PREVALENCE AND RISK FACTORS FOR *HELICOBACTER PYLORI* TRANSMISSION IN THE EASTERN CAPE PROVINCE: APPLICATION OF IMMUNOLOGICAL, MOLECULAR AND DEMOGRAPHIC METHODS.

By

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ABSTRACT

Helicobacter pylori (H. pylori) is a microaerophilic, Gram-negative motile curved rod that inhabits the gastric mucosa of the human stomach. The organism chronically infects billions of people worldwide and is one of the most genetically diverse of bacterial species. Infection with the organism potentially induces chronic gastritis and peptic ulcer disease. In addition, *H. pylori* plays a role in the etiology of gastric cancer and gastric MALT lymphoma. The risk of infection is increased in those living in the developing world, which has been ascribed to precarious hygiene standards, crowded households, and deficient sanitation common in this part of the world. Thus, the aim of this study was to identify the risk factors in the transmission of *H. pylori* in our environment, i.e. in Nkonkobe Municipality in the Eastern Cape Province, South Africa. Faecal samples were collected from 356 apparently healthy subjects, consisting of 168 males and 188 females aged from 3 months to ≥ 60 years (Mean = 31 years). A standardized questionnaire was applied, it described demographic characteristics including age, sex, household hygiene, socioeconomic status, area of residence, duration of stay in the area, sharing bath water, sharing tooth brush, habit of sucking thumb, medication currently being taken or medication taken within the past three months, source of water, type of toilet used, education and occupation. A sandwich-type enzyme immunoassay amplification technology (Amplified IDEIA™ Hp StAR™, Oxoid, UK) was used to analyze the faecal samples for the detection of *H. pylori* antigens using monoclonal antibodies specific for *H. pylori* antigens. To assess the possibility of faecal oral route with tap water as an intermediary link, PCR targeting the *ure*C (*glm*M), a highly conserved gene in *H. pylori*

was carried out to detect *H. pylori* DNA in faecal samples of already positive samples by HpSA test as well as in direct tap water used by the *H. pylori* positive subjects. QIAamp DNA stool mini kit was used to extract DNA from faecal samples. Tap water samples were then obtained using sterile bottles from areas inhabited by *H. pylori* positive subjects as determined by HpSA test and PCR. DNA extraction from water samples was done using UltraClean^{TM} Water DNA Isolation Kit (0.22µm) according to the manufacturer's instructions. PCR with primers specific for *H. pylori glm*M gene was carried out with both positive and negative controls incorporated. Fisher's exact test was used to assess the univariate association between *H. pylori* infection and the possible risk factors. Odds ratio (OR) and the corresponding 95% confidence interval (CI) were calculated to measure the strength of association using EPI INFO 3.41 package. P values of $< .05$ were required for significance. The precision rate of the diagnostic tests used was also determined. *H. pylori* antigen was detected in 316 of the 356 subjects giving an overall prevalence of 88.8%. Prevalence increased with age from 75.9% in children < 12 years age to 100% in the age group from 13 years to 24 years, also 100% prevalence of *H. pylori* was recorded in young adults aged 25-47 years and subjects aged ≥ 60 years ($P < .05$). *H. pylori* prevalence was higher in females than in males. Of 188 females who participated in the study, *H. pylori* antigen was detected in 172 (91.5%) versus 144 (85.7%) of 168 males ($P > .05$). Interestingly, *H pylori* antigen was detected more often (100%) in the high socioeconomic group than in those of low socioeconomic group (85.9%). Sixteen (66.7%) of twenty four faecal samples that had previously tested positive for the organism by HpSA test were confirmed positive by PCR. However none of the treated tap water samples tested positive for the organism by PCR. The present

study revealed a high prevalence of *H. pylori* in faecal samples of asymptomatic individuals in the Nkonkobe Municipality, an indication of active infection. The obtained results also revealed that direct treated tap water might not be playing a crucial role in the oral transmission of *H. pylori* in the studied population.

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LIST OF ABBREVIATIONS

BabA:	Lewis B Antigen Binding Adhesin
BHI:	Brain Heart Infusion.
BSA:	Bovine Serum Albumin.
CI:	Confidence Interval.
CSIR:	Council for Scientific and Industrial Research.
CWI:	Clean Water Index.
DNA:	Deoxyribonucleic Acid.
DWDS:	Drinking Water Distribution System.
ELISA:	Enzyme-linked Immunosorbent Assay.
FISH:	Fluorescent *in-situ* Hybridization
FN:	False Negative.
FP:	False Positive.
HpSA test:	*Helicobacter pylori* Stool Antigen Test.
HpSTAR:	*Helicobacter pylori* Rapid Stool Antigen Test.
IL:	interleukin.
iNOS:	Inducible Nitric Oxide Synthase.
MALT:	Mucosa Associated Lymphoid Tissue
MIC:	Minimum Inhibitory Concentration.
NFKB:	Nuclear Factor-kappa B.
NO:	Nitric Oxide.
NPV:	Negative Predictive Value.

OD:	Optical density
OR:	Odds Ratio
PCR:	Polymerase Chain Reaction.
PPIs:	Proton Pump Inhibitors.
PPV:	Positive Predictive Value.
RFLP:	Restriction Fragment Length Polymorphism.
SabA:	Sialic-acid Binding Adhesin
TLR2:	Toll-like receptor 2.
TLR5:	Toll-like receptor 5.
TN:	True Negative.
TP:	True Positive.
UBT:	Urea Breath Test.
UV:	Ultraviolet.
WHO:	World Health Organisation

DECLARATION

I declare that this thesis and the work contained herein is my original work and has not been presented to any other University for the award of any degree.

Name…….

Signature……

Supervisor's signature……

Date……

CHAPTER ONE

INTRODUCTION

1.1.0 BACKGROUND INFORMATION

Historically, scientists have fruitfully exploited microorganism for man's benefit. However some bacterial species have proved deadly to mankind, old and newly discovered bacterial pathogens are emerging with new forms of virulence and new patterns of resistance to antimicrobial agents of which *H. pylori* can not be disqualified. *H. pylori* chronically infect billions of people worldwide, reports have shown that its prevalence can reach up to 90% in developing nations. Infection with the bacterium causes chronic gastritis, peptic ulceration, gastric cancers and gastric mucosa associated lymphoid tissue (MALT) lymphoma (Warren *et al*., 1983; MacKay *et al*., 2003; Ahmed *et al*., 2007; Ceylan *et al*., 2007). *H. pylori* has been rated as a 'Class One' carcinogen by the World Health Organization (Peterson *et al*., 2000; Aguemon *et al*., 2005), the same category as cigarette smoking is to lung cancer.

It has been suggested that up to 95% of duodenal and 70% of gastric ulcers are attributable to *H. pylori* infection, and most cases occur in middle aged subjects (Rothenbacher, 2007), the highly productive age groups in societies. The increased risk of infection is especially high among those living in the developing world (Ahmed *et al.,* 2007). Infection can commence early in life (Aguemon *et*

al., 2000; Ndip *et al*., 2004; Steinberg *et al*., 2004; Ahmed *et al*., 2006) and endure lifelong if remedial actions are not administered.

The transmission pathways of *H. pylori* are still not clear. However, risk factors of transmission include precarious hygiene standards, over-crowding, and contaminated environment and water sources amongst others (Ndip *et al*., 2003). Oral-oral, faecal-oral, and person-to-person modes have been proposed as possible routes of transmission, either with or without transitional transmission steps during episodes of diarrhoea or gastro-oral contact in the event of vomiting. Previous serological studies have related a higher prevalence of antibodies against *H. pylori* in some professions (abattoir workers, shepherds and veterinary workers) to direct contact with *H. pylori* infected animals (Vaira *et al*., 1998; Papiez *et al*., 2003). Also, *H. pylori* has been isolated from the intestinal tract of dogs, cats and sheep (Dore *et al*., 2001). Research has also demonstrated that *H. pylori* can live for several days in milk and water in its infectious bacillary form and in river water for several months in a non-culturable but viable form (Sasaki *et al*., 1999; McKeown *et al*., 1999; Brown, 2000; Braganca *et al.*, 2007).

Although *H. pylori* are susceptible to a variety of antibiotics *in-vitro*, few antibiotics are currently being used to cure the infection. The limited choice of antibiotics coupled with the emergence of drug resistance especially metronidazole, amoxillin and clarythromycin (Arenz *et al*., 2006; Gerrits *et al.,* 2006; Aydin *et al*., 2007; Wolle, 2007), and the capacity for horizontal gene

exchange in *H. pylori* has substantially posed a challenge in the success of treatment regimes on a global scale.

1.2.0 PROBLEM STATEMENT AND HYPOTHESES

Ranked as a class one carcinogen (Peterson *et al*., 2000; Aguemon *et al*., 2005), *H. pylori* still continue to present itself as a serious health concern especially in the developing world. In an asymptomatic South African population studied (Fritz *et al*., 2006), *H. pylori* prevalence was observed to be 83.3% which is not significantly different from the 84% noted in another study conducted in Pretoria from asymptomatic subjects, with both cases PCR targeting the *glm*M gene of *H. pylori* being employed (Olivier *et al*., 2006). Infection with the bacterium causes chronic gastritis, peptic ulceration, gastric cancers and gastric MALT lymphoma.

Despite its clinical significance, there is still lack of consensus on the way this bacterium is transmitted (Konno *et al*., 2005; Braganca *et al*., 2007). The risk factors of transmission include poor socioeconomic status, precarious hygiene standards, over-crowding, and contaminated environment and water sources amongst others (Ndip *et al*., 2003). Nkonkobe Municipality is characterized by poor service delivery especially sanitation and water supply (Momba *et al*., 2006). There are prior studies that have highlighted the presence of *H. pylori* ranging from highly faecal polluted sewage water to tap water (Sasaki *et al*., 1999; Park *et al*., 2001; Brown *et al*., 2002; Fugimura *et al*., 2004; Queralt *et al*., 2005; Ahmed *et al.*, 2006; Ahmed *et al*., 2007; Braganca *et al*., 2007; Moreno *et al*., 2007). *H.*

pylori has the ability to maintain viability but in a nonculturable state in river water for several months in a coccoid form (Brown, 2000; Adams *et al.*, 2004; Fujimura *et al*., 2004). Culturability, has been recorded within 2 to 3 days of inhabiting natural waters (Gribbon *et al*., 1995; Adams *et al*., 2003). Biofilms also play a crucial role in maintaining the viability of *H. pylori* in water by providing localized environment suitable for survival during adverse conditions in water (MacKay *et al*., 1998). Therefore understanding the transmission route is a vital step towards controlling the spread of *H. pylori* and hence it's tribulations.

It is thus hypothesized that:

- The prevalence of *H. pylori* is high in the Nkonkobe Municipality.
- Household hygiene and water sources are risk factors for *H. pylori* transmission.

1.3.0 RESEARCH OBJECTIVES

1.3.1 Overall objective

- The overall objective of this study was to investigate the prevalence and risk factors for *H. pylori* transmission in the Nkonkobe Municipality.

1.3.2 Specific objectives:

To test our hypothesis we proposed the following specific objectives:

1. To detect *H. pylori* antigens in stool samples from asymptomatic subjects using HpSA test and hence determine the prevalence of the organism in the Nkonkobe Municipality.
2. To detect *H. pylori* DNA by PCR in faecal and water samples.
3. To correlate *H. pylori* antigenemia and DNA presence.
4. To determine household hygiene status in relation to *H. pylori* prevalence.
5. To ascertain the correlation between *H. pylori* prevalence and risk factors.

1.4.0 CHAPTER OVERVIEWS

The next chapter (Chapter 2) is composed of the literature review which has been extracted from two different publications (Dube *et al.,* 2009a; Dube *et al.,* 2009b). Chapter 3 is the methodology in which a detailed research design and methods are outlined to test the hypotheses proposed. Chapters 4 and 5 are composed of results and discussion respectively (part of which has been published, Dube *et al*., 2009c). The conclusion and proposed future studies form the last sections of Chapter 5.

CHAPTER TWO

LITERATURE REVIEW

2.1.0 INTRODUCTION

H. pylori, a cork-screw shaped, micro-aerophilic gram-negative coccobacillus (0.5µm wide by 2-4µm long), equipped with two to six flagella that are lophotrichously positioned, has chronically infected more than half of the world's population (Owen, 1998; Ndip *et al*., 2004; Ahmed *et al*., 2007).

The organism is one of the most genetically diverse of bacterial species. It was first successfully cultured in 1983 by Marshall and Warren from mucosal and stomach specimens of patients with gastritis (Warren *et al*., 1983). This genus *Helicobacter* was created in 1989 (Owen, 1998) and it belongs to the phylum *Proteobacteria*, class *Epsilon Proteobacteria*, order *Campylobacterales* and family *Helicobacteraceae*. Currently, this genus *Helicobacter* consists of over 20 recognized species; *H. pylori* and *H. felis* are the only species known to infect the human host with many species awaiting formal recognition (Fritz *et al*., 2006; Kusters *et al*., 2006).

Although most of the people harboring this organism are asymptomatic (Ndip *et al*., 2004), subsequent evidence has linked the bacterium in the pathogenesis and development of certain diseases such as gastric ulcers, chronic gastritis and stomach cancers (Konturek, 2003; MacKay *et al*., 2003; Braganca, 2007).

Colonisation of the human stomach by this organism usually occurs in the early stages of life (Ma *et al.,* 1998; Bassily *et al*., 1999), but persists lifelong in the absence of effective treatment. With the progress in research, intrinsic studies have sharpened consciousness in the clinical importance of *H. pylori*-linked diseases (Astrat *et al*., 2004; Ndip *et al*., 2004; Campbell *et al*., 2005; Farag *et al*., 2007). Since its discovery, overwhelming evidence has implicated it as an aetiologic agent of a spectrum of gastrointestinal diseases including gastric ulcers, chronic gastritis and stomach cancers (Holcombe *et al*., 1994; MacKay *et al*., 2003; Oleastro *et al*., 2006; Braganca, 2007).

Whilst a lot of development has been done to address transmission modes of *H. pylori*, its transmission pathways are still vague (Dowset *et al.*, 1999; MacKay *et al*., 2003; Fujimura *et al*., 2004; Deport and van der Merwe, 2007). Elucidation of the paramount role played by household hygiene and environmental factors in the transmission of bacterial pathogens is of great importance as a prophylactic move, both in developed and in developing countries. Several studies have highlighted the transmission incidence risk as ranging from simple to inevitable multifactor modes of transmission (Fugimura *et al.,* 2004; Konno *et al.,* 2004; Perry *et al*., 2006; van der Merwe *et al.,* 2007). Highly ostensible promoting features which are mostly associated with developing countries and lower socio-economic groups in the developed world include precarious hygiene standards, crowded households, deficient sanitation and contaminated environment and water sources (Ndip *et al*., 2004; Ahmed *et al*., 2007).

2.2.0 ECOLOGICAL NICHE OF *H. PYLORI*

The bug persists in an acidic gastric environment, typically provided by the corpus and antrum for the lifetime of the host. Also it can tolerate an alkaline environment with pH 9 being the limiting value (Jiang and Doyle, 1998). To mimic this environment, microbiologists have come up with marked differences of micro-aerobic atmospheres in a plausible task of culturing this pathogen (Ndip *et al*., 2003). Gas generation kit, a variable atmospheric incubator or an anaerobic jar refilled with 5–6% O_2, 10% CO_2, 80–85% N_2 gas mixture has been successfully used to mimic the required microaerophilic environments (Ndip *et al*., 2008). Media such as Columbia Agar Base or Brain Heart Infusion broth supplemented with blood or serum usually prove adequate for culture (Ndip *et al*., 2003). Research has also revealed that 99% of *H. pylori* cells can maintain respiratory activity for at least 250 days at 4°C as compared to loss of respiratory activity after 24 hours at 37°C (Gribbon *et al*., 1995).

2.3.0 SUCCESS OF *H. PYLORI* IN ITS ECOLOGICAL NICHE

Controversy still persists on the duration of the relationship between *H. pylori* and humans considering how this pathogen has adapted in having a complete life in the human stomach. However, co-evolution of *H. pylori* with humans over thousands of years has effectively refined the interactions that occur between bacterial and host effectors, transmission between hosts, survival during acidic stress within hosts, and avoidance of immune response (Blaser, 1997; Scott *et al*., 2007).

Gastric acidity and peristaltic muscle movement of the alimentary canal have the potential to preclude bacterial colonization of the human stomach. However, natural selection has enhanced this organism with some mechanisms to dodge these primary defences thereby initiating an infection (Peek, 2005). This organism makes use of a urea splitting enzyme (urease) to neutralise gastric acidity, as it traverse the gastric lumen (Campbell and Thomas, 2005). In addition to urea that can be bacterial derived or obtained from the host (Hoovey *et al.,* 2007), other enzymes such as catalase and oxidase are produced (Kusters *et al*., 2006). Locomotion and counteraction of peristalsis, another necessity for persistent infection is aided by the presence of two to six flagella (Brown, 2000). Urease activity and flagella secretion are coupled by the gene *FlbA* (Peek, 2005). Adherence is another requirement for prolonged colonisation of the stomach by *H. pylori* (Peek, 2005).

Inter-species and intra-species entero-coexistence has been highlighted in several studies, with competitive exclusion failing to take its toll (Gibson *et al.,* 1998; Nagorni, 2000; Akada *et al.,* 2003; Fritz *et al*., 2006; Samie *et al*., 2007). *H. pylori* has the capacity for horizontal gene exchange hence enabling genetic variability within the population. In addition, *H. pylori* show competency in the uptake of DNA from other *H. pylori* cells (Blaser and Atherton, 2004). Diversity of this organism can play an influential role in the survival of the population in its niche. Coexistence is enhanced by failure of competitive exclusion by *H. pylori* strains suggesting that these different strains occupy different gastro-mucosal

micro-niches (Akada *et al*., 2003). Flexibility or adaptability in this population, allows for maximised use of resources in a variety of niches (Blaser and Atherton, 2004). The size or availability of these gastro-mucosal micro-niches is affected by host genotype and age or physiology.

Inter-specific competition also influences survival of a population in a niche. Exposure of murine macrophages to pro-inflammatory cytokines or lipopolysaccharide (LPS) up-regulates inducible nitric oxide synthase (iNOS), which catalyses the conversion of L-arginine into nitric oxide (NO). The reaction between superoxide anions and NO results in a by product peroxynitrite, a strong oxidant and nitrating agent that is highly toxic to several enterogastric pathogens (Bryk *et al*., 2000; Allen, 2007).

2.4.0 PATHOGENESIS

2.4.1 Attachment in stomach of host

Infection by *H. pylori* is presumed to be from the gastric antrum and then extending down to the corpus after extensive mucosal damage (Akada *et al.*, 2003). Colonisation unavoidably stimulates nuclear factor-kappa B (NFKB) activation and interleukin-8 (IL-8) expression in gastric epithelial cells (Kim *et al.,* 2003). Toll-like receptor 2 (TLR2) and 5 (TLR5) recognize *H. pylori* and initiate signalling pathways that result in enhanced activation of NFKB; IL-8 is secreted by the host cells to attract components of the innate and adaptive immune systems to the site of infection. This polarises the immune response towards a Th1

response, further attracting inflammatory cells and T-lymphocytes. An effective CD4 +T-cell response is essential to clear *H. pylori*, however this organism has been shown to inhibit CD4+T-cell proliferation and arresting IL-2 cell-cycle progression resulting in avoidance of clearance thereby staging an infection (Gebert *et al.,* 2003; Sundrud *et al.,* 2004).

Initial infection by highly pathogenic strains possessing a cluster of genes known as the *cag* pathogenicity island result in altered expression of several genes associated with glycan biosynthesis especially β3GlcNAc T5, a GlcNAc transferase required for the biosynthesis of Lewis antigens (Marcos *et al*., 2008). Resultant over expression of β3GlcNAc T5 in human gastric carcinoma cell lines lead to increased sialyl–Lewis x expression, a specific kind of sugar molecule that these cells display on their surface as a flag to attract immune cells to the infection site (Nagorni, 2000; Bor-Shyang *et al.,* 2006; Marcos *et al*., 2008).

Among a number of adhesins, this organism uses bacterial adhesion protein called sialic-acid binding adhesin (SabA) to recognize a molecule associated with inflammation and a molecule known as Lewis B antigen binding adhesin (BabA) to adhere to the inflamed cells of the glandular lining (Mahdavi *et al*,. 2002; Bor-Shyang *et al.,* 2006; Baldwin *et al*., 2007). The ability of *H. pylori* to adjust its adherence properties to the level of inflammation it causes at the stomach surface could help explain how this bacterium maintains its persistence, decades-long infection in the stomach of millions worldwide.

2.4.2 Avoidance of the immune response of the host

Other than inhabiting superficial glycoprotein-rich mucosal niche meant to protect stomach cells from the secreted acids in the stomach cavity, a micro-distance from inflamed glandular cells (Mahdavi *et al.,* 2002; Delport and Merwe, 2007), the organism also avoids recognition by producing specific bacterial factors that stimulate selective expression of host genes and also by inducing an ineffective T-cell response. Genetic diversity of this organism also plays a paramount role in its persistence.

While the *rocF* gene is not essential for the initiation of an infection, it encodes arginase, an enzyme responsible for the hydrolysis of L-arginine to L-ornithine and urea. Reactive nitrogen intermediates are relatively ineffective against *H. pylori* (Bryk *et al*., 2000). Unavoidable, arginase allows the bacterium to evade host immune response by competing with macrophage iNOS for L-arginine. Due to bacterial cell deficiency in arginine synthesizing enzymes, this organism exploits the host's arginine to maintain the nitrogen balance (Hovey *et al*., 2007).

2.4.3 Ability to attain a viable but nonculturable state.

H. pylori has the ability to remain culturable in natural waters for 2 to 3 days at a low temperature (Gribbon *et al*., 1995; Adams *et al*., 2003). In prolonged adverse conditions, the organism can exist in all morphologies in a nonculturable state (Bode *et al*., 1993; Sorbeg *et al*., 1996; Atherton., 1997; Fujimura *et al*., 2004). The coccoid form induced by water is capable of colonizing the gastric mucosa

and causing gastritis in mice (Cellini *et al*., 1994; She *et al*., 2003), but whether *H. pylori* can revert from its coccoid to its infectious form in humans has not yet been determined (Owen., 1993). Nevertheless, the persistence of *H. pylori* cells in the environment in the viable but nonculturable state presents a public health hazard.

2.5.0 CLINICAL MANIFESTATION.

The organism is a major cause of upper gastrointestinal diseases such as gastritis, peptic ulcer and gastric cancer (Ahmed *et al*., 2007; Tanih *et al*., 2009). The primary disarray that follows initial colonization of the host is chronic active gastritis (Kusters *et al*., 2006). It has been suggested that up to 95% of duodenal and 70% of gastric ulcers are attributable to this infection and most cases occur in middle aged subjects (Rothenbacher, 2007). In the US, nearly all persons with duodenal ulcer are infected, and that persons without the infection will ever develop duodenal ulcer is highly unlikely. Although gastric ulcer is usually caused by these bacteria, about 30% of gastric ulcers in the US occur in persons without *H. pylori* and could be related to non steroidal anti-inflammatory drugs. Most gastric adenocarcinomas and lymphomas occur in persons with current or past infection with *H. pylori.*

The clinical outcome of long-term infection is variable and is considered to relate both to bacterial virulence factors (Gatta *et al*., 2003; Wang *et al.,* 2003; Monica *et al*., 2006) and host genotype. The vacuolating cytotoxin *VacA* (Ye *et al*., 2000)

and the *cag* pathogenicity island (Atherton., 1997, Bravo *et al*., 2002) are two identified virulence factors that are considered to have an important role in the pathogenesis of *H. pylori* infection. *VacA* gene comprises two variable regions, the s region which exists as an s1a, s1b, s1c, or s2 allele, and the m region, which occurs as an m1, m2a, or m2b allele (Figueiredo *et al*., 2000). *H. pylori VacA* type s1 strains appear to be more virulent than type s2 strains and are associated with higher risks for peptic ulcer disease, gastric atrophy, and gastric carcinoma (Figueiredo *et al*., 2000). The *VacA* s1 and *VacA* m1 strains are also strongly associated with a higher degree of inflammation and epithelial damage in the gastric mucosa. The intragastric distribution and severity of the chronic inflammatory process depends on a variety of factors, such as characteristics of the colonizing strain, host genetics and immune response, diet, and the level of acid production (Kusters *et al*., 2006).

Although the relation between *H. pylori* infection and gastric cancer has been well-established, some studies have shown a negative correlation of *H. pylori* infection and the development of gastric cancer in Africa. The high prevalence of infection, contrary with low rate of development into gastric cancer, well expressed as “African enigma”, is an ambiguity because the headway to atrophic gastritis in the African population does not differ from that reported in other regions (Segal *et al.,* 2001). The variants of the interferon-c gene (IFNGR1), which are more prevalent in Africans appear to play a significant role in the infection of human host contributing to a high prevalence even-though there is

relatively low pathogenesis of infection in Africa (Thye *et al*., 2003; Ndip *et al*., 2004).

2.6.0 EPIDEMIOLOGY OF *H. PYLORI* INFECTION.

Several studies have highlighted a high prevalence of this organism in the developing world including Africa (Bakka *et al*., 2002; Delport *et al*., 2006; Frenck *et al*., 2006; Fritz *et al.,* 2006; Mbulaiteye *et al*., 2006; Levin *et al.,* 2007; Ndip *et al.*, 2008; Dube *et al*., 2009c). Careful surveys have revealed that most persons in the developing world are infected with the bacterium with acquisition commencing at early stages of life. *H. pylori* infections have been documented in several studies done in developing countries, e.g. in Egypt, where the prevalence was estimated to be at 60% among patients greater than 6 years of age (Frenck *et al*., 2006). Recently, in a South African population studied by Fritz *et al*., (2006), *H. pylori* prevalence was observed to be 83.3% which is not significantly different to the 84% noted in another study conducted in Pretoria from asymptomatic individuals (Olivia *et al*., 2006). In a study population that included hospitalised patients conducted in Venda region, Polokwane, detection of *H. pylori* DNA in faecal samples by PCR revealed a 50.6% prevalence of the organism (Samie *et al*., 2006). However, on a separate study in Tanzania, seropositivity rose steeply with age from 76% in children aged 0-4 years to 99% in adults (Mbulaiteye *et al.*, 2006). Also, a similar trend was noted in Lybia where prevalence rose with age up to about 94% in age above 70 years (Bakka., 2002). In Cameroon, a high incidence of *H. pylori* was recorded in both asymptomatic and symptomatic

individuals using the HpSA technique (Ndip *et al.,* 2004). In Tunisia, high colonization rates have also been recorded among asymptomatic individuals (Ben-Ammar *et al.,* 2003). In a separate study, low socioeconomic level constituted as a main risk factor in asymptomatic Tunisian children (Maherzi *et al*., 2003).

Also of ambiguity, a serological study in Egypt revealed that increased education was significantly associated with an increased risk of infection among mothers (Bassily *et al*., 1999). However an improved hygiene-education programme could be necessary to adjust deep-rooted inherent behaviours in a plausible task to reduce microbial infections (Nala *et al.,* 2003).

In the Western countries, the pathogen generally affects about 20% of persons below the age of 40 years, 50% of those above 60 years, yet is uncommon in young children. The prevalence of *H. pylori* and the rate of infection are as well inversely related to socioeconomic status and sanitation (Bardhan, 1997; Kusters *et al*., 2006; Ahmed *et al*., 2007). In certain Western countries, immigration is responsible for isolated areas of high prevalence (http:www.helico.com/h_ epidemiology.html). However, prevalence of the pathogen correlates more with socioeconomic status rather than with ethnicity. In the U.S., the probability of being infected is greater for older persons (>50 years = >50%), minorities (African Americans 40-50%) and immigrants from developing countries (Latino

> 60%, Eastern Europeans > 50%). The infection is less common in more affluent Caucasians (< 40 years =20%).

2.7.0 RISK FACTORS AND TRANSMISSION OF *H. PYLORI*.

2.7.1 *H. pylori* in the environment

The microorganism cannot be cultured from drinking water distribution system (DWDS) using standard cultivation techniques, also cultivation from other environmental samples is difficult (Adams *et al*., 2003; Moreno *et al*., 2007). This limitation hinders conventional isolation and/or resource-limited laboratories from studying the epidemiology of *H. pylori* from environmental samples. Molecular and immunomagnetic separation, enzyme-linked immunoassay (ELISA), autoradiography, and FISH techniques (Horiuchi *et al*., 2001; Degnan *et al*., 2003; Braganca *et al*., 2007; Moreno *et al*., 2007; Giậo *et al*., 2008; Dube *et al*., 2009c) offer useful approaches for detecting the bacteria in such systems.

2.7.2 Plausible routes of transmission.

As one of the medical important bacterial pathogens, *H. pylori* transmission pathways are still vague (Konno *et al*., 2005; Braganca *et al*., 2007) and currently more than 50% of the world's population is infected. The prevalence of *H. pylori* infection and the rate of infection are inversely related to the standard of living and sanitary practice (Malaty *et al.,* 1998; Ahmed *et al*., 2007; Dube *et al*., 2009a). The risks of transmission include precarious hygiene standards, crowding, and contaminated environment and water sources (Bunn *et al*., 2002; Suerbaum

and Michetti, 2002). The possible routes of transmission include oral–oral and faecal–oral, either with or without transitional transmission steps (Vaira *et al.*, 2001; Ahmed *et al*., 2006) during episodes of diarrhea or gastro-oral contact in the event of vomiting (Deport and Merwe, 2007). Person to person transmission can be a plausible cause of infection (Brown *et al.,* 2002). Use of contaminated water including municipal tap water has also been suspected to have a high impact in the transmission of the organism (Ahmed *et al*., 2006). However, water purification, improved hygiene, reduced environmental contamination, immunization (vaccination) and antibiotic treatment have played an important role in reducing the morbidity and mortality of bacterial disease especially in the developed world where these are acceptable cultural practices.

2.7.3 Faecal contamination

In most infected persons, *H. pylori* can be cultured from the stool, providing evidence that spread by faecal-oral contact with infected persons is likely (Thomas *et al*., 1992; Dore *et al*., 2000). As the organism can survive for several days in water, water contamination with human excrement has the potential for transmission by the faecal-oral route (Sorbeg *et al*., 1996; Adams *et al*., 2003). In natural waters, the microorganism retains its spiral form and shows better culturability than when kept in a nutrient rich environment (Konishi *et al*., 2007). *H. pylori* cells remain culturable longer in cooler waters (<20^{o}C) than in warmer waters (>20^{o}C) (Gribbon *et al*., 1995; Adams *et al*., 2003). Research has also demonstrated that *H. pylori* can live for several days in milk and tap water in its

infectious bacillary form, and in river water for several months in the nonculturable but viable coccoid form (Brown, 2005; Dube *et al*., 2009b). As mentioned before, this form is capable of colonizing gastric mucosa and causing gastritis in mice (She *et al*., 2003).

H. pylori has been positively isolated or tested from faeces of adults and children using culture techniques or the *H. pylori* stool antigen tests (HpSA and HpSTAR kits) (Krausse *et al.*, 2008) and PCR (Klein *et al*., 1991; Thomas *et al*., 1992; Mapstone *et al.*, 1993; Lottspeich *et al*., 2007). In one study, the organism was successfully isolated from fresh stool specimens obtained from 12 of 25 known *H. pylori* infected patients (Ceylan *et al*., 2007). With the view that a positive correlation of infection and faecal pollution has been demonstrated (Queralt *et al.*, 2004), limited sanitation services in Africa could unavoidably lead to faecal contamination of the environment and water sources. Hence, playing habits like swimming in river or dam water should have the capacity to accelerate the rate of infection in an African population. If this bug can use water as a vehicle for transmission, then this assumption leaves a lot of questions unanswered pertaining to the prevalence of the organism in rural areas in Africa with poor sources of water.

2.7.4 *H. pylori* contaminated water sources

Contamination of drinking water sources can occur from raw sewage overflow, septic tanks, leaking sewer lines, land application of faeces/sludge, and from

partial treated wastewater (Konishi *et al.*, 2007). The use of contaminated drinking water, including municipal tap water, has long been suspected to have a high impact on the transmission of *H. pylori* (Ahmed *et al*., 2007; Braganca *et al*., 2007; Konishi *et al*., 2007), particularly in areas where the use of untreated water is common. The bacterium has been detected in river, creek, lake and well water (Hulte *et al*., 1996; Hegarty *et al*., 1999).

2.7.5 Biofilms aid persistence in water

Biofilms are a protective niche for several pathogenic bacteria, protecting them from stressful conditions (Giậo *et al*., 2008). According to the Centers for Disease Control and Prevention (Donlan *et al*., 2002), the new definition of a biofilm is a microbial derived sessile community characterised by cells that are irreversibly attached to a substratum or interface or to each other, embedded in a matrix of extra-cellular polymeric substances that they have produced, and exhibit an altered phenotype with respect to growth rate and gene transcription. Biofilms are found in aquatic and industrial water systems as well as in a large number of environments and medical devices relevant for public health. Microorganisms growing in a biofilm are highly resistant to antibiotics, disinfectants, and/or germicides, with the concentration required for 99% reduction up to 1000-fold higher than for planktonic bacteria.

In 2001, a group in Scotland provided the first evidence for the presence of *Helicobacter* species in biofilms in a water distribution system (Park *et al*., 2001).

In a recent study, all morphologic forms of the organism (spiral, rod and coccoid) were observed in drinking water biofilms (Braganca *et al*., 2007). A subsequent study on the persistence and cultivability of *H. pylori* in drinking-water biofilms showed that all cultivable bacteria could not be detected at any point in time, the cells could incorporate, undergo morphologic transformation, persist, and even agglomerate in biofilms for at least 31 days without a noticeable decrease in the total cell number or in the intracellular rRNA content (Giậo *et al*., 2008). The authors concluded that biofilms appear to provide a concentration mechanism allowing a concentrated bolus of microbial cells to escape; that they might not only be infectious but also might escape routine grab sample microbiological analyses.

2.7.6 Water disinfection

Oxidizing disinfectants like chlorine are most commonly used in drinking water (Margolin, 1997). In the viable but nonculturable coccoid form attained due to stressful environments (Cellini *et al*., 1994; Gribbon *et al*., 1995; Mouery *et al*., 2006), *H .pylori* resist the disinfection practices normally used in drinking water treatment (Atherton, 1997; Baker *et al*., 2002; Moreno *et al*., 2007). In chlorinated water, when compared with *E. coli*, the survival of *H. pylori* appears to be linked with superior resistance. Hence, if *H. pylori* tolerate disinfectants in water distribution systems, then transmission by waterborne route is feasible. Additionally, microorganisms like *Acanthamoeba castellanii* have been shown to

promote the survival of the pathogen under experimental conditions (Winiecka-Krusnell *et al*., 2002).

2.8.0 WATER SOURCES IN AFRICA

In Africa, supply of clean water is still a challenge toward the control of microbial infections, hence, focus on how best to improve water quality within the 200 m radius, a standard required by the World Health Organization (WHO), will be a step toward controlling the transmission of microorganisms via water. The intervention techniques used to treat water include physical removal of pathogens (for example, filtration, sedimentation, and adsorption), chemical treatment (for example, assisted sedimentation, chemical disinfection, and ion exchange), heat and ultra violet (UV) radiation.

The very high prevalence of *H. pylori* in Africa may be linked to water sources (Ndip *et al*., 2004). In this part of the world, domestic water sources range from indoor tap water to the most ancient forms, such as direct dam or river water (Thomas *et al*., 1992; Carbone *et al*., 2005) from which the presence of the antigen cannot be ruled out (Brown, 2000; Braganca *et al*., 2007). Bacteriological water quality relies primarily on the type of disinfectants used and the ability to sustain enough residual concentrations, meaning the concentration of biodegradable compounds in water as well as the prevailing water temperature and the piping material used (Baker *et al*., 2002).

Protected wells, groundwater, and rain water are other forms of domestic water. Whereas containers can be provided to store clean water for drinking and domestic use, this method is linked with risks of contamination, for example, during transportation, storage, and during domestic use (Figueiredo *et al*., 2002; Mouery *et al.*, 2006). Several studies have highlighted the presence of the microorganism or their DNA in water (Hulten *et al*., 1998; Queralt *et al*., 2004; Hegarty *et al*., 1999; Braganca *et al*., 2007; Ahmed *et al*., 2007; Konishi *et al*., 2007; Nayak and Rose, 2007).

A Council for Scientific and Industrial Research (CSIR) report estimated that only 21% of South African households have access to piped water within their homes. Additionally, 43.3% and 30% of the population in Mangwe and Beitbridge in Zimbabwe were found to be using unprotected water (Baker *et al.*, 2002). In Tanzania, 67% of both rural and urban populations were reported to be having access to clean and safe water (Margolin, 1997). Furthermore, a shortage of funds in African countries has an impact on the operation of sewage systems, which are below standard with rural populations using latrines, thereby unavoidably increasing chances of fecal contamination of water sources (Margolin, 1997). Because faecally contaminated water has the potential for transmission via the fecal-oral route (Sorbeg *et al*., 1996), most Africans might be at risk of becoming infected with the organism due to the primitive water sources that are still the main water sources in some communities.

Earlier studies demonstrated that water-borne transmission of *H. pylori* could be an important source of infection in developing countries, especially if the water supply is vulnerable to bacterial contamination and is untreated. In undesirable conditions, *H. pylori* turns to a nonculturable form (Gribbon and Barer, 1995), inevitably there is still a need to demonstrate the back conversion to a bacillary form to prove the involvement of the coccoid form in the transmission and waning nature of the infection. An earlier study, however, demonstrated that the coccoid forms do not completely lose their maintenance factors or properties and might be able to infect mice (Winiecka-Krusnell *et al.*, 2002; Azevedo *et al*., 2007), thereby providing insight on the possibility of transmission by the coccoid form. Use of boiled or filtered water would be an exploitative idea as it has been shown to reduce infection (Figueiredo *et al*., 2002). Although tangible evidence of this mode of transmission is lacking, water as a vehicle for the transmission of *H. pylori,* cannot be ruled out (Dube *et al*., 2009c). Hence, much attention is still necessary to gather more facts on this proposed route of transmission.

In African societies, education must be encouraged to help curb infection rates because several studies have highlighted an increased prevalence of the pathogen with low levels of education. Interestingly, a serological study in Egypt revealed that increased education was significantly associated with an increased risk of infection among mothers (Bassily *et al*., 1999).

2.9.0 ASSOCIATION BETWEEN *H. PYLORI* IN WATER AND CLINICAL INFECTION

Although *H. pylori* infection is ubiquitous, its mode of transmission remains an enigma. *H. pylori* is presumed to be transmitted orally. One way that *H. pylori* could be transmitted orally is by means of fecal matter through the ingestion of waste tainted food or water. In addition, the organism could be transmitted from the stomach to the mouth through gastro-esophagal reflux (in which a small amount of the stomach's contents is involuntarily forced up the esophagus) or belching, common symptoms of gastritis. The bacterium could then be transmitted through oral contact.

Evidence that the source of drinking water is likely to be related to *H. pylori* infection has emerged from studies showing that waterborne transmission may be important in areas of the world with high rates of infection and less than adequate water quality (Hulten *et al*., 1998; Youri, 1998; Park *et al*., 2001).

In 1997, the US Environmental Protection Agency Office of Ground Water and Drinking Water expressed concerns over possible waterborne transmission by adding *H. pylori* to its contaminant candidate list (Zhang *et al.*, 1996; Dube *et al*., 2009a). In developing countries, water remains the major source of transmission of enteric pathogens. In a study assessing the impact of town planning, infrastructure, sanitation and rainfall on the bacteriological quality of domestic water supplies in Lagos, Nigeria, faults in pipelines, probably resulting from

illegal tapping into distribution system and pipes aging, along with their location near or across a drainage system were strongly correlated with the high level contamination of pipe-borne water supplies ($p < .05$) (Goodman *et al*., 1996).

Although a study in Guatemala concluded that *H. pylori* is unlikely to be transmitted by water (Klein *et al.*, 1991), epidemiologic studies in many countries, such as Colombia (Carbone *et al*., 2005), rural China (McKeown *et al*., 1999), and Lima, Peru (Nayak and Rose, 2007), have shown that infection is related to *H. pylori* contaminated water sources. As cited by Nayak and Rose, 2007, presumptive evidence of waterborne transmission of *H. pylori* was first provided by Klein *et al*, who found that Peruvian children whose homes had an external water supply were three times more likely to be infected with the bacterium than children whose homes had an internal water source. Five years later, *H. pylori* DNA was detected in drinking water samples collected from the same areas. The 375-base pair fragment from the adhesin gene was amplified from 24 water samples; the 500-base pair fragment of the 16S ribosomal RNA and the 375-base pair fragment of the adhesin gene were amplified from 11 of the samples (Federal Register, 1997). The results of that study confirming the presence of *H. pylori* in drinking water in Peru suggest waterborne transmission of the pathogen in certain environments.

The finding of *H. pylori*-positive drinking and sewage water samples by PCR assays in Japan provided further evidence that waterborne transmission may be

important, especially in areas of the world having high rates of *H. pylori* infection and less than adequate water quality. *H. pylori* DNA was detected in well water used by persons who had acquired *H. pylori* infection in the past (Horiuchi *et al*., 2001). Among those having a history of drinking well water, *H. pylori* serological prevalence in residents who were at least 10 years of age (85.3%) was significantly higher than in those under 10 years of age (25%) or in those with no history of drinking well water (6.3%). In a continuation of the same study, *H. pylori* DNA was detected in well water obtained from all five wells from which the five seropositive members had drunk (Egwari and Aboaba, 2002).

Many North American arctic communities are characterized by risk markers associated with *H. pylori* infection, including inadequate water supply and sanitation systems. The prevalence of *H. pylori* infection was measured in two traditional communities in the central Canadian arctic using PCR to test for the presence of the bacteria in local water supplies (McKeown *et al*., 1999). Of the 256 subjects from the two communities, one hundred and thirty (50.8%) were positive for *H. pylori* IgG antibodies. Compared with seronegative individuals, the seropositive subjects were more likely to be male ($p = 0.01$). Antibody status did not differ with respect to age, community, alcohol or cigarette use, number of persons per household, gastrointestinal complaints or previous investigations, medications, or presence of blood group O, Lewis a-b+.

Additionally, *CagA* antibodies were detected in 78 (61.9%) of 126 *H. pylori*-seropositive subjects tested; on the other hand, 41 (35.3%) of 116 *H. pylori*-seronegative subjects were also *cagA* positive. Water samples taken from the water delivery truck in Chesterfield Inlet and from two lakes near Repulse Bay were positive for *H. pylori*. The authors concluded that the detection of *H. pylori* in local water supplies could point to a natural reservoir for the organism or to possible contamination from human sewage.

An association between *H. pylori* seropositivity and certain demographic, environmental, and lifestyle factors was determined (Brown *et al.*, 2002). A cross-sectional survey was administered to 3288 adults (1994 seropositive, 1019 seronegative, 275 indeterminate) from 13 villages in Linqu County, one of the poorest counties in one of the least economically developed provinces in China, Shandong Province. The source of drinking water varied between *H. pylori* positive and negative subjects, with the highest risk [odds ratio (OR) = 1.8, 95% confidence interval (CI): 1.4-2.3] and percent seropositive (72.2%) found in subjects who obtained their water from a shallow village well. An elevated OR was also associated with washing or bathing in a pond or ditch (OR = 1.6, 95% CI: 1.0-2.4; % seropositives = 71.5%)

In the US, a preliminary study by Hegarty and coworkers as cited by Thomas *et al*., 1992, found actively respiring micro-organisms binding monoclonal anti-*H. pylori* antibody in most surface and shallow groundwater samples tested (n = 62),

supporting a waterborne route of transmission for this organism. A later study using molecular methods confirmed that the presence of *H. pylori* in untreated well water correlates with infection in consumers *($p < .02$)* and with the presence of *Escherichia coli*, indicating fecal contamination (Steinberg *et al*., 2004). The authors concluded that consumption of untreated well water should be considered a risk factor for *H. pylori* infection.

In a study in Kazakhstan, a clean water index (CWI) was created based on the consistency of boiling water before drinking, the frequency of storing and reusing water, and the frequency of bathing and showering. *H. pylori* infection was inversely correlated with CWI [56%, 79%, and 95% for high, middle, and low, respectively ($p < .05$)]. Additionally, drinking river water had the highest risk of *H. pylori* infection (OR = 13.6, 95% CI = 1.8-102.4; $P < .01$, compared with tap water) (Brown *et al*., 2002).

Recently, an outrageous report was published of a very high prevalence of *H. pylori* infection among the South Indian population who were drinking tap water or well water ($p < .001$). Using the PCR assay, Ahmed *et al*. (2007) investigated the roles of household hygiene and water source in the prevalence and transmission of *H. pylori* infection in a selected population of 500 adults with upper gastrointestinal tract symptoms. The overall prevalence of *H. pylori* was 80%. The prevalence of infection among people who drank water from wells was 92% compared with 74.8% of those who drank tap water ($p < .001$). *H. pylori*

infection prevalence was higher in people with low clean water index (CWI) (88.2%) than in those with higher CWI (33.3%) ($p < .001$). The results of their study imply that the risk of acquisition and transmission of *H. pylori* can be prevented to a certain extent by following improved household hygienic practices, proper waste disposal measures, and the regular use of boiled water for drinking purposes.

In addition, in a census sample of 684 2-9-year-old children in Aldana, Nariño, a rural community in the Colombian Andes, *H. pylori* prevalence, as determined by the ^{13}C-urea breath test, was 69%, and prevalence increased from 53% in 2 year-olds to 87% in 9 year-olds. Among transmission-pathway proxies, the strongest predictor of *H. pylori* status was the number of persons who lived in the home, with the number of children apparently being of greater importance than the number of adults. Swimming a few times a year in rivers and streams (OR = 3.3, 95% CI = 1.2-6.4), or in swimming pools (OR = 3.6, 95% CI = 1.5-8.5) increased the odds of infection, as did using streams as a drinking water source (OR = 2.8, 95% CI = 1.2-6.8; $p < .001$), but not well or tap water (Carbone *et al*., 2005). In Spain, fluorescent in-situ hybridization (FISH) revealed the presence of *H. pylori* in two river water and one wastewater sample (Hulten *et al*., 1996; Karita *et al*., 2003).

2.10.0 HOUSEHOLD HYGIENE

In preventing waterborne diseases, hygiene is the starting point. High standards of hygiene can be maintained only if a steady supply of clean water and proper sanitation system are available. Several studies have been conducted to relate *H. pylori* prevalence with household hygiene (Malaty *et al*., 1998; Perry *et al*., 2006; Ahmed *et al*., 2007). Prevalence was observed to be higher within families using non-flush toilets, outdoor toilets, outdoor water taps, and use of river water (Ahmed *et al*., 2007). Most of the findings show that poor hygiene has a positive correlation with increased prevalence of the micro-organism.

Safe disposal of human excreta is the first step in preventing faeco-oral and other routes of disease transmission. Improved sanitation standards reduce contamination of the environment. In a study of *H. pylori* in China prevalence was elevated due to infrequent hand-washing before meals, washing less than half the time (OR=1.6, 95% CI: 1.0-2.5; % seropositive = 74.4%), and never washing (OR=3.8, 95% CI: 0.5-31.0; % seropositive = 87.5%) (Brown, 2000).

Poor sanitation, like the lack of sanitary services at home, is believed to be an important risk factor for *H. pylori* infection (Mendall *et al*. 1992; Malaty *et al*., 1998). Impaired hygiene during childhood especially in developing countries seems to be associated with a higher prevalence of the organism. Sharing cups, premastication of food for young children, sharing water for bathing and washing

hands and limited sanitary facilities have been shown to be having a positive correlation with increased prevalence of *H. Pylori* (Ahmed *et al*., 2007).

Several studies have proposed that most people acquire *H. pylori* infection during childhood (Goodman and Correa, 1995; Malaty *et al.*, 1998; Rowland, 2000). DNA fingerprinting studies have confirmed the similarity of strains from children and those from their mothers thereby suggesting a possibility of mother to child transmission (Delport *et al.,* 2006); interestingly, infants born to *H. pylori*–positive mothers showed a decreased risk of acquiring *H. pylori* infection during the first year of life (Konno *et al.,* 2005). Intraspecific transmission from mother to child has been linked to premastication of food (Mégraud *et al.*, 1995). In addition, use of common spoons, licking pacifiers or teats of feeding bottles by other subjects may result in the transmission of the organism (Rothenbacher *et al.,* 1999). Also a strong evidence for a transmission pathway from family members to children was recently observed (Ceylan *et al*., 2007).

However, controversy still exists regarding the role of the oral cavity as a route of transmission. In a recent study, it was deduced that the oral cavity is unlikely to contribute to the spread of this organism as oral cavities were found not favouring prolonged colonization by the organism (Olivier *et al*., 2006). In the same study, *H. pylori* was not detected from dental plaque samples by PCR even though 84% of the healthy subjects had tested positive to the antigen. Besides the oral-oral

route, the transmission of *H. pylori* could probably take place through the consumption of contaminated food.

2.11.0 LABORATORY DIAGNOSIS OF *H. PYLORI* INFECTION

Since the discovery of the organism in the early 80s, different techniques have been developed for diagnosing *H. pylori* in clinical specimen. These tests may be invasive or non-invasive (Shephered *et al.*, 2000; Tanih *et al.*, 2008). Endoscopy backed by gastric mucosal biopsy methods, histology, culture, or urease tests are forms of invasive test that could be used (Stromar *et al*., 2008). Non-invasive tests include the Urea Breath Test (UBT), Enzyme Immunosorbent Assay (ELISA), *H. pylori* Stool Antigen Test (HpSA), Rapid *H. pylori* Antigen Test (HpSTAR) and latex agglutination tests (Ndip *et al*., 2004; Krogfelt *et al*., 2005; Dube *et al*., 2009b).

2.11.1 Invasive tests

Gastric mucosal biopsy and string sample (string test) material provides better chance of successfully culturing the organism (Ndip *et al.*, 2003; Velapatiño *et al*., 2006). Due to probability of a patchy distribution of the organism in its niche, multiple biopsies from the atrum and corpus of the host increase chances of obtaining a reliable diagnosis (Ndip *et al*., 2003; Ndip *et al*., 2007b; Ndip *et al*., 2008). To access a better yield, the patient should have undergone several weeks without taking antibiotics or antisecretory drugs such as proton pump inhibitors (PPI) and omeprazole.

Histology

This can be accomplished by haematoxylin and eosin stains. To detect low levels of infection, and for clarity on characteristic morphology of *H. pylori*, additional stains that include Giemsa, Genta, Immunostaining, Dieterle, Acridine orange, Gimenez Warthin-Starry silver, McMullen and Creosyl violet are used (Logan *et al*., 2001; Gatta *et al*., 2003; Ndip *et al*., 2003; Levin *et al*., 2007). These staining techniques predominantly allow examination of prepared slides under the light microscope.

Culture

The culturing technique has been mostly challenged by the inability of the organism to remain viable for a longer period outside its host. In an unfavourable environment the organism turns into a coccoid form which is viable but nonculturable. However successful cultures of *H. pylori* have been grown from biopsies, string samples and stool specimen (Thomas *et al*., 1992; Parsonnet *et al*., 1999; Dore *et al*., 2000; Velapatiño *et al*., 2006). After collection, a biopsy sample is homogenised in 0.9% saline solution prior to inoculation (Ndip *et al*., 2003); for the string sample, homogenising by vortexing in transport medium containing brain heart infusion (BHI) broth, 20% glycerol and 1% Skirrow supplement is adequate before inoculation (Velapatiño *et al*., 2006).

Several agar recipes for isolating and culturing *H. pylori* are available. BHI agar containing Skirrow supplement (Oxoid) , Columbia colistin-nalidixic acid agar

with Dent supplement (Oxoid), Skirrow *Campylobacter* medium, Columbia agar, Chocolate agar, Marshall brain heart infusion (BHI) medium, Mueller–Hinton and Wilkins–Chalgren agar with horse blood and Belo Horizonte medium supplemented with 2, 3, 5 - triphenyltetrazolium chloride are other culture mediums that have been successfully utilized in culturing *H. pylori* (Velapatiño *et al*., 2006). However, Oxoid BHI agar supplemented with 5% horse blood, 1% (v/v) IsoVitalex (BBL, Microbiology systems, Becton Dickenson, Cowley) with and without antibiotics according to either the Skirrow's medium or to the Dent and McNulty medium has been recommended for general use (Owen, 1995; Dent and McNulty, 1998). As cited in Ndip *et al*., (2003), Morgan and coworkers stated that the agar based culturing medium can be substituted by selective enrichment medium that bears Brucella broth (Difco) supplemented with 10% foetal calf serum, 1% (v/v) Iso-Vitalex, polymyxin B sulphate (1000 units/mL), vancomycin (10µg/mL) and amphotericin B (2µg/mL).

Incubation

H. pylori is microaerophilic, therefore, other than using an incubation temperature of 37^{o}C, microaerobic conditions should exist for its growth. Outside the human host, atmospheric composition ranging from 5-6% O_2, 7-12% CO_2, 0-85% H_2 and 0-85% N_2 are conducive for *H. pylori* growth (Ndip *et al*., 2003; Velapatiño *et al*., 2006; Ndip *et al*., 2007a; Ndip *et al*., 2007b). Incubation periods of up to 10 days are satisfactory to maximize growth particularly if a biopsy sample is taken after an antibiotic treatment.

Urease test

This test makes use of the ability of the organism to produce urease. When *H. pylori* infected biopsy sample is added to urea containing medium, ammonia a by product, influence a pH-driven colour change that forms the basis of this diagnostic test (Midolo *et al*., 2000; Levin *et al*., 2007). The CLO test and rapid urease tests (RUT-home made) are of similar sensitivity and specificity i.e. 90% and 100% respectively (Logan and Walker, 2001; Levin *et al*., 2007). False-negative results may be obtained, current reports link these circumstances to the use of suppression medication especially PPIs and other antibiotics.

2.11.2 Non-invasive tests

Urea Breath Test (UBT)

This test is non-quantitative and it determines current infection as it relies on urease activity produced by the bacteria. False negatives can arise if there are too few bacteria in the stomach of infected host to produce detectable urease especially during or after a treatment regiment, also in the case of infection with different bacteria that also produce urease. Generally, either $_{13}C$ or $_{14}C$ is used. The labelled urea is hydrolysed by the urease enzyme in the stomach of an infected host, and the resulting CO_2 is absorbed across the gastric mucosa into the blood circulatory system, and then excreted through the lungs as expired air.

Serology

This is a useful tool for detecting *H. pylori* in either fresh or stored serum. Serological tests are non-quantitative and detect immunoglobulin (IgG) or IgA or IgM antibodies to *H. pylori* infection (Bassily *et al*., 1999; Brown., 2000; Mbulaiteye *et al*., 2006). The circulating antibodies to *H. pylori* can be detected by enzyme linked immunosorbent assay (ELISA) or latex agglutination tests. The resultant colour change due to the formation of an antigen-antibody complex forms the basis of most of these tests (Oderda *et al*., 2001).

While a serological tests based on antibody detection can be ideal and has been used widely in Africa (Bassily *et al*., 1999; Aoki *et al*., 2004; Longo-Mbenza *et al.,* 2006; Mohammad *et al*., 2007), it does not offer direct evidence of current infection resulting in failure to confirm the presence of the antigen. This is critical, especially with infants borne from *H. pylori* positive mothers where there is a high chance of trans-placental transfer of the IgG (Bassily *et al*., 1999). It also makes it difficult to differentiate between passively acquired and actively produced antibodies to the organism. In addition, no single antigen is recognised by sera from all subjects, this implies that the precision of serological tests relies on antigens used; hence local validation of the test becomes a requirement.

Stool antigen tests

A simple sandwich-type enzyme immunoassay amplification technology is used to analyze stool samples as per the manufacturer's instructions. It uses

monoclonal antibodies specific for *H. pylori* antigens. To avoid the problem of strain variation, an *H. pylori* strain found in different geographical regions and dietary groups is selected to produce bacterial sonicates (Larka *et al*., 1999). The main advantages of this technology are that it detects the present infection and it can be easily employed for large scale epidemiological studies of acquisition of *H. pylori* in all population groups. In addition, the colour change can be assessed visually in the micro-plate (HpSA test) or strip(HpSTAR test). These tests have been used successfully worldwide with high sensitivity, specificity and accuracy (Frenck *et al*., 2006; Lottspeich *et al.*, 2007; Krause *et al.*, 2008; Dube *et al*., 2009c). Its disadvantages include limited use with patients on medication containing antimicrobial agents, proton pump inhibitors and bismuth preparations due to suppressed growth of the organism thereby leading to false negative results. Also there is a possibility of detecting live and dead cells by this method or partially digested cells.

The newly introduced Rapid *H. pylori* Antigen Test (HpSTAR), is fast and results may be obtained within 20 minutes. However this test has shown an acceptable sensitivity of 77% while its specificity and accuracy is over 80% in an adult population (Krausse *et al*., 2008). However, sensitivity of 96.1% and specificity of 98.5% along with positive (PPV) and negative predictive values (NPV) of 96.1% and 98.5 % respectively have been recorded (Sykora *et al.*, 2003). In a recent study involving Egyptian children, the HpSTAR test had a sensitivity of 93%, specificity of 88%, PPV of 88% and NPV of 93% (Frenck *et al*., 2006). In

the same study involving Egyptian children, the HpSA test had a sensitivity of 71%, specificity of 76%, PPV of 73 and NPV of 74%. In addition, the major advantage of HpSTAR over the HpSA test is that it is easy to perform. However, HpSTAR is only qualitative while HpSA test with the combination of the micro-plate reader is quantitative.

2.11.3 Polymerase chain reaction (PCR).

PCR is a highly sensitive method in clinical diagnosis and has been widely used in a number of epidemiological studies (Smith *et al.*, 2002). Molecular techniques have helped in detection of *H. pylori* from water, gastric biopsies, stools, luminal aspirates, vomitus, saliva, dental plaque and other oral cavities that can provide prolonged habitat for the organism as it gains it's way to infect the host (Burocua *et al*., 1999; Kidd *et al*., 2001; Ndip *et al*., 2003; Astrat *et al*., 2004; Watson *et al*., 2004; Queralt *et al*., 2005).

Novel, efficient methods of stool specimen processing and specific DNA extraction have recently been developed rendering high sensitivity to the PCR method (Shuber *et al.*, 2002). Despite its high sensitivity, this method has challenges of overcoming PCR inhibitors in clinical specimen especially faecal samples. Several strategies have been employed to deal with failure of amplication due to PCR inhibitors, these include dilution of the DNA eluate, addition of BSA as well as using purification procedures or kits which are

commercially available (Burucoa *et al*., 1999; MacKay *et al*., 2003; Oikarinena *et al*., 2009).

PCR protocol that amplifies the *ure*C (*glm*M) gene of *H. pylori* has been targeted to detect *H. pylori* due to its highly conserved nature in this organism (Stone *et al*., 1997; Burucoa *et al.*, 1999). However, several genes have been targeted in detecting *H. pylori,* the 23S rRNA gene, 16S rRNA, *ure*A and *ure*B among others (Amann *et al*., 2000; Horiuchi *et al*., 2001; Park *et al*., 2001; Yilmaz *et al*., 2007).

This bacterium contains two *23S rRNA* genes and mutations are generally found in both copies, however, heterogeneity has been described. Mutation in one copy of the *23S rRNA* gene may be easily transferred to the other *23S rRNA* gene by efficient homologous DNA recombination under selective pressure (Yilmaz *et al.,* 2007).

While *H. pylori* has a single copy of *adhesin* or *ureA* gene, reports have been made that PCR with 16S rRNA primers is more sensitive in detecting *H. pylori* than PCR with ureA primers (Horiuchi *et al.,* 2001). While the *16S rRNA* gene is also a highly conserved gene and has been targeted in several studies, microorganisms often contain more than one copy of the *16S rRNA* gene, if this is not taken into account during analysis there will be an over representation of species containing more than one *16S rRNA* gene and also there is heterogeneity

(up to 5%) in *16S rRNA* genes within the same organism that has been reported (Amann *et al*., 2000).

2.11.4 Validation of diagnostic tests

Validation of tests is important as a determinant of suitable methods to be employed for improved quality of research. The factors that may affect test validity include patient age, gender and geographic location (Frenck *et al.,* 2006). In one of the few validation tests done in Africa, the UBT and HpSTAR stool antigen kit were found to be reliable non-invasive tests for *H. pylori* diagnosis among children and adult populations both attaining high sensitivity and specificity (Shepherd *et al*., 2000; Ndip *et al*., 2004; Frenck *et al.*, 2006). However, the main advantage of HpSA over UBT is that, active infection could be ascertained with anti-genemia. Stool antigen tests were introduced more recently hence there is still a paucity of information on test accuracy. Collection and proper handling of stool samples present logistic challenges on population-based studies; for example, there should be assurance that no other source of contamination by the antigen is undermined e.g. through collection devices.

The gold standards for validating some of the diagnostic tests for *H. pylori* infection are still a challenge. Other than being invasive, biopsy-based techniques mainly used as a gold standard, do not cater for the possibility of patchy distribution of the organism in the human stomach (Brown, 2000). To reduce this uncertainty, multiple biopsies can be taken from the antrum and corpus (Krogfelt

et al., 2005). Also, high standard disinfection practices need to be exercised to avoid latrogenic transmission of the bug during sampling.

2.12.0 TREATMENT

Recommended first-line drugs for *H. pylori* eradication are triple therapy consisting of two antibiotics, clarithromycin, amoxicillin and ciprofloxacin or metronidazole in combination with a proton pump inhibitor (PPI) (Aydin *et al.,* 2007). Bismuth-based quadruple therapy is also a first-line if not a second line option regimen in areas with remarkably high rates of clarithromycin and metronidazole resistance (Wolle *et al*., 2007; Tanih *et al*., 2009). Triple therapies based on levofloxacin and/or rifabutin mainly with combination of amoxicillin are options if multiple eradication failure occurs.

In addition to patient noncompliance, clarithromycin resistance is the most important cause of failure of these eradication therapies. In a recent study, two-week eradication therapy with pantoprazole plus amoxicillin and clarithromycin was shown to achieve a satisfactory success rate (92.8%) in clarithromycin-sensitive cases. While the 2-week therapy was more successful, the success rates of eradication therapies of both 1-week (26.7%) and 2-week duration (60%) were disappointing in clarithromycin-resistant cases (Aydin *et al*., 2007).

The minimum inhibitory concentration (MIC) of clarithromycin is low and is relatively unaffected by lowering the pH (Kaya *et al*., 2007). The drug reaches a

high concentration in gastric mucosa with a high degree of binding to *H. pylori* ribosomes (Baglan, 2006). *H. pylori* eradication therapy with a PPI plus amoxicillin and clarithromycin for one week is the most popular treatment regimen. Pantoprazole-based triple therapy is effective for *H. pylori* eradication in clarithromycin sensitive cases, highly effective *H. pylori* eradication protocols are needed for clarithromycin-resistant ones (Aydin *et al*., 2007). However, recent studies from many countries have reported the failure of these regimens because of antibiotic resistance.

Seven different point mutations (A2115G, G2141A, A2142G, A2142C, A2143G, A2143C, and A2142T) in the peptidyltransferase region of the V domain of the 23S rRNA gene have been found to be associated with resistance to clarithromycin (Occhialini *et al*., 1997). A later study described another point mutation, T2182C, associated with clarithromycin resistance in twelve clarithromycin-resistant *H. pylori* isolates (Khan *et al*., 2004). Resistance of *H. pylori* to clarithromycin is mainly due to an adenine-to-guanine transition at positions 2142 and 2143, and to an adenine-to-cytosine transversion at position 2142, which is included in the peptidyltransferase loop of the 23S rRNA gene. The most frequently (98%) observed mutations are A2142G and A2143G, with the A2142C mutation being much rarer (1.6%). Other mutations (A2115G, G2141A, and T2717C) have been described but appear to be infrequent (Kaya *et al*., 2007).

CHAPTER THREE

MATERIALS AND METHODS

3.1.0 RESEARCH DESIGN

This study encompassed different asymptomatic families residing in the Nkonkobe Municipality in South Africa. Patients visiting Victoria Hospital as well as Happy Rest Clinic donated stool samples after signing the consent form. Demographic data was obtained by means of a standard questionnaire. Based on the presence of the antigen in stool samples (high OD value), water was collected from area of residence of corresponding subjects for molecular analysis of *H. pylori* DNA by PCR.

3.2.0 ETHICAL ISSUES

This study was approved by the Department of Health in the Eastern Cape Province and the Goven Mbeki Research and Development Centre (GMRC) at the University of Fort Hare (Appendix 1). All subjects participating in this study signed a consent justifying their consent to participate in the study. In the case of minor children, the parent/guardian signed the consent form.

3.3.0 SELECTION OF SUBJECTS

The aim of the study was explained to the subjects and their consent to participate obtained. (Consent form: Appendix 2). Eligible members were enrolled based on the questionnaire data, those with a history of gastro duodenal ulcer, with

recurrent chronic complaints of the upper digestive tract for more than two months or those currently using anti-ulcer medications were excluded. Participation in the study was voluntary. Information collected from all participants was entered into a computer and confidentiality maintained.

3.4.0 QUESTIONNAIRE

A questionnaire is a good tool that has been employed in various aspects of epidemiological studies to gather information. In this study a standard questionnaire (Appendix 3) was administered to the subjects to obtain socio-demographic and economic data. Data such as age, sex, socioeconomic status, history of any gastric or duodenal diseases, habit of sucking fingers, eating raw vegetables, sharing bedroom, sharing bath water, sharing toothbrush, water source and type of toilet used was asked from each subject. In the case of children, the parent or guardian was responsible for answering the questionnaire.

3.5.0 COLLECTION OF SAMPLES

3.5.1 Stool samples

Each eligible participant provided a stool sample by using a sterile collection device, which fitted over the toilet sit. A sterile wooden spoon was used to transfer the sample directly into a collection bottle. Immediately after collection, faecal samples were transported in ice and stored at -20^{o}C until use.

3.5.2 Water samples

Treated water from taps/tanks used by the participants who tested positive for the organism were collected using sterilized (autoclaved) 1L bottle containers that were transported in ice to the laboratory. Water samples were filtered using a sterile 0.22µm pore size cartridge/nylon membrane filters (supplied with the DNA extraction kit) which (catridge/nylon membrane filters) were then stored at -20^{o}C until use.

3.6.0 DETECTION OF *H. PYLORI* ANTIGEN BY THE HpSA TEST

A sandwich-type enzyme immunoassay amplification technology (Amplified IDEIA^{TM} Hp StAR^{TM} Oxoid,UK) was used to analyse stool samples as per the manufacturer's instructions. It uses monoclonal antibodies specific for *H. pylori* antigens. The main advantages of this technology is that it detects present infection and also the combination of ELISA technology and HpSA test highlights the level of infection; also this method is not invasive. In addition, the colour change can be assessed visually. This test has been used successfully worldwide (Shepherd *et al*., 2000; Ndip *et al*., 2004; Frenck *et al*., 2006; Ceylan *et al*., 2007). Its disadvantages include limited use with patients on medication containing antimicrobial agents, proton pump inhibitors and bismuth preparations due to suppressed growth of the organism thereby leading to false negative results. Also there is a possibility of detecting live and dead cells by this method or partially digested cells.

Briefly, supernatant of faecal suspension and a horseradish peroxidase (HRP) labeled monoclonal antibodies (Antibody conjugate) were added to the wells of microplate coated with monoclonal antibodies specific for *H. pylori* antigens, and then the preparation was incubated for 60 minutes at 25^{o}C with shaking. Washing was done to remove any unbound antibody conjugate. This was followed by addition of a colourless single-component enzyme substrate (Tetramethylbenzidine-TMB) and incubated for 10 minutes at 25^{o}C. This was followed by addition of a stop solution. Absorbance was measured spectro-photometrically at 450 nm. The results were assigned as positive or negative on the basis of the manufacturer's recommended cut off values. The absorbance values $\geq$ 0.190 were assigned as positive while values $<$ 0.190 were assigned as negative.

3.7.0 DETECTION OF *H. PYLORI* DNA IN STOOL AND WATER SAMPLES BY POLYMERASE CHAIN REACTION (PCR).

PCR is highly sensitive and hence allows diagnosis even if the organism is available in low quantities. Despite its high sensitivity, this method may have challenges of overcoming PCR inhibitors in clinical specimens' especially faecal samples. Several strategies were employed to deal with failure of amplification due to PCR inhibitors; these include dilution of the DNA eluate, addition of BSA as well as using purification kits which are commercially available.

In this study, a QIAamp DNA Stool Mini Kit (Qiagen) was used to extract DNA from HpSA positive stool samples according to the manufacturer's instructions. Briefly, about 200 mg of frozen faecal samples were aseptically transferred to separate 2 ml microcentrifuge tubes in ice and 1.4 ml of lysis buffer ASL was added to each sample and homogenized by vortexing for one minute. The suspension was incubated for 5 minutes at 70^{o}C after which it was vortexed for 14 seconds. This was followed by centrifugation at 10, 000 x g for 2 minutes to pellet stool particles and 1.2 ml of the supernatant was pipetted into new 2 ml microcentrifuge tubes followed by the addition of one inhibitEx tablet to each tube. The tubes were vortexed until the tablet was completely suspended after which the suspension was incubated at room temperature for one minute.

The suspension was then centrifuged at 10, 000 x *g* for 6 minutes. The resultant supernatant was pipetted into a new 1.5 ml microcentrifuge tube and centrifuged at 10, 000 x *g* for 6 minutes. Two hundred µl supernatant was then pipetted into a new microcentrifuge tube containing 15 µl proteinase K and vortexed for 10 seconds. Two hundred µl of buffer AL was added and the suspension vortexed for 15 seconds followed by incubation at 70^{o}C for 10 minutes.

After incubation, 200 µl of ethanol (96%) was added to the lysate and mixed by vortexing. The resultant lysate was carefully transferred to a labeled QIAamp spin column and centrifuged at 10, 000 x *g* for 2 minutes. The QIAamp spin column was then inserted in a new 2 ml collection tube; 500 µl buffer AW1 was then

added into the spin column, closed and centrifuged at 10, 000 x *g* for 2 minutes. The QIAamp spin column was then inserted into another new 2 ml collection tube and 500 µl of buffer AW2 added. The cap was closed, and centrifuged at 10, 000 x *g* for 6 minutes. The QIAamp spin column was transferred to a new 2 ml collection tube and centrifuged at 10, 000 x *g* for 2 minutes, after which the QIAamp spin column was inserted into a new labeled 1.5 ml microcentrifuge tube and 200 µl buffer AE was then pipetted directly onto the QIAamp membrane. The spin column was closed and incubated for 5 minutes at room temperature. This was followed by the final centrifugation at 10, 000 x *g* for 2 minutes to elute DNA that was stored at -80^{o}C until use.

DNA from water samples was extracted using MO BIO Laboratories UltraClean^{TM} water DNA isolation kit (0.22µm) according to the manufacturer's instructions. Briefly, on arrival at the laboratory, water samples were filtered to trap microorganisms by the 0.22 µm filter membrane. The filter membrane was aseptically removed using sterile forceps and inserted in the labeled water bead tube that was kept in ice until use.

Four ml of bead solution was then added to the water bead tube and vortexed for 30 seconds; 500 µl of solution WD1 was added to each water bead tube and vortexed for 30 seconds. The water bead tubes were then tightly secured horizontal to the vortex adapter and vortexed at maximum speed for 10 minutes after which it was centrifuged at 2500 x *g* for one minute.

The supernatant (3.4 ml) was then transferred to a clean 15 ml centrifuge tube and 600 µl of solution WD2 was added followed by a brief vortexing for 5 seconds. The suspension was then incubated for 5 minutes at 4^{o}C followed by centrifugation at 2500 x *g* for 4 minutes. The supernatant (4 ml) was transferred to another clean 15 ml tube, and 8 ml of solution WD3 was added to the supernatant and vortexed for 5 seconds. The supernatant was then loaded into a 50 ml spin filter tube and centrifuged at 2500 x *g* for 2 minutes. The flow through was discarded and 3 ml of solution WD4 was added followed by centrifugation at 2500 x *g* for 3 minutes. The flow through was discarded again and the filter tube centrifuged again at 2500 x *g* for 5 minutes. The spin filter was then transferred into a new 50 ml tube followed by addition of 3 ml of solution WD5 to the center of the white filter membrane.

The tubes were then centrifuged at 2500 x *g* for 2 minutes to elute DNA. To concentrate the DNA, 300 µl of 5M NaCl was added to 3 ml DNA elutes and mixed. Six ml of 100% ice-cold ethanol was then added and mixed. The suspension was then centrifuged at 2500 x *g* for 20 minutes and the supernatant decanted. The residual ethanol was dried in a speed vac and 200 µl of sterile nuclease free water was added to re-suspend precipitated DNA. The DNA was then stored at -80^{o}C until use.

Eluates obtained were subjected to PCR as previously reported by Burucoa *et al.* (1999). A PCR amplification method for a portion of the *ureC* (*glm*M) gene was

used to verify the presence of the organism. A positive control for the PCR assay, was *H. pylori* DNA obtained from known *H. pylori* strain NCTC 11638 that had been cultivated on Columbia Agar Base supplemented with horse-blood (Ndip *et al*., 2003). A negative control constituted all the components of the reaction mixture except DNA. The reaction was performed directly on eluates obtained from stool and water samples. The reaction was performed with primers whose nucleotide sequence was derived from a known sequence of the *glm*M gene: Forward 5'-TTTGGGACTGATGGCGTGAGGGGTAA-3' and Reverse 5'-GGACATT CAAATTCACCAGGTTTTGAG-3'. Amplification reaction mixtures (50µl) contained 10 mM Tris-Cl (pH 8.3), 50 mM KCl, 3 mM $MgCl_2$, 2.5 U of Hotstar DNA polymerase (Fementas), 200 µM deoxynucleoside triphosphate mix, 0.2 µM of each primer, and 1µl of eluate from stool sample, and 5µl of DNA eluates from water samples respectively. Amplification was carried out in a DNA thermocycler (Mastercycler Personal System, German). After an initial denaturation of target DNA at 95°C for 5 min, thermal cycling for each set of primers was 95°C for 1 min, 50°C for 1 min, and 72°C for 1 min for a total of 40 cycles. The final cycle included extension for 5 min at 72°C.

3.8.0 GEL ELECTROPHORESIS

Ten microliters of PCR products was analyzed by electrophoresis on a 0.8% (wt/vol) agarose gel, stained with ethidium bromide, at 110V for 45 min in 0.5X Tris-acetate-EDTA (TAE) buffer. The PCR products were examined using a

transilluminator (UVP Gel Documentation System) in parallel with molecular size markers, GeneRuler™ 1 Kb plus DNA Ladder (Fementas).

3.9.0 STATISTICAL ANALYSIS

Fisher's exact Chi-square test was used to assess the univariate associations between *H. pylori* infection and the possible risk factors. Odd ratio (OR) and corresponding 95% confidence interval were calculated to measure the strength of associations using EPI INFO 6.04 package (Centres for disease Control and Prevention, Antlanta, GA, USA). P-Values <0.05 was considered for statistical significance. The specificity, sensitivity, positive predictive value (PPV), and negative predictive value (NPV) were calculated to assess precision of the different diagnostic tests used, the following formulas were used.

$$\text{PPV} = \frac{\text{Number of True Positive}}{\text{Number of True Positives + Number of False Negatives}}$$

$$\text{NPV} = \frac{\text{Number of True Negative}}{\text{Number of True Negatives + Number of False Negative}}$$

$$\text{SPECIFICITY} = 1 - \frac{\text{Number of False Positive}}{\text{Number of False Positive + Number of True Negative}}$$

$$\text{SENSITIVITY} = 1 - \frac{\text{Number of False Negative}}{\text{Number of True Positive + Number of False Negative}}$$

CHAPTER FOUR

RESULTS

4.1.0 PREVALENCE AND RISK FACTORS OF *H. PYLORI* TRANSMISSION

Three hundred and fifty six subjects (168 males and 188 females) were enrolled in the study to detect *H. pylori* antigens in faecal samples. The variables linking epidemiological risk factors and prevalence of the organism as analyzed by the univariate analysis are shown in Table 1. *H. pylori* antigen was detected in 316 subjects, yielding an overall prevalence of 88.8%. The prevalence increased with age from 75.9% in children less than 12 years to 100% in the age group 13-24 years, young adults aged 25-47 years and subjects aged ≥ 60 years ($P < .05$; OR = 0.52, 95% CI: 0.08-2.75). The prevalence was higher in females than in males. Of 188 females who participated in the study, *H. pylori* antigen was detected in 172 (91.5%) against 144 (85.7%) of 168 males ($P > .05$; OR = 0.56, 95% CI: 0.11-2.58), which did not reach statistical significance.

H. pylori prevalence was higher in those who used river/other water (94%) than in those who used tap water (87%) ($P > .05$; OR = 2.47, 95% CI: 0.30- 1.14). The organism was more prevalent when more than two persons shared a bed (94.9%) than in one person per bed (84.2%) ($P > .05$; OR = 0.29, 95% CI: 0.02-2.81). Interestingly, *H. pylori* antigen was detected more (100%) in subjects of the high socioeconomic group than in those of the low socio-economic group (85.9%).

However, this did not reach statistical significance (P > .05; OR = 0.36, 95% CI: 0.01-2.97).

Table 1. Association between epidemiological risk factors and *Helicobacter pylori* infection (univariate analysis)

Variable	Total subjects	*H. pylori*-positive	OR (95% Cl)	χ^2-test	P-value
Age (Years)	(N)	N (%)	0.52 (0.08-2.75)	9.973	0.04
≤12	116	88 (75.9)			
13-24	12	12 (100)			
25-47	108	108 (100)			
48-60	84	72 (85.7)			
≥60	36	36 (100)			
All	356	316 (88.8)			
Gender			0.56 (0.11-2.58)	0.742	0.389
Male	168	144 (85.7)			
Female	188	172 (91.5)			
Water source			2.05(0.29-1.21)	0.714	0.387
River	69	64(92.8)			
Tap	287	248(86.4)			
Toilet type			1.21(0.23-5.61)	0.080	0.777
Non-flush	228	204(90)			
Flush	128	112(88)			
Socioeconomic status			0.36(0.01-2.97)	1.981	0.371
Low	256	220 (85.9)			
Medium	72	68 (94.4)			
High	28	28 (100)			
Suck fingers			4.9(2.57-9.39)	30.01	<0.001
Yes	259	238(91.9)			
No	97	68(70.1)			

CI, Confidence interval; OR, Odd ratio; P-value, value for significance (OR=4.9, 95% CI: 2.57-9.39)

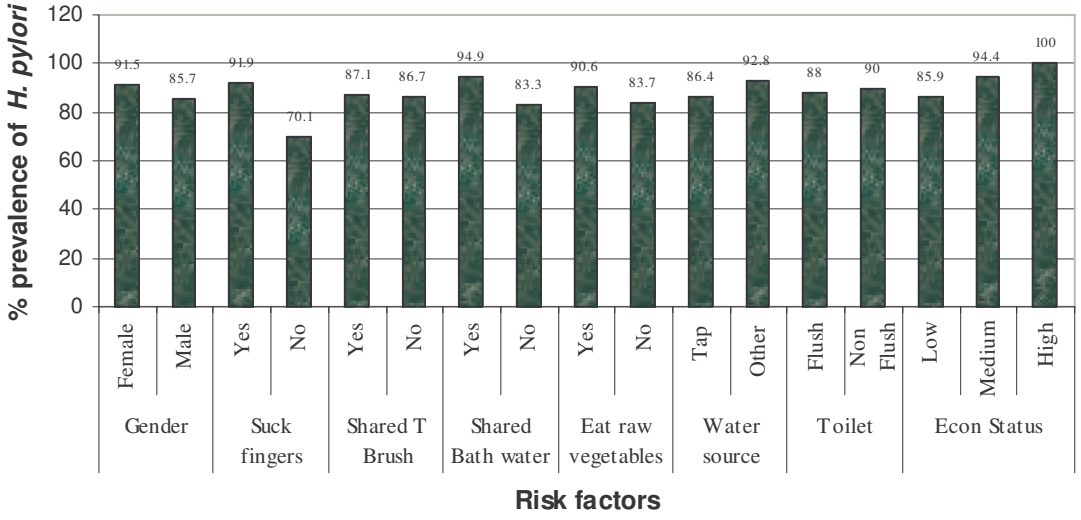

Figure.1. Association between percentage *Helicobacter pylori* prevalence and risk factors.

Figure.1 above also shows that a high percentage of 91.9% infected with the organism had a habit of sucking fingers and the OR of being *H. pylori* positive was significantly high (P<0.001; OR=4.9, 95% CI: 2.57-9.39). Sharing tooth brush was also linked with a high prevalence of *H. pylori* even though it was not statistically significant (P>0.05). Eating raw vegetables increased the risk of accessing the infection (OR=1.92, 95% CI: 0.98-3.76, P=0.04).

4.2.0 QUANTITATIVE ANALYSIS OF LEVEL OF INFECTION.

The level of infection increased with age (Figure. 2). Infection levels increased gradually from the first few months of life to about 25 years of age after which a marked increase in the level of infection was observed. High levels of *H. pylori* infection were also recorded in subjects residing in rural areas as compared to low levels of infection in those in semi-urban areas (Figure. 3).

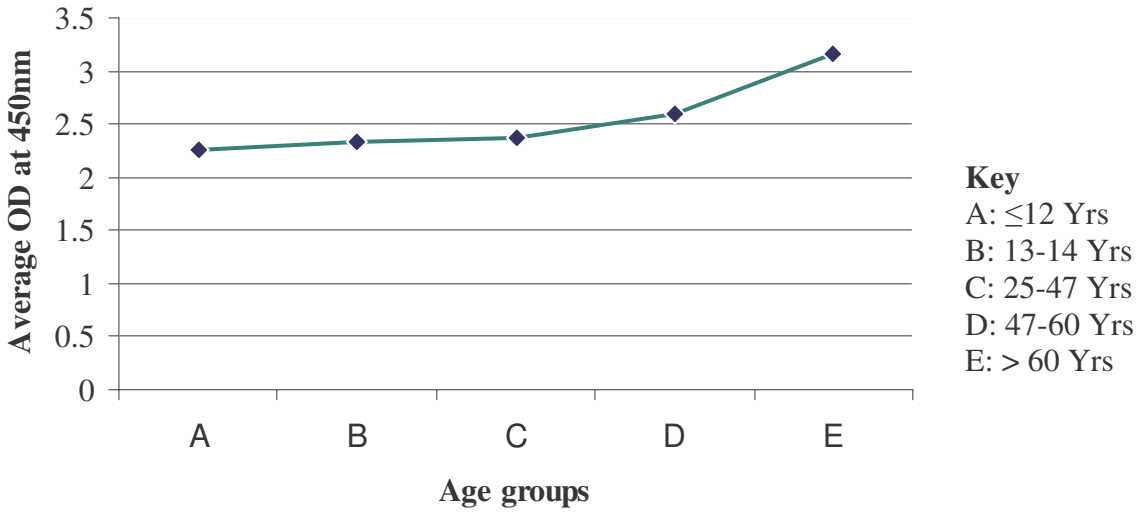

Figure. 2 Average Optical Density (OD) of antigenemia at 450nm per age group

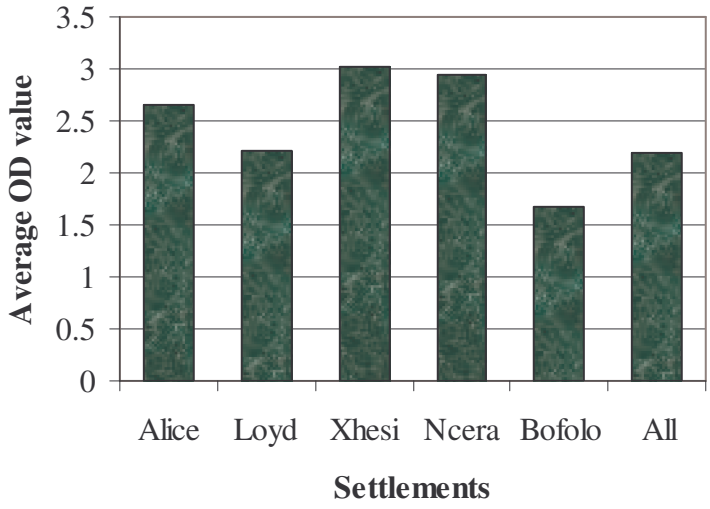

Figure. 3 Average Optical Density (OD) of antigenemia at 450nm per area.

4.3.0 DETECTION OF *H. PYLORI* DNA BY PCR.

Faecal samples for DNA analysis were selected based on their high optical density (OD) values (spectrophotometrically) of being positive by the HpSA test.

The target was the 1.1 Kb fragment of the *glm*M gene known to be highly conserved in *H. pylori*. To reduce chances of false negative results due to PCR inhibitors contained in faecal samples, bovine serum albumin (BSA) was added at a concentration of 0.1µg/µL as a constituent of the 50µL PCR reaction mixture. Sixteen (66.7%) of twenty four faecal samples that had previously tested positive for the organism by HpSA test were confirmed positive by PCR (Figure.4 and Table 2).

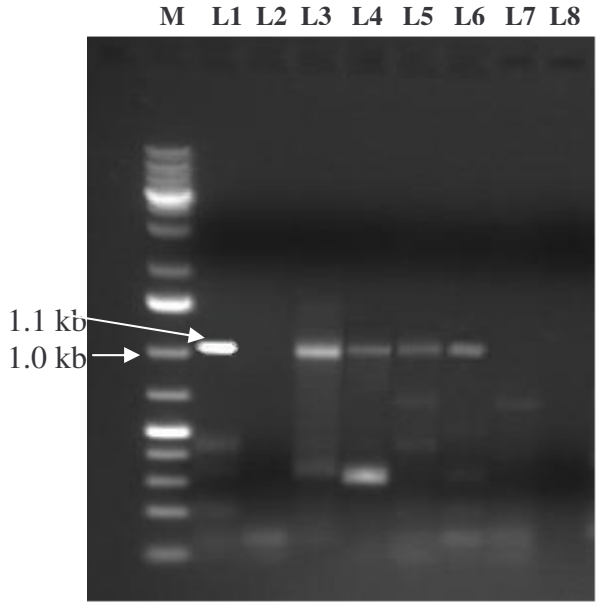

Figure. 4. Gel photograph showing specific detection of *H. pylori* DNA in stool specimens. M-1 Kb plus molecular weight marker, L1-positive control, L8-negative control, L2-L7, DNA from stool samples.

Table 2. HpSA test and detection of *glm*M gene by PCR in human faecal samples.

Test	Faecal Sample Number																									
	R107	R18	R29	R32	R43	R48	R70	R101	R127	R144	R191	R206	R213	R219	R248	R261	R285	R297	R305	R316	R330	R343	R349	R354	+ve Control	-ve Control
HpSA test	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
PCR (*glm*M gene)	+	+	-	+	-	+	+	-	+	+	+	-	+	-	+	+	-	+	+	+	+	-	-	+	+	-

Water samples analyzed were all collected directly from taps at the point of use. None of the samples tested positive (Table. 3). Both the positive and negative controls were always accurate in the two separate incidences when PCR was done after sampling (Figure. 5).

Table 3. PCR analysis of DNA eluates extracted from direct tap water samples at the point of use in Nkonkobe Municipality.

	Water samples						
Rep/Primer	Alice	Xhesi	Ncera	Loyd	KwaSakhi	+ve Control	-ve Control
Week 1. *glm*M gene-PCR.	-	-	-	-	-	+	-
Week 2. *glm*M gene-PCR.	-	-	-	-	-	+	-

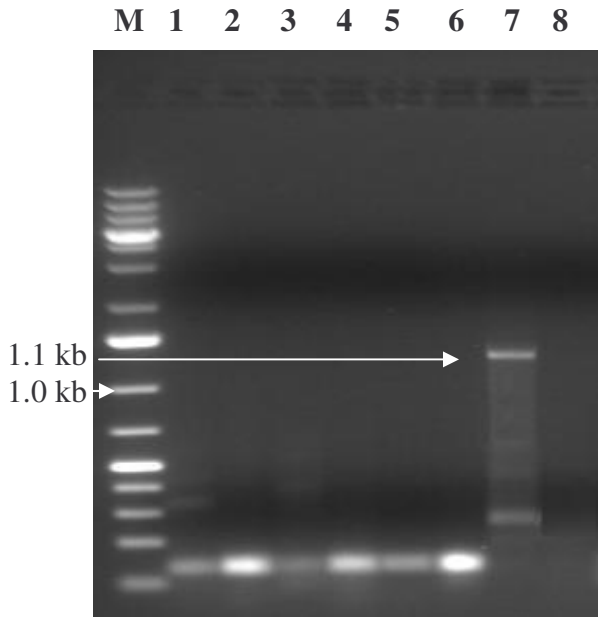

Figure. 5. Gel photograph showing failure to detect *H. pylori* DNA from treated tap water samples. M-1 Kb plus molecular weight marker, L1-L6, DNA from water samples, L7 and L8, positive and negative controls respectively.

4.4.0 PRECISION RATE OF THE DIAGNOSTIC TESTS USED.

With regard to the HpSA test, the post-test probability of presence or absence of the antigen was calculated based on the three hundred and fifty six HpSA results obtained (168 from males faecal samples and 188 from female faecal samples). Three hundred and twenty (320) samples were true positives (TP), four (4) samples were false positives (FP), thirty two (32) samples were true negatives (TN) and none was false negative. This test had a sensitivity and specificity of 100% and 88.8% respectively. The positive predictive value (PPV) and negative

predictive value (NPV) of this method were both 100%. The sensitivity, specificity and predictive values of the HpSA test are shown in Table 4.

Detection of *H. pylori* from faecal samples by PCR had a low sensitivity (66.6%), the specificity of the method was 100%. Sixteen (16) samples were TP, zero were FP, the PPV was also 66.6%. The NPV was 0% since all the samples were positive for the organism by HpSA test as shown in Table 4. The values for the use of PCR in the detection of *H. pylori* DNA in water samples are also presented in Table 4.

Table 4. Performance of the HpSA test and PCR for detection of *H. pylori* antigen in faecal and water samples.

TEST	Type of sample	TP	FP	TN	FN	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
HpSA	Faecal sample	320	4	32	0	100	88.8	100	100
PCR	Faecal samples	16	0	0	8	66.6	100	66.6	0
PCR	Water samples	0	0	20	0	100	100	0	100

CHAPTER FIVE

DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.1.0 DISCUSSION

Reports have indicated substantial evidence for the acquisition of *H. pylori* primary infection at early age, both in developed and developing countries (Pelser *et al*., 1997; Ndip *et al.*, 2004; Alborzia *et al*., 2006; Ahmed *et al.*, 2007). The mode of *H. pylori* transmission however remains controversial. In this study, the HpSA test was used to detect *H. pylori* antigen. The test is a reliable non-invasive method for detecting *H. pylori* antigens in stool specimens because active infection can be delineated. This method has also been shown to have high sensitivity and a positive predictive value for the detection of *H. pylori* infection, especially in asymptomatic individuals (Ndip *et al.*, 2004; Shepherd *et al.*, 2000), which is in line with the results of the present study.

The prevalence of *H. pylori* of 88.8% (316 of 356) found in this study is higher than the 40%-76% reported in other studies conducted in developing countries (Pelser *et al.*, 1997; Figueroa *et al*., 2002). Our study supports the findings of Rothenbacher and Brenner (1999) because most of our subjects had no clinical symptoms. Additionally, variants of the interferon-*y* gene (IFNGR1), which was however not investigated in this study but which have been reported to be more prevalent in Africans and play a significant role in human *H. pylori* infection (Thye *et al.*, 2003), might contribute to explain the high prevalence and the

relatively low pathogenesis of *H. pylori* in Africa. Of interest may also be a shift in immune response from Th1 to Th2 responses following co-infection with parasites and or bacterial infections in early childhood, which may persist into adult-hood (Mbulaiteye *et al.*, 2006), a situation common in our environment that may to some extent explain the paradoxical lower gastric cancer risk in this population.

Prior studies have reported the importance of age, gender, poor household living conditions, and low socioeconomic status as predictors for the acquisition of *H. pylori* infection in children (Bardhan, 1997; Alborzia *et al*., 2006). In our study, the frequency of infection increased with age from 75.9% in children (≤ 12 years), to 100% in teenagers (13-24 years), 100% in young adults (25-47years), and 100% in old people (≥ 60 years). This difference was statistically significant (P = .04). The OR (OR=0.52, 95% CI: 0.08-2.75), however indicated that an association was likely between an increase in age and *H. pylori* colonization. That is, children are equally at risk of acquiring infection as others. This finding corroborates previous studies (Holcombe *et al*., 1992; Pelser *et al*., 1997; Ndip *et al*., 2004). In their study, Holcombe *et al*. (1997) documented that *H. pylori* infection is common from an early age in the developing world where most children are colonized by the age of 10 years. In a study in South West Cameroon, Ndip *et al*. (2004) reported a high prevalence (71.05%) of *H. pylori* colonization in children 7-10 years old. A high prevalence in the teen years was also recorded in a study in Tanzania (Mbulaiteye *et al*., 2006).

The findings of this study therefore provide an emphasis on the direction of research encompassing the mode of transmission of *H. pylori* among human subjects. Since infection initiates early in life and endures lifelong if not treated, prevalence of 100% in teens suggest that to get more meaningful results backed by proper controls, risk factors should be assessed in young children especially from birth till time of infection. This is supported by the study done by Jun-ling *et al*. (1998). In their study, a high prevalence of 85% at the age group 3-4 and 67% at the age group 11-12 respectively were recorded. The findings of this study are consistent with prior studies of *H. pylori* prevalence in highly endemic areas in the developing world.

H. pylori colonization was higher in females than in males, but did not reach statistical significance (P = .039). This result is not in harmony with the findings of previous reports (Moayyedi *et al*., 2002; Ndip *et al*., 2004), in which a higher prevalence rate was reported in males than in females. Kaltenthaler *et al*. (1995) reported that *H. pylori* infection is generally higher in males than in females and suggested that this might relate to young boys having poorer hygiene than young girls because infection is acquired at an early age. We therefore think that our divergent observation in this study could be due to the different sample sizes used (more females participated than males).

In this study, we interestingly found an inverse relation between *H. pylori* prevalence and socio-economic status, but this difference was not statistically

significant (P = .371). The OR (OR = 0.360, 95% CI: 0.01-2.97) showed that socio-economic status was not associated with a high prevalence of *H. pylori*. This result is not in agreement with prior studies (Ndip *et al*., 2004; Alborzia *et al*., 2006). We are constrained to think that the trend observed in our present study may be misleading because our sample sizes were largely disproportionate, with 256 subjects belonging to the low socioeconomic group as opposed to 28 in the high socioeconomic group.

Household crowding has been reported to be a risk factor for infection (Alborzia *et al*., 2006; Fritz *et al.*, 2006) and bedroom sharing was a major factor in these studies. In our study, we found a higher prevalence with an increase of people sharing the same bed although this too was not statistically significant (P = .272). The OR (OR = .29; 95% CI: 0.02-2.81) indicated that bed sharing is not a likely risk factor for colonization, which is in contrast to the studies of Alborzia *et al*. (2006) and Frietz *et al*. (2006). This difference may be due to the small sample size used in our study. Aguemon *et al*. (2005) in their study in Benin also did not find bedroom sharing as a risk factor, which lays credence to our present study.

Subjects referred for upper gastrointestinal endoscopy in Hyderabad, South India, who ate raw vegetables were found to be at a higher risk of infection (Ahmed *et al*., 2006). A similar suggestion had been made previously that consumption of raw vegetables could have led to a high risk of infection in Chile (Hopkins *et al.*, 1993). A comparable observation was made in this study; consumption of raw

vegetables exposed the population at a higher risk (OR=1.92; 95% CI: 0.98-3.76) and was found to be statistically significant as a risk factor of *H. pylori* infection. (χ^2=4.29, P-value=0.04). These findings supports the notion of oral-oral or faecal oral route with or without intermediate vectors of transmission which is thought to be the primary route of transmission (Vaira *et al*., 2001; Delport *et al.,* 2006).

H. pylori prevalence was lower (86.4%) in those who used tap water compared with those who used river water (92.8%), but this was not statistically significant (P = .387) (OR = 2.05; 95% CI: 0.29-1.21). Similarly, those who used a flush toilet had a lower prevalence (88%) compared with those who used non-flush toilet (90%), but again, this was not statistically significant (P = .777) (OR = 1.21; 95% CI: 0.23-5.61). The odd ratios indicated, however, that those who use river water and non-flush toilets are more likely to be colonized by the organism.

Within countries, there may be a similarly wide variation in the prevalence between more affluent urban populations and rural population (Aguemon *et al*., 2005). A lack of proper sanitation, drinking water and basic hygiene, as well as poor diet and overcrowding, all play a role in determining the overall prevalence of infection (Ndip *et al*., 2004).

Detection of the antigen in faecal samples by the HpSA test and PCR provided solid evidence of a possible faecal-oral route with or without transmission vectors in the studied population. Sixteen (66.7%) of twenty four faecal samples that had

previously tested positive for the organism by HpSA test were confirmed positive by PCR (Table 2). The HpSA test recorded a sensitivity of 100%, far much higher than a sensitivity of 66.6% recorded by PCR in the detection of *H. pylori* from the same faecal samples. This data is consistent with the findings of other researchers (Shuber *et al.*, 2002; Lottspeich *et al*., 2007; Falsafi *et al*., 2009).

The differences noted in the outcomes or sensitivity of the two techniques on faecal samples could be the influence of PCR inhibitors and probably, the patchy distribution of this bacterium in faecal samples. Since the HpSA and PCR were not done concurrently, freezing and thawing of samples might have also influenced the results of the later experiments using PCR. Also, an unexpected difference in the amplification rate of PCR products was observed from sample to sample. This could be due to low target DNA or differences in the quality and quantity of inhibitors that escaped the PCR inhibitor removal system of the DNA extraction procedure.

A variety of inhibitors have been characterized in faecal samples e.g. glycogen, fats, phenolic compounds, cellulose constituents of bacterial cells, heavy metals and non-target nucleic acids (Monteiro *et al*., 1997; Lofstrom *et al*., 2004). In this study, non-target nucleic acids might have been the most influential inhibitor since total genomic DNA extract was being analyzed. With the awareness that the selection of nucleic acid extraction method is a trade-off between sensitivity of the nucleic acid extraction method and the efficiency of removal of PCR

inhibitors (Hale *et al*., 1996), culturing and purifying bacterial colonies before DNA extraction could be of importance. However, culturing *H. pylori* from stool samples has proved difficult with divergent results (Thomas *et al*., 1992; MacKay *et al*., 2003; Ndip *et al*., 2003). Biopsies obtained from the antrum and corpus of the human stomach could be of help. However the major disadvantage of biopsy method over direct stool sample is that it is invasive, expensive, uncomfortable and therefore not suitable for epidemiological studies especially involving children (MacKay *et al*., 2003).

Some studies have highlighted the presence of *H. pylori* in water ranging from highly faecal polluted sewage water to tap water (Sasaki *et al*., 1999; Park *et al*., 2001; Brown *et al*., 2002; Fugimura *et al*., 2004; Queralt *et al*., 2005; Ahmed *et al.*, 2006; Ahmed *et al*., 2007; Braganca *et al*., 2007; Moreno *et al*., 2007). This organism has been found to survive in river water for several months in a coccoid form which is viable but non-culturable (Brown, 2000; Adams *et al.*, 2004). Biofilms also play a crucial role in maintaining the viability of *H. pylori* in water by providing a localized environment suitable for survival during adverse conditions (MacKay *et al*., 1998). Biofilms are an association of species entrapped within a polysaccharide matrix (Momba *et al*., 2000). Park *et al*. (2001) successfully isolated *H. pylori* from biofilms in municipal water pipelines treated with chlorine.

Based on the data collected by means of a questionnaire used in this study, the high prevalence of the antigen in faecal samples (which may be related to water sources such as tap, dam, river, borehole) unavoidably led to the hypothesis that, water sources play a role in the transmission of *H. pylori* in the Nkonkobe Municipality. To test this hypothesis, PCR was employed to detect *H. pylori* DNA in positive faecal samples of the study subjects residing in Nkonkobe Municipality; and water samples obtained from water for domestic use within human settlements within the municipality. However, *H. pylori* DNA was not detected in any of the tap water samples collected in Nkonkobe Municipality even though a recent study had highlighted a faecal coliform count ranging up to 25 CFU/100 mL at the point of use, thereby highlighting failure of rural water treatment plants to supply safe drinking water (Momba *et al*., 2006). To be certain about these findings, several control experiments having positive and negative controls were done to confirm the results. In their study, Queralt *et al*. (2004) provided similar findings when they failed to detect *ure*A gene of *H. pylori* in 19 spring water samples with low levels of faecal contamination, ranging from >0.1 to 2.5 x10^2 CFU/100 mL. This is in line with the results of the present study since the urease (u*re*C) gene also known as the *glm*M gene, a highly conserved gene in this organism was the target.

Also another study conducted in a high-endemic area in Dhaka, Bangladesh, using a highly sensitive Real-Time PCR assay, revealed negative results in the detection of *H. pylori* DNA from drinking and environmental water (Janzon *et al*., 2009).

The probable explanation for failure to detect the organism could be that since this organism is not an intestinal pathogen, there is the likelihood that it has a low concentration in stool samples; in conjunction with terminated reproductivity or loss of viability outside the human host, its concentration in water will be too low (Moreno *et al*., 2007).

However, other studies have illustrated success in detection of *H. pylori* in water including chlorinated drinking water (Krumbiegel *et al*., 2004; Queralt *et al*., 2005; Braganca *et al*., 2007). It may seem that other predisposing factors play an important role in the detection of *H. pylori* DNA in water therefore enhancing water as a probable vehicle for *H. pylori* transmission.

5.2.0 CONCLUSION

From the results obtained in this study, the following conclusions can be drawn:

1. This study has revealed a high prevalence of *H. pylori* antigens (88.8%) from faecal specimen of asymptomatic individuals with acquisition commencing during early childhood (infection was detected at 4 months).
2. There is a possible association between *H. pylori* prevalence and; poor socioeconomic status and poor household hygiene including sharing bath water, habit of eating raw vegetables and sucking fingers.
3. Our hypothesis that water is a risk factor for *H. pylori* transmission in the study locality was not confirmed. This however does not rule out water as a vehicle of

H. pylori transmission as any contamination during water collection or during storage avails *H. pylori* to infect its human host.

4. Results demonstrate the usefulness and high sensitivity of the HpSA antigen test as a noninvasive diagnostic tool of *H. pylori* infection in the studied population.
5. *H. pylori* DNA was detected in HpSA positive faecal samples an indication of active infection in the study population.

5.2.1 PROSPECTIVE STUDIES/RECOMMENDATIONS

Similar studies need to be done in areas that use surface wells, river, dams, boreholes and tap water to ascertain the role of water in the transmission of *H. pylori* by using more elaborate and discriminatory molecular based approaches such as PCR-RFLP and Real Time PCR. The combination of DNA sequencing will bring better and meaningful results as far as discriminatory index is concerned. Increasing sample size and probably sampling throughout the year will improve the epidemiological characterization of *H. pylori*. We also recommend that to attain much more meaningful results, the study of risk factors and modes of transmission of *H. pylori* should be focused from birth until time of infection. That is, a cohort study will reveal details of the dynamics of *H. pylori* infection in the studied population.

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APPENDICES

APPENDIX 1

ETHICAL CLEARANCE

Two ethical clearances for this study were obtained from the Goven Mbeki Research and Development Center (GMRC) at the University of Fort Hare and the Department of Health in the Eastern Cape Province.

University of Fort Hare
Together in Excellence

GOVAN MBEKI RESEARCH AND DEVELOPMENT CENTRE
Private Bag X1314, ALICE, 5700; Tel: 040-6022319; E-fax: 086 628 2842
E-mail: pstrijdom@ufh.ac.za or rflanegan@ufh.ac.za

16 January 2008

TO WHOM IT MAY CONCERN

I declare that I have reviewed the attached Research Protocol with attachments of Prof Roland N Ndip, entitled "Prevalence and transmission of *Helicobacter pylori* in the Eastern Cape Province: Impact of water sources and household hygiene", which will be conducted under the auspices of the University of Fort Hare, Alice, South Africa.

The research, which does involve subjugation of humans as research objects, has been judged to be relevant, designed in accordance with accepted scientific practices and norms, as well as – particularly – in harmony with universally accepted international standards and ethical practice in its use of human persons as subjects of research and is in the opinion of the reviewer likely to be successful in achieving its objective.

The researcher has designed purpose-specific informed consent forms which are simple, properly designed and user-friendly in order to protect the interests of human subjects, enabling their understanding of all implications of consent to participate.

Yours sincerely

Dr Petrus DF Strijdom
Acting Dean of Research & Development

ECDoH-Res0002_

Ikamva elihle

Eastern Cape Department of Health

Enquiries: Zonwabele P. Merile
Tel No: 040 609 3408
Fax No. 040 609 3784
Date: 12th June 2008
e-mail address: zonwabele.merile@impilo.ecprov.gov.za

Dear Prof. Roland N. Ndip

Re: Prevalence and transmission of Helicobacter pylori in the Eastern Cape Province: Impact of water sources and household hygiene

The Department of Health would like to inform you that your application for conducting a research on the abovementioned topic has been approved based on the following conditions:

1. During your study, you will follow the submitted protocol and can only deviate from it after having a written approval from the Department of Health in writing.
2. You are advised to ensure observe and respect the rights and culture of your research participants and maintain confidentiality and shall remove or not collect any information which can be used to link the participants. You will not impose or force individuals or possible research participants to participate in your study. Research participants have a right to withdraw anytime they want to.
3. The Department of Health expects you to provide a progress on your study every 3 months (from date you received this letter) in writing.
4. At the end of your study, you will be expected to send a full written report with your findings and implementable recommendations to the Epidemiological Research & Surveillance Management. You may be invited to the department to come and present your research findings with your implementable recommendations.

Your compliance in this regard will be highly appreciated.

DEPUTY DIRECTOR: EPIDEMIOLOGICAL RESEARCH & SURVEILLANCE MANAGEMENT

31-ENE-1992; 12:48 From: To:+1000 P.2

APPENDIX 2

CONSENT FORM

Title of Study: **Prevalence and transmission of *Helicobacter pylori* in the Eastern Cape Province: Impact of water sources and household hygiene.**

I,…….. (Name) having full capacity to consent for myself, an adult, or a child named …… (subject's name) and having attained my …….. birthday, do herby consent to my/his/her participation in the research study: Mentioned above. Understanding disease and Transmission. " under the direction of Prof. Roland Ndip . The methods and means by which the study will be conducted and the risks which may be reasonably expected have been explained to me by ……... I have been given the opportunity to ask questions concerning this investigational study, and any such questions have been answered to my full and complete satisfaction.

Subject's or guardian's Signature: ……. Date:…….

Permanent Address: ……Tel:…….
…….
…….
Witness'sName: …….
Witness's Signature: …… Date: …….
Study Number: …….

NOTE; You may at any time during the course of this study withdraw this consent and remove yourself/your child from the study without prejudice.

ASSENT FORM AGREEMENT FOR INDIVIDUALS 2 THROUGH 17 YEARS OF AGE

Title of Study: Prevalence and transmission of *Helicobacter pylori* in the Eastern Cape Province: Impact of water sources and household hygiene.

PURPOSE: Stool sample will be taken for *H. pylori* detection.

PARTICIPATION: You do not have to be part of this study, participation is voluntary. If you do agree to be in the study, your participation can help find what germs are causing you to be sick.

WHAT YOU WILL EXPERIENCE:

RISKS: There are no risks since you will pass the stool normally.

BENEFITS: There is no direct benefit to you (you child) aside from the satisfaction that your (your child's) participation may help to better understand this type of disease in South Africa in the future.

ASSURANCE OF CONFIDENTIALITY OF VOLUNTEERS'S IDENTITY: Records relating to your participation in the study will remain confidential. Your name will not be used in any report resulting from this

study. All computerized records and laboratory specimens will only have a unique study number, not your name.

I,……...(Name) am …….. years old and do hereby assent to being in the research study: " Genotypes of *H. pylori* circulating in South Africa: Understanding disease and Transmission " under the direction of Prof Roland Ndip. You may at any time change your mind and withdraw yourself from the study.

Subject's name: ……..
Subject's signature:…….Date:……
Permanent Address:…….Tel:……..
…….
…….
Witness's Signature: ……. Date: ……..

Study Number: …….

APPENDIX 3

QUESTIONNAIRE

THE QUESTIONS MAY BE ANSWERED BY TICKING IN THE APPROPRIATE BOX OR BY WRITING IN THE SPACE PROVIDED

I.D. No: ……Today's date:…….

SECTION A: INDIVIDUAL

1. Name: …….
2. Date of Birth: ……
3. Sex: …….
4. Country of Birth: ....…….
5. If you were born out of South Africa, at what age did you move to S. Africa? ……..

SECTION B: THE FAMILY AND HOME

6. Usual address: ……

   ……
   …….
   ……

7. How long have you lived at this address?

   Less than 6 months ☐
   6 months but less than 2 years
   2 years but less than 5 years ☐
   5 years or longer

8. If less than 2 years, please give previous address: ☐

   ☐

   …….
   ……
   ……..

9. How long did you live at the previous address? ……

10. Please provide the details of people who lived with you in your family home using the table below.

What is the relationship of This person to you?	Age	Gender Male/Female	How long has this person lived with you?

11. What is the country of birth of your parent(s)?

    Mother: …… Father:……..

12. If born outside South Africa, at which age did your parents move to South Africa?

    Mother: …… Father: …….

SECTION C: SOME MORE INFORMATION ABOUT YOUR HOME

13. How many rooms are there in total (not including toilets, bathrooms or half landings)? ……..

14. How many of these are bedrooms?

15. How many people share a bedroom?

16. When you were 5 years of age or younger, did you share a bedroom?

    Yes ☐ No ☐

17. What was your age when solid/semi-solid foods were first introduced into your diet? ….……

18. As a baby/infant did you suck your thumb/fingers?

    Yes ☐ No ☐

SECTION D: SOME MORE INFORMATION ABOUT SOCIAL LIFE

19. In your house do you share or use one another's toothbrushes?

    Yes ☐ No ☐ Don't know ☐

20. Do you bath in the same water?

    Yes ☐ No ☐

21. Are you on any regular medication?

    Yes ☐ No ☐

22. If yes, please specify the medication and it's purpose:
    ……...……
    ……
    ……

23. Have you had any course of antibiotics for any reason in the last 3 months?

    Yes ☐ No ☐

24. Eating

    a) Do you eat home cooked food or outlet cooked food?
       ……..

    b) Do you like eating raw vegetables?
       ……..

    c) If yes, you get them from:

       Street vendors ☐ Modernized suppliers ☐

25. Drinking water:

    a) Until 5 years of age, did you drink borehole, tap water or well water?
       ……

    b) Between 6 and 20 years? …….

c) At present if you are an adult? ……

26. Lavatory:

a) Until the age of 5, did you use flush or non flush toilet? ……

b) Between 6 and 20 years of age? ……

c) At present if you are an adult? …….

27. Drinking habit:

At what age did you:

a) Start drinking alcohol? ……

b) Drank little/occasional? …….

c) Stop drinking alcohol? ……..

d) Never drank alcohol? …….

28. Smoking habit?

At what age did you

a) Start smoking? ……How many pulls a day? ……

b) Stopped smoking?…….

c) Never smoked. …….

29. Have you ever had a gastric or duodenal disease before?

Yes ☐ No ☐

30. If Yes, was it (please tick correct)

Gastric ulcer ☐ Gastritis ☐ Duodenal ulcer ☐ Others ☐

31. What is your education level (please tick correct)?

Basic ☐ Secondary ☐ Tertiary ☐

32. What is your economic status (please tick correct)?

High ☐ Medium ☐ Low ☐

33. What is your occupation? .....

APPENDIX 4

PUBLICATIONS, CONFERENCE PRESENTATION AND MANUSCRIPT IN PREPARATION.

1. Publications.

a) Dube C., Tanih N.F. and Ndip R.N. (2009a). *Helicobacter pylori* in water sources: A global environmental concern. Reviews on Environmental Health. 24(1):1-14.

b) Dube C., Tanih N.F., Clarke A.M., Mkwetshana N., Green E. and Ndip R.N. (2009b). *Helicobacter pylori* infection and transmission in Africa: Household hygiene and water sources as plausible factors exacerbating spread. African Journal of Biotechnology. 8(22):6028-6035.

c) Dube C., Nkosi T.C., Clarke A.M., Mkwetshana N., Green E. and Ndip R.N. (2009c). *Helicobacter pylori* antigenemia in an asymptomatic population of Eastern Cape Province, South Africa: Public health implications. Reviews on Environmental Health. 24(3):249-255.

2. Conference presentation

Dube C., Nkosi T.C., Clarke A.M., Mkwetshana N., Green E. and Ndip R.N. (2009). *Helicobacter pylori* antigenemia in an asymptomatic population of the Eastern Cape Province of South Africa: an indication of socioeconomic status:

Paper presented at the Bio2Biz SA 2009 and SASM 09 Conference, ICC, Durban, South Africa, 20-23 September 2009.

3. Manuscript in preparation.

Detection of *Helicobacter pylori* DNA by PCR in faecal samples and tap water in the Nkonkobe Municipality.

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Helicobacter pylori in Water Sources: A Global Environmental Health Concern

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Abstract: *Helicobacter pylori* are Gram-negative micro-aerophilic motile curve rods that inhabit the gastric mucosa of the human stomach. The bacterium chronically infects billions of people worldwide and is one of the most genetically diverse of bacterial species. More than half of the world population in both developed and developing countries are infected with this organism. Infection usually occurs without overt clinical symptoms, particularly in poor communities. If untreated, the infection can last for decades without causing symptoms. In some communities, however, infection with the organism causes peptic and duodenal ulcers , gastritis, duodenitis, and gastric cancers. How *H. pylori* initially enters the stomach is not known, but contaminated food particles and water are suspected, with the former physically shielding it from stomach acid. Similarly, the route of transmission of this pathogen is unknown. Several reports have suggested the possibility of waterborne transmission as the organism can survive for a few days in fresh cold water, salt water, distilled water, and tap water. Knowledge of the epidemiology and mode of transmission of *H. pylori* is important to prevent its spread and may be useful in identifying high risk populations.

Keywords: *Helicobacter* epidemiology, waterborne transmission, genetic factors,

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INTRODUCTION

Helicobacter pylori are Gram-negative micro-aerophilic motile curve rods that inhabit the gastric mucosa of the human stomach. The bacterium chronically infects billions of people worldwide and is one of the most genetically diverse of bacterial species /1/. Infection with the organism has been shown to follow geographic, age, race, ethnic, and socio-demographic patterns, with a higher prevalence in developing than in developed countries /2-4/.

Infection with this bacterium is generally asymptomatic, and the majority of those infected do not develop clinical symptoms. The known clinical diseases associated with infection, gastritis, duodenal ulcer, gastric lymphoma, and gastric cancer /1,4,5/, constitute a global public health problem in developed and developing countries.

The route of transmission of this pathogen is still unknown /4/. Understanding the route is important if public health measures to prevent its spread are to be implemented. Person-to-person transmission seems most likely as the only place where this pathogen has been isolated is the human gastrointestinal tract /4/. Research on direct person-to-person transmission is, however, failing to provide substantial evidence to account for all

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Review

Helicobacter pylori infection and transmission in Africa: Household hygiene and water sources are plausible factors exacerbating spread

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***Helicobacter pylori* (*H. pylori*) is a microaerophilic motile curve rod that inhabits the gastric mucosa of the human stomach. The organism chronically infects billions of people worldwide and is one of the most genetically diverse of bacterial species. Infection with the bacterium which leads to chronic gastritis, peptic ulceration, gastric cancers and gastric malt lymphoma has been reported to follow a pattern linked to geographic and socio-demographic factors. Studies have documented a higher prevalence in Africa than elsewhere although the pathological outcomes do not correlate with infection. *H. pylori* transmission pathways are still vague, but the risks of transmission include precarious hygiene standards, over-crowding and contaminated environment and water sources amongst others. The possible routes of transmission include oral-oral, faecal-oral and person- to -person, either with or without transitional transmission steps during episodes of diarrhoea or gastro-oral contact in the event of vomiting. Use of contaminated water including municipal tap water has also been suspected to have a high impact in the transmission of the organism. To generate the data presented in this paper, we conducted an internet based search on relevant literature pertaining to *H. pylori* epidemiology in general and Africa in particular. Sites such as Pubmed, AJOL, Scopus and Goggle scholar were mainly used. This paper therefore attempts to appraise the role of household hygiene and water sources in the transmission of this organism in the developing world context.**

Keywords: *H. pylori,* Africa, prevalence, transmission, socio-economic factors, environmental factors, hygiene.

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Helicobacter pylori Antigenemia in an Asymptomatic Population of Eastern Cape Province, South Africa: Public Health Implications

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Abstract: Helicobacter pylori is a major cause of such upper gastrointestinal diseases as gastritis, peptic ulcer, and gastric cancer. The risk of infection is increased in those living in the developing world, which has been ascribed to precarious hygiene standards, crowded households, and deficient sanitation common in this part of the world. Fecal samples were collected from 356 apparently healthy subjects, consisting of 168 males and 188 females aged from 3 months to > 60 years (Mean = 31 years). A standardized questionnaire describing demographic characteristic including age, sex, household hygiene, socioeconomic status, and so on was applied. A sandwich-type enzyme immunoassay amplification technology (Amplified IDEIA™ Hp StAR™, Oxoid, UK) was used to analyze the fecal samples for the detection of//, *pylori* antigens using monoclonal antibodies specific for *H. pylori* antigens. Fisher's exact test was used to assess the univariate association between *H. pylori* infection and the possible risk factors. Odds ratio (OR) and the corresponding 95% confidence interval (CI) were calculated to measure the strength of association using EPI INFO 3.41 package. P values of < .05 were required for significance. *H. pylori* antigen was detected in 309 of the 356 subjects giving an overall prevalence of 86.8%. Prevalence increased with age from 75.9% in children < 12 years age to 100% in young adults aged 25-47 years and subjects aged > 60 years (P < .05). *H. pylori* prevalence was higher in females than in males. Of 188 females who participated in the study, *H. pylori* antigen was detected in 172 (91.5%) versus 144 (85.7%) 168 males (P > .05). Interestingly, *H pylori* antigen was detected more often (100%) in the high socioeconomic group than in those of low socioeconomic group (85.9%) (P > .05). The results of this study have revealed a high prevalence of *H. pylori* antigens in fecal samples of asymptomatic individuals in the Nkonkobe municipality, an indication of active infection. Socioeconomic status, contaminated water, and poor sanitation may play a role in *H. pylori* transmission in this population. This finding is of public health and epidemiologic significance.

Keywords: Helicobacter pylori, antigenemia, socioeconomic status, sanitation, epidemiology

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