc Natl Acad Sci U S A 1997 Jul 8;94(14):7595-7599.

Segal ED, Tompkins LS. Transformation of *Helicobacter pylori* by electroporation. BioTechniques 1993 Feb;14(2):225-226.

Segal ED. Electroporation of *Helicobacter pylori*. Methods Mol Biol 1995;47:179-184.

Segal I, Otley A, Issenman R, Armstrong D, Espinosa V, Cawdron R, et al. Low prevalence of *Helicobacter pylori* infection in Canadian children: a cross-sectional analysis. Can J Gastroenterol 2008 May;22(5):485-489.

Senkovich OA, Yin J, Ekshyyan V, Conant C, Traylor J, Adegboyega P, et al. *Helicobacter pylori* AlpA and AlpB bind host laminin and influence gastric inflammation in gerbils. Infect Immun 2011 Aug;79(8):3106-3116.

Sethi A, Chaudhuri M, Kelly L, Hopman W. Prevalence of *Helicobacter pylori* in a First Nations population in northwestern Ontario. Can Fam Physician 2013 Apr;59(4):e182-7.

Seyda T, Derya C, Fusun A, Meliha K. The relationship of *Helicobacter pylori* positivity with age, sex, and ABO/Rhesus blood groups in patients with gastrointestinal complaints in Turkey. Helicobacter 2007 Jun;12(3):244-250.

Sgouros SN, Bergele C. Clinical outcome of patients with *Helicobacter pylori* infection: the bug, the host, or the environment? Postgrad Med J 2006 May;82(967):338-342.

Shashi K., Satinder K., and Bharat P. A complete review on: Liposomes. International Research Journal of Pharmacy 2012;3(7):10-16.

She RC, Wilson AR, Litwin CM. Evaluation of *Helicobacter pylori* Immunoglobulin G (IgG), IgA, and IgM serologic testing compared to stool antigen testing. Clin Vaccine Immunol 2009 Aug;16(8):1253-1255.

Sheu BS, Sheu SM, Yang HB, Huang AH, Wu JJ. Host gastric Lewis expression determines the bacterial density of *Helicobacter pylori* in *babA2* genopositive infection. Gut 2003 Jul;52(7):927-932.

Shi R, Xu S, Zhang H, Ding Y, Sun G, Huang X, et al. Prevalence and risk factors for *Helicobacter pylori* infection in Chinese populations. *Helicobacter* 2008 Apr;13(2):157-165.

Shiota S, Suzuki R, Yamaoka Y. The significance of virulence factors in *Helicobacter pylori*. J Dig Dis 2013 Jul;14(7):341-349.

Shokrzadeh L, Baghaei K, Yamaoka Y, Shiota S, Mirsattari D, Porhoseingholi, A.and Reza Zali, M. Prevalence of *Helicobacter pylori* infection in dyspeptic patients in Iran. Gastroenterology insights 2012;4(1).

Silva AL, Alexandrino F, Verissimo LM, Agnez-Lima LF, Egito LC, de Oliveira AG, et al. Physical factors affecting plasmid DNA compaction in stearylamine-containing nanoemulsions intended for gene delivery. Pharmaceuticals (Basel) 2012 Jun 18;5(6):643-654.

Simons K, Sampaio JL. Membrane organization and lipid rafts. Cold Spring Harb Perspect Biol 2011 Oct 1;3(10):a004697.

Singh DY, Prasad NK. Double liposomes mediated dual drug targeting for treatment of *Helicobacter pylori* infections. Pharmazie 2011 May;66(5):368-373.

Singh V, Trikha B, Nain CK, Singh K, Vaiphei K. Epidemiology of *Helicobacter pylori* and peptic ulcer in India. J Gastroenterol Hepatol 2002 Jun;17(6):659-665.

Smeets LC, Kusters JG. Natural transformation in *Helicobacter pylori*: DNA transport in an unexpected way. Trends Microbiol 2002 Apr;10(4):159-62; discussion 162.

Statistics Canada. Life expectancy, at birth at 65 years by privinces and territories. 2012; Available at: http://www.statcan.gc.ca/tables-tableaux/sum-som/l01/cst01/health72a-eng.htm. Accessed 10/16, 2014.

Steer HW, Colin-Jones DG. Mucosal changes in gastric ulceration and their response to carbenoxolone sodium. Gut 1975 Aug;16(8):590-597.

Steer HW. Ultrastructure of cell migration throught the gastric epithelium and its relationship to bacteria. J Clin Pathol 1975 Aug;28(8):639-646.

Stein CA, Subasinghe C, Shinozuka K, Cohen JS. Physicochemical properties of phosphorothioate oligodeoxynucleotides. Nucleic Acids Res 1988 Apr 25;16(8):3209-3221.

Stein M, Bagnoli F, Halenbeck R, Rappuoli R, Fantl WJ, Covacci A. c-Src/Lyn kinases activate *Helicobacter pylori* CagA through tyrosine phosphorylation of the EPIYA motifs. Mol Microbiol 2002 Feb;43(4):971-980.

Stein M, Rappuoli R, Covacci A. Tyrosine phosphorylation of the *Helicobacter pylori* CagA antigen after cag-driven host cell translocation. Proc Natl Acad Sci U S A 2000 Feb 1;97(3):1263-1268.

Stewart JC. Colorimetric determination of phospholipids with ammonium ferrothiocyanate. Anal Biochem 1980 May 1;104(1):10-14.

Stolte M, Meining A. The updated Sydney system: classification and grading of gastritis as the basis of diagnosis and treatment. Can J Gastroenterol 2001 Sep;15(9):591-598.

Storm, G. and Crommelin, D. J.A. Liposomes: quo vadis? research focus 1998;1(1):19.

Styer CM, Hansen LM, Cooke CL, Gundersen AM, Choi SS, Berg DE, et al. Expression of the BabA adhesin during experimental infection with *Helicobacter pylori*. Infect Immun 2010 Apr;78(4):1593-1600.

Suerbaum S, Michetti P. *Helicobacter pylori* infection. N Engl J Med 2002 Oct 10;347(15):1175-1186.

Suerbaum S, Smith JM, Bapumia K, Morelli G, Smith NH, Kunstmann E, et al. Free recombination within *Helicobacter pylori*. Proc Natl Acad Sci U S A 1998 Oct 13;95(21):12619-12624.

Suzuki M, Mimuro H, Kiga K, Fukumatsu M, Ishijima N, Morikawa H, et al. *Helicobacter pylori* CagA phosphorylation-independent function in epithelial proliferation and inflammation. Cell Host Microbe 2009 Jan 22;5(1):23-34.

Suzuki RB, Lopes RA, da Camara Lopes GA, Hung Ho T, Speranca MA. Low *Helicobacter pylori* primary resistance to clarithromycin in gastric biopsy specimens from dyspeptic patients of a city in the interior of Sao Paulo, Brazil. BMC Gastroenterol 2013 Dec 4;13:164-230X-13-164.

Tabernero J, Shapiro GI, LoRusso PM, Cervantes A, Schwartz GK, Weiss GJ, et al. First-in-humans trial of an RNA interference therapeutic targeting VEGF and KSP in cancer patients with liver involvement. Cancer Discov 2013 Apr;3(4):406-417.

Talebi Bezmin Abadi A, Taghvaei T, Mohabbati Mobarez A, Vaira G, Vaira D. High correlation of babA 2-positive strains of *Helicobacter pylori* with the presence of gastric cancer. Intern Emerg Med 2013 Sep;8(6):497-501.

Tegtmeyer N, Wessler S, Backert S. Role of the cag-pathogenicity island encoded type IV secretion system in *Helicobacter pylori* pathogenesis. FEBS J 2011 Apr;278(8):1190-1202.

Telmesani AM. *Helicobacter pylori*: prevalence and relationship with abdominal pain in school children in Makkah City, western Saudi Arabia. Saudi J Gastroenterol 2009 Apr;15(2):100-103.

Terradot L, Bayliss R, Oomen C, Leonard GA, Baron C, Waksman G. Structures of two core subunits of the bacterial type IV secretion system, VirB8 from Brucella suis and ComB10 from *Helicobacter pylori*. Proc Natl Acad Sci U S A 2005 Mar 22;102(12):4596-4601.

Thamphiwatana S, Gao W, Obonyo M, Zhang L. In vivo treatment of *Helicobacter pylori* infection with liposomal linolenic acid reduces colonization and ameliorates inflammation. Proc Natl Acad Sci U S A 2014 Dec 9;111(49):17600-17605.

Thomas JE, Gibson GR, Darboe MK, Dale A, Weaver LT. Isolation of *Helicobacter pylori* from human faeces. Lancet 1992 Nov 14;340(8829):1194-1195.

Tkachenko MA, Zhannat NZ, Erman LV, Blashenkova EL, Isachenko SV, Isachenko OB, et al. Dramatic changes in the prevalence of *Helicobacter pylori* infection during childhood: a 10-year follow-up study in Russia. J Pediatr Gastroenterol Nutr 2007 Oct;45(4):428-432.

Tomasini ML, Zanussi S, Sozzi M, Tedeschi R, Basaglia G, De Paoli P. Heterogeneity of cag genotypes in *Helicobacter pylori* isolates from human biopsy specimens. J Clin Microbiol 2003 Mar;41(3):976-980.

Tomatari, F. Haghi, A. Mohabbati Mobarez, M. Amini, D. Hosseini, and A. Talebi Bezmin Abadi. Resistance to Metronidazole and Clarithromycin in Dyspeptic Patients in Iran. Iranian Red Crescent Medical Journal 2010;12.

Torres J, Leal-Herrera Y, Perez-Perez G, Gomez A, Camorlinga-Ponce M, Cedillo-Rivera R, et al. A community-based seroepidemiologic study of *Helicobacter pylori* infection in Mexico. J Infect Dis 1998 Oct;178(4):1089-1094.

Torres J, Perez-Perez G, Goodman KJ, Atherton JC, Gold BD, Harris PR, et al. A comprehensive review of the natural history of *Helicobacter pylori* infection in children. Arch Med Res 2000 Sep-Oct;31(5):431-469.

Torres LE, Melian K, Moreno A, Alonso J, Sabatier CA, Hernandez M, et al. Prevalence of *vacA*, *cagA* and *babA2* genes in Cuban *Helicobacter pylori* isolates. World J Gastroenterol 2009 Jan 14;15(2):204-210.

Traikia M, Warschawski DE, Recouvreur M, Cartaud J, Devaux PF. Formation of unilamellar vesicles by repetitive freeze-thaw cycles: characterization by electron microscopy and 31P-nuclear magnetic resonance. Eur Biophys J 2000;29(3):184-195.

Tsuda M, Karita M, Morshed MG, Okita K, Nakazawa T. A urease-negative mutant of *Helicobacter pylori* constructed by allelic exchange mutagenesis lacks the ability to colonize the nude mouse stomach. Infect Immun 1994 Aug;62(8):3586-3589.

Tsuda M, Karita M, Nakazawa T. Genetic transformation in *Helicobacter pylori*. Microbiol Immunol 1993;37(1):85-89.

Tsujii M, Kawano S, Tsuji S, Fusamoto H, Kamada T, Sato N. Mechanism of gastric mucosal damage induced by ammonia. Gastroenterology 1992 Jun;102(6):1881-1888.

Tu Z. An improved system for competent cell preparation and high efficiency plasmid transformation using different *Escherichia coli* strains. Electronic Journal of Biotechnology 2005;8(1).

Tummuru MK, Cover TL, Blaser MJ. Cloning and expression of a high-molecular-mass major antigen of *Helicobacter pylori*: evidence of linkage to cytotoxin production. Infect Immun 1993 May;61(5):1799-1809.

Turner A, Crompton S, and Langlois S. Statistics Canada. 2013; . Accessed 10/3, 2013.

Ueda J, Gosho M, Inui Y, Matsuda T, Sakakibara M, Mabe K, et al. Prevalence of *Helicobacter pylori* infection by birth year and geographic area in Japan. Helicobacter 2014 Apr;19(2):105-110.

Umamaheshwari RB, Jain NK. Receptor-mediated targeting of lipobeads bearing acetohydroxamic acid for eradication of *Helicobacter pylori*. J Control Release 2004 Sep 14;99(1):27-40.

Vaira D, Zullo A, Hassan C, Fiorini G, Vakil N. Sequential Therapy for *Helicobacter pylori* Eradication: The Time is Now! Therap Adv Gastroenterol 2009 Nov;2(6):317-322.

Vale FF, Vitor JM. Transmission pathway of *Helicobacter pylori*: does food play a role in rural and urban areas? Int J Food Microbiol 2010 Mar 31;138(1-2):1-12.

Valle J, Kekki M, Sipponen P, Ihamaki T, Siurala M. Long-term course and consequences of *Helicobacter pylori* gastritis. Results of a 32-year follow-up study. Scand J Gastroenterol 1996 Jun;31(6):546-550.

van der Woude MW, Baumler AJ. Phase and antigenic variation in bacteria. Clin Microbiol Rev 2004 Jul;17(3):581-611, table of contents.

van Doorn LJ, Figueiredo C, Sanna R, Plaisier A, Schneeberger P, de Boer W, et al. Clinical relevance of the *cagA*, *vacA*, and *iceA* status of *Helicobacter pylori*. Gastroenterology 1998 Jul;115(1):58-66.

van Meer G, de Kroon AI. Lipid map of the mammalian cell. J Cell Sci 2011 Jan 1;124(Pt 1):5-8.

van Vliet AH, Wren BW. New levels of sophistication in the transcriptional landscape of bacteria. Genome Biol 2009;10(8):233.

Van Zanten SJ, Dixon MF, Lee A. The gastric transitional zones: neglected links between gastroduodenal pathology and *helicobacter* ecology. Gastroenterology 1999 May;116(5):1217-1229.

Vasu K, Nagaraja V. Diverse functions of restriction-modification systems in addition to cellular defense. Microbiol Mol Biol Rev 2013 Mar;77(1):53-72.

Vaucheret H, Fagard M. Transcriptional gene silencing in plants: targets, inducers and regulators. Trends Genet 2001 Jan;17(1):29-35.

Veldhuyzen van Zanten SJ, Pollak PT, Best LM, Bezanson GS, Marrie T. Increasing prevalence of *Helicobacter pylori* infection with age: continuous risk of infection in adults rather than cohort effect. J Infect Dis 1994 Feb;169(2):434-437.

Venerito M, Krieger T, Ecker T, Leandro G, Malfertheiner P. Meta-analysis of bismuth quadruple therapy versus clarithromycin triple therapy for empiric primary treatment of *Helicobacter pylori* infection. Digestion 2013;88(1):33-45.

Vieira DB, Carmona-Ribeiro AM. Cationic lipids and surfactants as antifungal agents: mode of action. J Antimicrob Chemother 2006 Oct;58(4):760-767.

Vincent P. Transmission and acquisition of *Helicobacter pylori* infection: evidences and hypothesis. Biomed Pharmacother 1995;49(1):11-18.

Voyageur, C.J. and Calliou, B. (2000) Various shades of red: Diversity within Canada's Indigenous community London Journal of Canadian Studies, 16, 109-124.

Wada A, Yamasaki E, Hirayama T. *Helicobacter pylori* vacuolating cytotoxin, VacA, is responsible for gastric ulceration. J Biochem 2004 Dec;136(6):741-746.

Walder J. Antisense DNA and RNA: progress and prospects. Genes Dev 1988 May;2(5):502-504.

Wang D, Jing NH, Lin QS. Stearylamine liposome as a new efficient reagent for DNA transfection of eukaryotic cells. Biochem Biophys Res Commun 1996 Sep 13;226(2):450-455.

Wang H, Meng J, Jia M, Ma X, He G, Yu J, et al. oprM as a new target for reversion of multidrug resistance in Pseudomonas aeruginosa by antisense phosphorothioate oligodeoxynucleotides. FEMS Immunol Med Microbiol 2010 Dec;60(3):275-282.

Wang J, Blanchard TG, Ernst PB. Host Inflammatory Response to Infection. In: Mobley HLT, Mendz GL, Hazell SL, editors. *Helicobacter pylori*: Physiology and Genetics Washington (DC): ASM Press; 2001.

Wang J, Wang WH, Li J, Liu FX. Celecoxib inhibits *Helicobacter pylori* colonization-related factors. World J Gastroenterol 2010 Feb 21;16(7):846-853.

Wang Y, Roos KP, Taylor DE. Transformation of *Helicobacter pylori* by chromosomal metronidazole resistance and by a plasmid with a selectable chloramphenicol resistance marker. J Gen Microbiol 1993 Oct;139(10):2485-2493.

Warren JR, Marshall BJ. Unidentified curved bacilli on gastric epithelium in active chronic gastritis. Lancet 1983 Jun 4;1(8336):1273-1275.

Watada M, Shiota S, Matsunari O, Suzuki R, Murakami K, Fujioka T, et al. Association between *Helicobacter pylori cagA*-related genes and clinical outcomes in Colombia and Japan. BMC Gastroenterol 2011 Dec 22;11:141-230X-11-141.

Weaver KE. Emerging plasmid-encoded antisense RNA regulated systems. Curr Opin Microbiol 2007 Apr;10(2):110-116.

Weel JF, van der Hulst RW, Gerrits Y, Roorda P, Feller M, Dankert J, et al. The interrelationship between cytotoxin-associated gene A, vacuolating cytotoxin, and *Helicobacter pylori*-related diseases. J Infect Dis 1996 May;173(5):1171-1175.

Wen Y, Feng J, Scott DR, Marcus EA, Sachs G. A cis-encoded antisense small RNA regulated by the HP0165-HP0166 two-component system controls expression of ureB in *Helicobacter pylori*. J Bacteriol 2011 Jan;193(1):40-51.

West AP, Millar MR, Tompkins DS. Effect of physical environment on survival of *Helicobacter pylori*. J Clin Pathol 1992 Mar;45(3):228-231.

Winter JA, Letley DP, Cook KW, Rhead JL, Zaitoun AA, Ingram RJ, et al. A role for the vacuolating cytotoxin, VacA, in colonization and *Helicobacter pylori*-induced metaplasia in the stomach. J Infect Dis 2014 Sep 15;210(6):954-963.

World gastroenterology organisation global guideline: *Helicobacter pylori* in developing countries. J Dig Dis 2011 Oct;12(5):319-326.

World Health Organization. 2013; Available at: www.who.int/topics/health_services_indigenous/en/. Accessed 10/3, 2013.

Xiang Z, Censini S, Bayeli PF, Telford JL, Figura N, Rappuoli R, et al. Analysis of expression of CagA and VacA virulence factors in 43 strains of *Helicobacter pylori* reveals that clinical isolates can be divided into two major types and that CagA is not necessary for expression of the vacuolating cytotoxin. Infect Immun 1995 Jan;63(1):94-98.

Yamaoka Y. Increasing evidence of the role of *Helicobacter pylori* SabA in the pathogenesis of gastroduodenal disease. J Infect Dev Ctries 2008 Jun 1;2(3):174-181.

Yamaoka Y. Mechanisms of disease: *Helicobacter pylori* virulence factors. Nat Rev Gastroenterol Hepatol 2010 Nov;7(11):629-641.

Yamaoka Y. Pathogenesis of *Helicobacter pylori*-Related Gastroduodenal Diseases from Molecular Epidemiological Studies. Gastroenterol Res Pract 2012;2012:371503.

Yamaoka Y. Roles of *Helicobacter pylori* BabA in gastroduodenal pathogenesis. World J Gastroenterol 2008 Jul 21;14(27):4265-4272.

Yanagihara K, Tashiro M, Fukuda Y, Ohno H, Higashiyama Y, Miyazaki Y, et al. Effects of short interfering RNA against methicillin-resistant *Staphylococcus aureus* coagulase in vitro and in vivo. J Antimicrob Chemother 2006 Jan;57(1):122-126.

Yang D, Pornpattananangkul D, Nakatsuji T, Chan M, Carson D, Huang CM, et al. The antimicrobial activity of liposomal lauric acids against Propionibacterium acnes. Biomaterials 2009 Oct;30(30):6035-6040.

Yim JY, Kim N, Choi SH, Kim YS, Cho KR, Kim SS, et al. Seroprevalence of *Helicobacter pylori* in South Korea. Helicobacter 2007 Aug;12(4):333-340.

Yin Y, He LH, Zhang JZ. Successful isolation of *Helicobacter pylori* after prolonged incubation from a patient with failed eradication therapy. World J Gastroenterol 2009 Mar 28;15(12):1528-1529.

Yu J, Leung WK, Go MY, Chan MC, To KF, Ng EK, et al. Relationship between *Helicobacter pylori babA2* status with gastric epithelial cell turnover and premalignant gastric lesions. Gut 2002 Oct;51(4):480-484.

Yucel T, Aygin D, Sen S, Yucel O. The prevalence of *Helicobacter pylori* and related factors among university students in Turkey. Jpn J Infect Dis 2008 May;61(3):179-183.

Zambon CF, Navaglia F, Basso D, Rugge M, Plebani M. *Helicobacter pylori babA2*, *cagA*, and *s1 vacA* genes work synergistically in causing intestinal metaplasia. J Clin Pathol 2003 Apr;56(4):287-291.

Zeng M, Mao XH, Li JX, Tong WD, Wang B, Zhang YJ, et al. Efficacy, safety, and immunogenicity of an oral recombinant *Helicobacter pylori* vaccine in children in China: a

randomised, double-blind, placebo-controlled, phase 3 trial. Lancet 2015 Oct 10;386(10002):1457-1464.

Zevit N, Levy I, Shmuely H, Samra Z, Yahav J. Antibiotic resistance of *Helicobacter pylori* in Israeli children. Scand J Gastroenterol 2010 May;45(5):550-555.

Zhang BB, Li Y, Liu XQ, Wang PJ, Yang B, Bian DL. Association between *vacA* genotypes and the risk of duodenal ulcer: a meta-analysis. Mol Biol Rep 2014 Nov;41(11):7241-7254.

Zhang C, Tang N, Liu X, Liang W, Xu W, Torchilin VP. siRNA-containing liposomes modified with polyarginine effectively silence the targeted gene. J Control Release 2006 May 15;112(2):229-239.

Zhang S, Moise L, Moss SF. *H. pylori* vaccines: why we still don't have any. Hum Vaccin 2011 Nov;7(11):1153-1157.

Zhang Y, Liu H, Zhou K. Lack of correlation of *vacA* genotype, *cagA* gene of *Helicobacter pylori* and their expression products with various gastroduodenal diseases. Chin Med J (Engl) 2001 Jul;114(7):703-706.

Zhang ZW, Dorrell N, Wren BW, Farthingt MJ. *Helicobacter pylori* adherence to gastric epithelial cells: a role for non-adhesin virulence genes. J Med Microbiol 2002 Jun;51(6):495-502.

Zhou L, Zhang J, Chen M, Hou X, Li Z, Song Z, et al. A comparative study of sequential therapy and standard triple therapy for *Helicobacter pylori* infection: a randomized multicenter trial. Am J Gastroenterol 2014 Apr;109(4):535-541.

Zhou X, Su J, Xu G, Zhang G. Accuracy of stool antigen test for the diagnosis of *Helicobacter pylori* infection in children: A meta-analysis. Clin Res Hepatol Gastroenterol 2014 Mar 10.

Zhou X. SHP-2 tyrosine phosphatase--the target of CagA. Trends Microbiol 2002 Apr;10(4):164.

Zhu Y, Zhou X, Wu J, Su J, Zhang G. Risk Factors and Prevalence of *Helicobacter pylori* Infection in Persistent High Incidence Area of Gastric Carcinoma in Yangzhong City. Gastroenterol Res Pract 2014;2014:481365.

Zou QH, Li RQ. *Helicobacter pylori* in the oral cavity and gastric mucosa: a meta-analysis. J Oral Pathol Med 2011 Apr;40(4):317-324.