Molecular and phenotypic characterization of *Helicobacter pylori* isolates from patients with gastroduodenal pathologies in the Eastern Cape Province of South Africa

By

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A thesis submitted in fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

(MICROBIOLOGY)

In the Department of Biochemistry/Microbiology

Faculty of Science and Agriculture

University of Fort Hare.

2011

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DECLARATION

I, Tanih Nicoline Fri declare that the thesis for the award of a Doctor of Philosophy degree in Microbiology at the University of Fort Hare, hereby submitted by me, has not been previously submitted for a degree at this or any other university and that it is my original work in design and execution, and that all the reference materials contained therein have been duly acknowledged.

Signature……...

Supervisor's signature……...

Co-supervisor's signature……..

Date……...

DEDICATION

To my father, Mr. Tanih David, mother, Mrs Tanih Beatrice

and my siblings

ACKNOWLEDGEMENTS

My profound gratitude goes to my supervisor, Prof. Roland N. Ndip, and co-supervisor, Dr. Lucy Ndip of the Department of Biochemistry and Microbiology, University of Buea, Cameroon for their mentorship, sustained interest, and diligent supervision of this work. My gratitude also goes to Mr. Green E, Dr. Mkwetshana N and Dr. Clarke AM for their helpful suggestions throughout this investigation. Many thanks to Mr. Okeleye BI and Mr. Buta N for their assistance during a stage of this study. Special thanks to Dr. Naidoo N and the nurses of the Gastroenterology Unit, Livingstone Hospital, Port Elizabeth and the patients enrolled in the study for making available to us gastric biopsies. I am equally grateful to Mr. Clarke B for his technical support throughout this study.

I am grateful to Prof. Lawrence Weaver for funding part of my study and placing his laboratory at my disposal for part of this work. My gratitude is equally extended to Martin McMillan for his patience in helping me gain skills in current techniques of biomolecular sciences. My thanks go to all members in the Department of Child Health, University of Glasgow, Scotland for their assistance during my stay there.

My appreciation also goes to the National Research Foundation for funding this study, through a grant to Prof. Roland N. Ndip; and to the staff of Govan Mbeki Research and Development Centre for their cooperation and co-ordination in all financial issues pertaining to this study. I was supported by a bursary from the Govan Mbeki Research and Development Center (GMRDC), through Prof. Roland N. Ndip as the grant holder. Special thanks to the Department of Biochemistry and Microbiology of the University of Fort Hare for the

laboratory facilities placed at my disposal, and the Microbial Pathogenicity and Molecular Epidemiology Research Group for their technical support.

My heartfelt thanks is extended to my parents; Mr. Tanih David Azongho and Mrs. Tanih Beatrice Anwi, and equally to Mr. and Mrs. Tembon, Dr. Charles Massango, Mrs. Lindsay Achimbi Fonong for their support and inspiration, my friends and family especially siblings for their love, understanding, kindness, patience, and support throughout the period of this study.

My utmost gratitude goes to Almighty God for His guidance and abundant wisdom bestowed on me throughout this investigation.

LIST OF ABBREVIATIONS

AIDS	Acquired Immune Deficiency Syndrome
BHI	Brain Heart Infusion
cagA	Cytotoxin associated gene
cDNA	Complementary DNA
CDC	Centers for Disease Control and Prevention
CLSI	Clinical and Laboratory Standards Institute
CT	Computed tomography
CFU/ML	Colony forming units per milliliter
Cla	Clarithromycin
CO_2	Carbon dioxide
^{13}C	Carbon-13
^{14}C	Carbon-14
DNA	Deoxyribose nucleic acid
dNTP	Deoxynucleotide triphosphate
DU	Duodenal Ulcer
ELISA	Enzyme Linked Immunosorbent Assay
GERD	Gastro-esophageal reflux disease.
g/L	gram per litre
GU	Gastric Ulcer
HCl	Hydrochloric acid
H_2O_2	Hydrogen peroxide
HSP	Henoch-Schonlein Purpura
IARC	International Agency for Research on Cancer
IgG	Immunoglobulin G
IL-1	Interleukin-1
iceA	Induced by Contact to Epithelium
LPS	Lipopolysaccharide
LiPA	Line probe assay
MALT	Mucosa Associated Lymphoid Tissue
MDR	Multidrug resistance

$MgCl_2$	Magnesium Chloride
MIC	Minimum Inhibitory Concentration
MLST	Multilocus sequence typing
mRNA	messenger RNA
Mg	Milligram
Mtz	Metronidazole
NADPH	Nicotinamide Adenine dinucleotide phosphate
NCCLS	National Committee for Clinical Laboratory Standard
NCTC	National type culture collection
NIH	National institutes of Health
NSAID	Non-steroidal anti-inflammatory drug
NUD	Non Ulcer dyspepsia
PBP	Penicillin binding proteins
PCR	Polymerase chain reaction
PFGE	Pulsed Field Gel Electrophoresis
PPI	Proton pump inhibitor
PU	Peptic Ulcer
PUD	Peptic Ulcer Disease
qPCR	Quantitative Polymerase Chain Reaction
RAPD	Randomly Amplified Polymorphic DNA
RFLP	Restriction Fragment Length Polymorphism
RNA	Ribose nucleic acid
RT–PCR	Reverse transcriptase PCR
SDS-PAGE	Sodium Dodecyl Sulphate Poly-Acrylamide Gel Electrophoresis
TAE	Tris Acetate
TFF1	Trefoil factor 1
ul	Micro liter
µg/mL	Microgram per milliliter
vacA	Vacuolating cytotoxin A
WHO	World Health Organization
X^2	Chi Square

GENERAL ABSTRACT

Helicobacter pylori is an important human pathogen known to chronically infect billions of people worldwide, causing a number of gastric related diseases. Prevalence of this organism is very high in Africa and has been reported to vary between and even within countries. *H. pylori* eradication using the two antibiotics regimen and a proton pump inhibitor often fails due to increasing drug resistance. Studies in South Africa have demonstrated the presence of this organism in the study area. This study investigated the prevalence of *H. pylori* in the Eastern Cape Province of South Africa; determined the antimicrobial susceptibility patterns of isolates; the molecular basis of the resistance pattern of isolates; the most prevalent genotype and the sequence diversity of the genes involved in virulence.

We examined 254 consecutive patients who were referred to Livingstone Hospital, Port Elizabeth with gastric related morbidities between June 2008 to December 2008 for endoscopy and determined the prevalence of infection with respect to age, sex, endoscopic diagnosis, ethnic background and lifestyle**.** Two gastric biopsies were collected (one from the antrum and the other from the corpus) and the organism was isolated on Columbia agar base. Determination of antimicrobial susceptibility/resistant patterns of the isolates to the current antibiotics employed in treatment were executed. Genotyping was carried out using PCR based approach to determine the prevalence of virulence genes (*cagA*, *vacA* and *iceA*) while the molecular basis of resistance and diversity of virulence genes were determined by sequencing the amplified products.

Presumptive isolates were further confirmed by PCR targeting the *glmM* gene. The overall prevalence of *H. pylori* was 66.14% (168/254). Prevalence was highest (100%) amongst patients with duodenitis (1/1), gastric cancer (GC) (4/4) and gastric erosion (5/5) as they were all positive for the organism. However, patients with gastritis (75%; 6/8), gastric ulcer (GU) (70.8%; 17/24), duodenal ulcer (DU) (65%; 26/40), non-ulcer dyspepsia (NUD) (64.7%%; 55/85), and gastro- oesophageal reflux disease (GERD) (64%; 29/45) also had high prevalence rates of the organism. The organism was not isolated (0%) from patients with gastro-duodenitis and atypical oesophageal reflux disease respectively. The prevalence of infection was highest amongst the coloured (66.92%; 87/130) and lowest in whites (59.52%; 25/ 42).

Susceptibility was determined using the Kirby-Bauer disc diffusion and agar dilution methods. Data was analyzed and marked susceptibility of isolates was observed for ciprofloxacin (100%) and amoxicillin (97.5%). Isolates also demonstrated good activity to clarithromycin (80%) and gentamicin (72.5%). However, marked resistance (95.5%) was observed for metronidazole. The MIC ranged from 0.0625–8 µg/mL. The lowest MIC with a range of 0.0625 - 1µg/mL was recorded for ciprofloxacin while the highest (5–8µg/mL) was noted for gentamicin. Multidrug resistance was a common phenomenom encountered in this study. Thirty-two (17.02%) isolates showed multidrug-resistance to metronidazole and erythromycin ($MET^{R}ERT^{R}$). The least resistance pattern was $CLA^{R}TET^{R}AMX^{R}MET^{R}GEN^{R}ERT^{R}$ (O.53%) and ERT^{R} (O.53%).

Polymerase Chain Reaction using specific primer sequences was used to identify the presence of virulence genes. *cagA* was identified in 90% of the strains investigated. Fifty-eight of the 100 strains had the *vacA* signal sequence genotype s1 and 26 had subtype s2. Combined *vacA* s1/s2 was detected in 16 of the strains. *vacA* middle region analysis showed that 8(8%) strains were m1 while 50 were m2. Combined *vacA* m1/m2 was detected in 36 of the strains. s1m2 (20%) and s2m2 (20%) genotypes were the most common allelic combinations of the *vacA* gene among our strains. Multiple *vacA* genotypes were detected in this study, amongst which the most prevalent was s1m1m2 (61%) 28/46. *IceA1* was present in 2 (2%) of our strains while *iceA2* was present in 58 of all the samples analyzed.

Sequenced data indicated that *rdxA* and *frxA* truncation was found only in metronidazole-resistant strains. Mutation in the *rdxA* gene may contribute more significantly than *frxA* gene to the high level of resistance to metronidazole. Two point mutations (A2142G and A2143G) in the 23SrRNA genes of clarithromycin- resistant strains were detected. The findings from this study support the need to continue monitoring the antibiotic susceptibility of *H. pylori* in the Eastern Cape Province of South Africa to guide empiric treatment of such infection.

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CHAPTER ONE

GENERAL INTRODUCTION

The human gastrointestinal tract is colonized by an abundance of bacteria, which are in constant interaction with the epithelial lining usually leading to an intricate balance between tolerance and immunological response. There is ample evidence that the abundant presence of bacteria thus play a role in the maintenance of human health, as well as in the induction of chronic inflammatory diseases of the gastrointestinal tract (Kuipers and Michetti, 2005). *Helicobacter pylori (H. pylori)* is recognized as a chronic colonizer of the human stomach. It is a small, curved or spiral, highly motile, gram-negative bacillus. It chronically infects billions of people worldwide, is one of the most genetically diverse of bacterial species, and has been implicated as the major cause of various diseases since the Nobel-winning discovery by Warren and Marshall in 1982 (Marshall and Warren, 1983)**.**

Ample evidence now exists linking the bacterium to the pathogenesis of chronic gastritis, peptic ulceration, adenocarcinoma of the distal stomach, mucosa associated lymphoid tissue lymphoma (MALT) and primary gastric non-Hodgkin's lymphoma, as well as in a number of extra gastric diseases (Permin & Anderson, 2005; Ahmed *et al*., 2007). While majority of infections are asymptomatic (>70%), the association of its colonisation with chronic gastritis, peptic ulcer diseases, and gastric malignancies is well documented in both adults and children (Suerbaum and Michetti, 2002; Ndip *et al.,* 2008). The evidence of its association with gastric cancer has led to the organism being classified as a class 1 carcinogen by the World Health Organisation (WHO) (IARC, 1994).

Infection with *H. pylori* has been shown to follow a geographic and socio-demographic distribution. Interestingly however; the infection rate in various populations does not parallel the incidence of morbidity and mortality caused by the infection (Ahmed *et al*., 2007). Infection with this organism cause considerable morbidity, and impose a major health burden upon health care systems. There are particular concerns in developing countries, where the prevalence of infection is often markedly higher than seen in the developed world. In various regions of sub-Saharan Africa, for example 61-100% of the population may harbour the pathogen with young children (aged<10years) usually forming the age group with the highest prevalence (Asrat *et al*., 2004; Ndip *et al.*, 2004). The highest prevalence rates are associated with low socioeconomic status, overcrowding, poor hygienic and unclean water supplies (Graham and Graham, 1998; Dube *et al*., 2009a). In general, in developing countries, more than 50% of children are infected by the age of 10 years, with the prevalence of infection rising to 80% in young adults and lasting for years or decades (Ndip *et al*., 2004). In contrast, in the majority of developed countries, children become infected at a rate of less than 1% a year (Graham and Graham, 1998).

Gastric infections with the organism are common and ubiquitous in Africa (Segal *et al*., 2001). The infection usually starts in the gastric antrum and spread to the corpus after extensive mucosal damage. In Africa, *H. pylori* infection early in life is strongly associated with pernicious anaemia, growth faltering and increased susceptibility to other food and water borne pathogens, especially among children from the poorest societal strata (Thomas *et al*., 2004). No consensus exists on the mode of transmission of this organism. Transmission probably occurs mostly by faecal-oral and oral-oral routes and via recently contaminated food and water, and unclean hands (Kersulyte *et al*., 1999; Dube *et al*., 2009b; Tanih *et al*., 2010a).

A number of methods for the diagnosis of infection caused by the organism have been developed. These methods include invasive assays, which require the tissue obtained by endoscopy (histologic examination, culture, biopsy urease test, and PCR and other molecular biology based methods); and non-invasive assays, which detect either an immunologic response (e.g., specific antibodies against *H. pylori*) or metabolic products of *H. pylori* urease activity (urea breath test). Microbiological isolation is the theoretical 'gold standard' for identifying *H. pylori* infection (Me´graud *et al*., 1999). The prevalence of multi-drug resistant strains makes it increasingly likely that culture and antibiotic sensitivity testing may become a prerequisite for patients with persistent infection after initial or repeated treatment failure (Krogfelt *et al.,* 2005; Tanih *et al*., 2010b).

Direct and multilocus sequence typing has indicated that different *H. pylori* genotypes predominate in different human populations. In particular, African strains seem to be distinct from those of other continents (Falush *et al*., 2003). This diversity probably reflects a combination of factors including: mutation, recombination among divergent lineages, gene transfer from unrelated species and preferential transmission among family members (Akada *et al*., 2003). Infection with one strain of the organism does not protect against subsequent co-infection with a different strain; hence a high rate of polyclonal infection results. Polyclonal infection is quite common and frequent in developing countries and it allows for exchange of DNA between different strains, which could promote the spread of genes encoding important virulence factors or resistance to antibiotics (Logan and Walker, 2002). *H. pylori* is not a new bacteria species and, by virtue of its urease enzyme and other products, it has become well adapted to its unique niche within the gastric mucus. It also has genetic heterogeneity (no two strains are identical), and studies have suggested that this diversity may allow each strain to

become uniquely adapted to each host to an extent that, for some subjects, it may be considered as a commensal bacteria (Logan and Walker, 2002).

The ability to characterize and distinguish different *H. pylori* strains is important for understanding transmission and genome evolution. These efforts have been greatly aided by complete genome sequences of four *H. pylori* strains (Tomb *et al*., 1997) and advances in molecular genotyping, ranging from RAPD fingerprinting to multilocus sequence typing (MLST) (Akopyanz *et al*., 1992; Achtman *et al*., 1999; Colding *et al.,* 1999). *H. pylori* DNA is best obtained by culture of bacteria obtained during endoscopy or by string test, but can also be obtained directly by extraction from gastric biopsies or gastric juice (Bravo *et al.,* 2002; Wang *et al.,* 2003; Chattopadhyay *et al*., 2004). The ability to genotype and phenotype *H. pylori* is extremely important, making possible, for example, characterization of transmission in families and in larger human populations; detection of antibiotic resistance in patients whose treatment has failed, and thereby choice of better therapy without need for second endoscopy (Megraud *et al.,* 1999); detection of mixed infections (Kersulyte *et al*., 1999); detection of virulence-associated genes in asymptomatic carriers, thereby allowing estimation of risks of later complications to them or to their children (Mackay *et al.,* 2003).

The clinical outcome of long-term infection is variable and is considered to relate to bacterial virulence factors (van Doorn *et al.,* 1999; Wang *et al.,* 2003) along with host genotype, physiology and environmental factors (Holcombe, 1992; El-Omar *et al.,* 2000). Many studies have shown geographic differences in predominant *H. pylori* genotypes, based either onvirulence associated genes such as *vacA* and *cagA* or "housekeeping genes"(van Doorn *et al.,*1999; Bravo *et al.,* 2002). Several *H. pylori* genes that are related to the risk of disease have been identified some of which include *vacA, cagA, iceA* and several other

"housekeeping genes" such as *ureA*, *ureC*, *ureAB*, *flaA, flaB, atpA, efp, mutY, ppa, trpC, ureI, yphC* etc (Atherton, 1997; van Doorn *et al.,* 1999; Blaser and Berg, 2001) which may not be directly linked to disease.

The cytotoxin-associated gene, *cagA,* a marker for the *cag* pathogenicity island (PAI), is present in many but not all *H. pylori* strains. Its presence is associated with more severe clinical outcomes (Ando *et al.,* 2002; Chattopadhyay *et al*., 2004). Numerous laboratory studies have shown that proteins encoded by *cagA* and other PAI genes contribute to virulence in promoting severe inflammatory responses to infection and altering cell fate and epithelial developmental programs. The *vacA* gene is far from the *cag* PAI. At least some forms of *vacA* protein generate vacuoles in epithelial cells, disrupt tight junctions between epithelial tissues, interfere with antigen processing, etc. (Cover and Blanke, 2005). The *vacA* gene is present in all *H. pylori* strains and contains two importantly variable regions, s and m (van Doorn *et al.,* 1999).

There is global variation in the distribution of *vacA* alleles in different ethnic populations (Wang *et al*., 2003). Four families of the *vacA* alleles have been so far identified based on the signal encoding peptide, encoded by the s region (s1a, s1b, s1c, and s2); and three families based on the middle (m) region (m1, m2a, and m2b) (van Doorn *et al.,* 1999; Wang *et al.,* 2003). Most *vacA* s1 strains are *cagA* positive, and most *vacA* s2 strains are *cagA* negative (Ando *et al.,* 2002), which suggests that these two loci comprise part of a co-adapted gene complex (Bukanov and Berg, 1994). Certain differences among strains can impact on colonization or disease – among them: abilities to induce synthesis of cytokine (IL-8) and thereby severe inflammatory responses, and to form vacuoles in host tissues, traits that depend on the cag pathogenicity island and s1-type alleles of the *vacA* toxin gene,

respectively; adherence to carbohydrate Lewis B and other carbohydrate structures; survival after brief acid exposure, as in the gastric lumen and the genetically distinct ability to grow under mildly acidic conditions, as in the gastric mucin (pH ~5), where most *H. pylori* reside *in vivo* (Akada *et al*., 2003).

Long-term outcomes considerably vary among different human hosts from no overt pathology, to peptic ulcer disease (occurring in 20% of the infected population), atrophic gastritis and gastric cancer. The severity of inflammation appears to relate to host and bacterial genetics as well as to other factors particularly acid secretion (Logan and Walker, 2001). Genetic diversity of virulence factors and antigenic profiles of various *H. pylori* strains include enhanced motility, production of urease, catalase and phospholipase C, specific adherence to gastric epithelial cells and production of vacuolating cytotoxin A (*vacA*). A genetic difference in the individual immune responses to the pathogen, for example linked to IL-1 gene cluster polymorphisms may result in failure to eradicate the infection and lead to chronic mucosal damage (Permin and Andersen, 2005).

Presently, it is no surprise that *H. pylori* is the first bacterium, and the second infectious organism after hepatitis B virus to be classified as a class I carcinogen according to WHO criteria (IARC, 1994). This important statement made gastric cancer the outcome of an infectious disease (Parsonnet, 2000). Furthermore, *H. pylori* also appears to have potential benefits to the host. This includes decreasing the risk of diarrhoeal disease in children (Rothenbacher *et al.,* 2000), an observation which, however, has been contradicted by others (Passaro *et al.,* 2001). It is suggested that carriage of *H. pylori* may protect against infection by exogenous intestinal pathogens. All persons who carry the organism essentially have inflammatory cell infiltration in the gastric mucosa, which, in adults, is associated with peptic

ulceration. With those whom the infection is in the corpus, an additional potential benefit is the limitation of acid production by the effect of cytokines such as interleukin (IL-1) on parietal cell function. This effect has been suggested to decrease the risk of more severe gastro-oesophageal reflux disease in patients with an insufficiently lower oesophageal sphincter, which may protect against the long-term consequences of reflux disease, in particular Barret's oesophagus and oesophageal adenocarcinoma (De Martel *et al.,* 2005). These associations have designated *H. pylori* as a slow acting bacterium that exerts its main clinical outcomes years to decades after initially haven colonized a host, and whose effects can be both beneficial and deleterious to its host.

The National Institutes of Health, Bethesda, Maryland, USA has recommended routine treatment of patients with infection (NIH, 1994). The most successful and universal treatment is the triple therapy, which comprises a proton pump inhibitor and a combination of two antibiotics. Eradication of the organism is the first therapeutic approach that constitutes a reliable long-term prophylaxis of peptic ulcer relapse, accelerating ulcer healing and reducing the rate of ulcer complications (Yuen *et al*., 2005). The eradication of *H. pylori* has been shown to result in ulcer healing, prevention of peptic ulcers recurrence and maybe also a reduction in the prevalence of gastric cancer in high risk populations (Sepulveda and Coelho, 2002). Moreover, eradication of the organism improves the quality of life of ulcer patients (Yuen *et al*., 2005); a most pathological state associated with infected patients. *H. pylori* infection is often treated with a combination of two antibiotics and a proton pump inhibitor (PPI) that is, either metronidazole (Mtz), clarithromycin, and amoxicillin (Chaudhuri *et al*., 2003; Tanih *et al.,* 2010b). Unfortunately, the increase in antibiotic resistance is starting to affect the efficacy of treatment, and, in spite of the impact of *H. pylori*, preventive vaccination strategies still do not exist (Kusters *et al*., 2006). Metronidazole resistance is

common and is important clinically as a primary cause of failure of Mtz based anti-*Helicobacter* therapies (Megraud *et al*., 1999; Buta *et al*., 2010). Resistance has also been observed for clarithromycin, amoxicillin and tetracycline (Deloney and Schiller 2000; Ndip *et al*., 2008).

Resistance of the organism to antibiotics is a growing global concern which needs public health attention. In Africa, the problem of resistance presents a tremendous challenge (Ndip *et al*., 2008). New antimicrobial regimens are therefore being developed to overcome the problem of antibiotic resistance in bacterial pathogens, such as combination of antibiotics with plant extract, honey and other natural products that possess antimicrobial activity (Ndip *et al*., 2007 a, b; Tanih *et al.,* 2009). Plant and Plant products have shown great promise in the treatment of several intractable infectious diseases, including opportunistic AIDS infections. Plants are natural blueprints for the development of new drugs (Iwu *et al.,* 1999). Screening of plants has shown that higher plants represent a potential source of several novel and potentially important antibiotic prototypes (Afolayan, 2003). Anti-*H. pylori* activity has been recorded in several indigenous medicinal plants in different parts of the world (Akinyemi *et al*., 2005). Also, honey has been found to be effective in the treatment of gastro-enteritis. The possibility of using honey orally, for the treatment of *H. pylori* infections, seems worthy (Singh, 2000).

1.1 Rationale of the study

H. pylori is a Gram negative curved rod that inhabits the gastric mucosa of the human stomach. The organism chronically infects billions of people worldwide, displaying great heterogeneity and a major cause of peptic ulcer disease and gastric cancer in many societies. It alters gastric physiology, increases susceptibility to other food and waterborne pathogens, and the risk of infant malnutrition especially among the very poor (Thomas *et al.,* 2004). The ability to genotype and phenotype *H. pylori* is extremely important. It makes possible the characterization of the pathogen, its transmission in human populations and the detection of antibiotic resistance in patients. It also allows for the detection of virulence-associated genes in asymptomatic carriers, and the understanding of genome evolution thereby allowing estimation of risks of later complications (Akopyanz *et al*., 1992).

H. pylori infection seems to be very common in South Africa, as expected in developing countries. For example, Pelsar *et al.* (1997) documented a high prevalence (67 - 84%) of *H. pylori* antibodies in children in Bloemfontein, while Mosane *et al*. (2004) also documented *H. pylori* IgG antibodies in South African mothers and their children. Furthermore, Kidd *et al.* (2001) and some other researchers (Letley *et al.,* 1999; Kidd *et al.,* 1999) have carried out a number of studies on *H. pylori* in Cape Town which have portrayed the importance of this organism in this environment. Ally *et al.* (1999) in their study in Soweto reported a high prevalence of this organism in their study population. Recently, Fritz *et al*. (2006) observed a high *H. pylori* prevalence of 83.3% which is not significantly different to the 84% noted in another study conducted in Pretoria from asymptomatic individuals. Samie *et al*. (2007) reported an *H. pylori* prevalence of 50.6% in their study in Venda, North of South Africa. In their study, Dube *et al*. (2009b) equally documented a high prevalence (86.8 %.) of *H. pylori*

antigens in the stools of asymptomatic individuals in the Eastern Cape Province. Based on limitations in the tests used in these studies, this may well underestimate the real frequency.

The genotypes of *H. pylori* strains of a region are likely to affect the patterns of human disease, and the predominant genotypes of African *H. pylori* strains seem to differ from those of other geographic regions. Indeed, predominant genotypes may also differ within Africa. *H. pylori* strains from Africa in general and South Africa in particular have not been studied to any significant extent. In South Africa, the study of *H. pylori* has received a very cursory attention. There is therefore a paucity of data on the genotypes of *H. pylori* in Africa as a whole and reports of South African populations are particularly fragmentary given the ethnic diversity in this population (McNulty *et al.,* 2004; Samie *et al*., 2007).

Studies on antimicrobial susceptibility/ resistance patterns of *H. pylori* are lacking in South Africa. A Pubmed search indicated only five most recent studies in 2000. Acknowledging that the susceptibility patterns of microorganisms can change with time and geographical location (Obi *et al*., 1998; Ndip *et al*., 2005); given that the organism has been shown to be associated with substantial morbidity and mortality (Sherif *et al*., 2004) coupled with the increase in treatment failure due to emerging resistance to currently used antibiotics (Ndip *et al*., 2008), it will be valuable to determine the current susceptibility/ resistance patterns of *H. pylori* strains circulating in South Africa to guide empiric treatment and prevent the emergence of further resistance.

Furthermore, current reports on *H. pylori* prevalence and associated morbidity are lacking in the Eastern Cape Province which is predominantly rural, plaqued with factors which may favour spread of the organism. This study was therefore aimed at investigating the prevalence of *H. pylori* in patients with gastric related morbidities in the Eastern Cape Province, the

genotypes of the strains, especially in terms of *vacA, cagA* and *iceA* virulence genes, "housekeeping gene" sequences *ureC(glmM),* patterns of antibiotic susceptibility and resistance, and the genes involved in antibiotic resistance.

1.2 Hypotheses:

The following hypotheses were investigated:

1. The incidence of *H. pylori* is high among patients with gastrodudenal pathologies.
2. There is a high frequency of antibiotic resistance.
3. *H. pylori* genotypes that predominate in South Africa are distinct from those predominating elsewhere in the world.

1.3 Overall objective:

The overall objective was to investigate the diversity of *H. pylori* strains in the Eastern Cape Province, in terms of virulence and antibiotics resistance markers.

1.4 Specific Objectives

The specific objectives of this study are:

(i) To determine the prevalence of *H. pylori* in symptomatic patients.

(ii) To ascertain the prevalence of *H. pylori* strains as a function of patient ethnic group.

(iii) To determine the antibiotic susceptibility patterns of isolates.

(iv) To characterize the genes involved in antibiotic resistance.

(v) To characterize the frequencies of particular types of *vacA, cagA* and *iceA* alleles in *H. pylori* strains, as a function of disease status.

(vi) To characterize by sequence typing the overall genotypes of *H. pylori* strains.

1.5 Scope of thesis

The proceeding chapters deals with specific objectives which this study investigated except for chapter 2 which embodies a systematic review on *H. pylori* literature, cutting across taxanomy, culture, morphology, prevalence and epidemiology, virulence determinants, clinical manifestations, antibiotic susceptibility and resistance profiles, diagnosis and control. This chapter has produced three published review article (Tanih *et al.,* 2008; Tanih *et al.,* 2009; Tanih *et al*., 2010a).

Phenotypic characterization of *H. pylori* strains using culture based method for isolation and identification of *H. pylori* to determine the prevalence in the study population is the subject of Chapter three. Prevalence of *H. pylori* is influenced by geographic and socio-demographic factors. In this chapter, the prevalence of this organism with respect to patient ethnic group and disease status in the Eastern Cape Province is investigated.

Resistance to antimicrobial agents in the treatment of *H. pylori* presents a global challenge. In chapter 4, a phenotypic study to elucidate the antibiotic susceptibility patterns of isolates to the recommended antibiotics used for empiric treatment in South Africa is conducted. This is expected to provide useful data to guide empiric treatment and curtail the spread of resistant strains.

The molecular characterization of the strains using Polymerase Chain Reaction (PCR) based method is contained in Chapter 5. Many studies have focused on the molecular epidemiology of *H. pylori* with emphasis on the prevalence of virulence genes *vacA, cagA* and *iceA* in

different geographic regions. However there is a paucity of information on these genes in the Eastern Cape Province. The knowledge generated here will provide an insight on the prevalence of these genes in this environment hence an estimation of the risks of later complication in individuals infected with these strains.

Sequence analysis of *H. pylori vacA*s1 and *vacA*m2 as well as genes involved in drug resistance for metronidazole (*rdxA, frxA*) and clarithromycin (23SrRNA) isolates is investigated in Chapter 6. *H. pylori* is noted for its great diversity which differs from one geographic region to another. This study therefore sought to investigate the differences found in this environment using PCR and sequence based typing as this can impact on the clinical management of patients.

In summary, each chapter mentioned above contains its own abstract, introduction, research methods, results, discussion, conclusions, and references. At the end of all of these, a general discussion, conclusions and recommendations that forms chapter seven was recapitulated. The data emanating from chapters 3-5 have been published as indicated in appendix 4.

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CHAPTER TWO

LITERATURE REVIEW

2.1 Taxonomy

H. pylori was first observed in 1983 by Marshall J. Barry and Warren J. Robin as *Campylobacter*-like organisms (formerly *C. pyloridis*) in the stomach of patients with type B gastritis (Marshall and Warren, 1983). The nomenclature, *Helicobacter* was first established in 1989. *Campylobacter, Helicobacter, Wolinella, Arcobacter* and *Flexispira* belong to a single phylogenetic group that is distinct from other Gram-negative bacteria based on *16SrRNA* sequencing, DNA hybridisation, genus-specific probes, cell wall protein and lipid characterization, serological and biochemical analysis (Owen, 1998; Kuster *et al.,* 2006).

Campylobacter and *Helicobacter* are the most clinically important members of the rRNA superfamily. The recently defined family, campylobacteriaceae contains the genera *Campylobacter* and *Arcobacter. Helicobacter, Wolinella* and *Flexispira* are now included in a phylogenetically distinct family Helicobacteriaceae (Forbes *et al*., 1998). There are more than 17 species of *Helicobacter* based on rRNA sequencing (Owen, 1998; Kuster *et al.,* 2006). Only four of these species are currently considered to be human pathogens; *H. pylori*, *H. felis, H. cinaedi* and *H. fennelliae* (Forbes *et al*., 1998; Fritz *et al.,* 2006**)**. However, reports on the isolation of *H. felis, H. cinaedi* and *H. fennelliae* in human gastric biopsy specimens are rare. *H. pylori* remain the principal *Helicobacter species* in humans with high infection rates in both the developing and developed world (Fritz *et al*., 2006).

General characteristics common to the superfamily include pleomorphic helical (spiral or curved) gram-negative microaerophilic organisms; characteristics that facilitate penetration

and colonization of mucosal environment (e.g. motile by polar flagella; corkscrew shaped); coccoid strains when exposed to oxygen or upon prolonged culture; organisms neither ferment nor oxidize carbohydrates; and they have low G+C base ratio (Owen, 1998).

The *Helicobacters* have the following characteristics: their cells have blunted/rounded ends in gastric biopsy specimens; on agar medium the cells become rod-like and coccoid on prolonged culture; abundant quantities of urease are produced only by gastric strains; abundant quantities of mucinase and catalase are produced; they are highly motile by lophotrichous flagella (tufts of flagella at poles of cells), which sometimes have a characteristic terminal bulb; and they have smooth cell wall with unusual fatty acids (Owen, 1998). *H. cinaedi* and *H. fennelliae* have single polar flagellum. *H. cinaedi and H. fennelliae* can be isolated from male homosexuals; rodent, and are often transmitted through sexual practices. They colonise human intestinal tract and cause diseases like proctitis, proctocolitis, enteritis and bacteraemia (Forbes *et al*., 1998).

2.2 Morphology of *H. pylori*

H. pylori is a gram negative bacterium measuring 2.5 – 3.5µm in length and 0.5–1.0µm in diameter (Brown, 2000; Ndip *et al.,* 2003; 2004). It is observed mainly in the spiral form *in-vivo*, having spiral periodicity in fresh cultures, and undergoes a morphological change to atypical forms as cultures age. A common atypical form is spherical which could be as a result of the bacterium minimizing contact with an unfavourable environment as well as after prolonged *in vitro* culture or antibiotic treatment (Owen, 1998). These forms have been associated with loss of culturability and are probably the resistant forms. Other forms of the organism reported in culture, and occasionally *in-vivo* include spherical, V-shaped, U-shaped (Ox-bowed) and straightened forms. In the normal spiral morphology, the bacterium has 4-6

unipolar flagella and is most actively motile although some cultures may appear to be non-motile in hanging drop preparations (Owen, 1998; Ndip *et al.,* 2003).

2.3 Growth Requirements

H. pylori is a fastidious microorganism hence its requirements for growth are complex. Microaerophilicity is an important feature of the organism with optimal growth at O_2 levels of 2 to 5% and the additional need of 5 to 10% CO_2 and high humidity (Kuster *et al.,* 2006). Many laboratories utilize standard microaerobic conditions of 85% N_2, 10% CO_2, and 5% O_2 for *H. pylori* culture. Growth occurs at 34 to 40°C, with an optimum of 37°C (Ndip *et al*., 2003; 2008). *H. pylori is* considered to be a neutralophile although its natural habitat is the acidic gastric mucosa. The bacterium will survive brief exposure to pH of less than 4, but growth occurs only at the relatively narrow pH range of 5.5 to 8.0, with optimal growth at neutral pH (Owen, 1998; Brown, 2000; Dube *et al.,* 2009a).

Complex growth media are required for growth of this organism. Often these media are supplemented with blood or serum (Ndip *et al.,* 2003). These supplements may act as additional sources of nutrients and possibly also protect against the toxic effects. Commonly used solid media for routine isolation and culture of *H. pylori* consist of Columbia or Brucella agar supplemented with either (lysed) horse or sheep blood or, alternatively, newborn or fetal calf serum. For isolation, selective antibiotic mixtures are available (Owen, 1998). Dent supplement consists of vancomycin, trimethoprim, cefsoludin, and amphotericin B, whereas Skirrow supplement consists of vancomycin, trimethoprim, polymyxin B, and amphotericin B (Ndip *et al.,* 2003). These supplements can be gotten commercially. Liquid media usually consist of Brucella, Mueller-Hinton, or brain heart infusion broth supplemented with 2 to 10% calf serum or 0.2 to 1.0% -cyclodextrins, often together with either Dent or Skirrow's

supplement (Ndip *et al.,* 2003). Isolation of *H. pylori* from gastric biopsy samples is difficult and not always successful. Cultures should be inspected from day 3 to day 14. *H. pylori* forms small (1-mm), translucent, smooth colonies (Ndip *et al.,* 2003).

2.4 Pathogenesis of *H. pylori* infection

2.4.1 Colonization of the gastric mucosa.

Colonization of *H. pylori* usually commences in the stomach (Kuster *et al.,* 2006). The stomach is protected by a mucosal barrier that prevents gastric secretions and other destructive agents from injuring the epithelial and deeper layers of the stomach wall (Akada *et al.,* 2003; Radosz-Komoniewska *et al*., 2005). The integrity of the mucosal layer is maintained by tight cellular junctions and the presence of protective mucus layer. Prostaglandin is derived from the cell membrane lipids and serves as a chemical messenger that protect the stomach lining by improving blood flow, increasing bicarbonate secretion, and enhancing mucus production (Porth, 2002). A major cause of gastric irritation and ulcer development is infection with *H. pylori* (Ally *et al*., 1999; Brown, 2000; Blaser and Berg, 2001). *H. pylori* is an infectious agent that thrives in the acid environment of the stomach and disrupts the mucosal barrier that protects the stomach from the harmful effects of its digestive enzymes. The organism adheres only to the mucus-secreting cells of the stomach; it does not usually colonize other parts of the gastrointestinal tract (Figueiredo *et al.,* 2002; Kuster *et al.,* 2006). The exceptions are areas such as Barrett's oesophagus and a duodenal ulcer site in which the normal epithelial layer has been replaced with gastric mucosa. *H. pylori* degrade mucin and has the capacity to interfere with the local protection of the gastric mucosa against acid. It also may produce toxins that directly damage the mucosa and produce ulceration in other ways (Blaser and Berg, 2001; Porth, 2002). Once mucosal barrier is disrupted, gastric secretions and other destructive agents can proceed in injuring and deepening the layers of

the epithelial and stomach wall. *H. pylori* has evolved several mechanisms to evade primary host defences such as acidity and peristalsis in order to establish persistent infection within the stomach (Ernst *et al.,* 2006). This organism elaborates a number of enzymes such as urease, catalase, oxidase, hydrogenase etc (Radosz-Komoniewsk *et al.,* 2005). Catalase helps the organism to survive in the host by preventing the formation of oxygen metabolites from hydrogen peroxide in neutrophils (Figueiredo *et al*., 2005).

The urease enzyme is highly conserved in *Helicobacter species* and two major subunits of this enzyme have been identified (*ureA* and *ureB*). Urease is a necessary factor for the establishment of chronic infection with *H. pylori* (Owen, 1998). This accessory protein, catalyses the cleaving of urea into ammonia and hydrogen carbonate, achieving a local neutralization of the acid pH in the cytoplasm and on the periplasm (Tanahashi *et al.,* 2000; Peek, 2005; Suarez *et al.,* 2006). Thus, the pathogen can successfully survive in the gastric lumen (pH 1-2) for a short time before it penetrates into the bicarbonate-buffered mucus layer of the gastric mucosa, its real habitat. The mucus layer has a pH gradient reaching from the epithelial cell surface (pH 7) to the lumen (pH 2), and the pathogen reacts chemotactically to this gradient (Haas, 2002). Isolates that lack the ability to produce urease correspondingly fail to colonize rodent models of infection indicating the importance of this conserved enzyme (Peek, 2005).

Active motility is a prerequisite for successful colonisation by this organism in an animal model (Kuster *et al.,* 2006)**.** Motility within the gastric mucosa is aided by a bundle of unipolarly inserted rotating four to six polar flagella that are comprised of two major structural subunits: *FlaA* and *FlaB* (Owen, 1998). The flagella posses a sheath probably to prevent depolymerization in an acid milieu. The organism remains mainly in the mucus,

while a subpopulation adheres to specific receptors of gastric epithelial cells. The genes encoding these two flagella components are located at distant sites on the *H. pylori* chromosome and are transcriptionally regulated by different promoters (O′Toole *et al.,* 2000). Similar to urease production, motility is required for persistent infection, and recent data have shown that a component of the flagella secretion apparatus which regulates flagella biosynthesis also regulates urease activity (Peek*,* 2005; Kuster *et al.,* 2006).

H. pylori adhere to the gastric epithelial cells *in vivo* an action that is important for nutrient acquisition and for resisting shedding of the organism from the mucus gel layer (Kuster *et al*., 2006). Several *H. pylori* strains and host ligands involved in adherence have been identified, as *SabA, OipA, AlpA*, and *AlpB*, including the BabA2 outer membrane protein, which is encoded by the *bab* (blood group antigen binding) genes (Maeda and Mentis, 2007). The BabA2 protein can bind fucosylated polysaccharides, which are blood antigens known as Lewis blood antigens (Ilver *et al.,* 1998; Sheu *et al.,* 2003). These antigens have been found both on the surface of the mucous membrane and in *H. pylori* lipopolysaccharide (LPS).

H. pylori hemagglutinin encoded by hpa has the tendency to bind to sialic acid-containing components of erythrocyte membranes and an exoenzyme S-like protein that binds phosphatidylethanolamine and gangliotetraosylceramide *in vitro*. *H. pylori* also has the tendency to bind to nonsialylated ligands, like laminin, fibronectin, various collagens, heparin sulfate, sulfatide, and trefoil factor 1 (TFF1) (Kuster *et al*., 2006). The O-antigen of *H. pylori* LPS contains different human Lewis antigens, including Lewis x, Lewis y, Lewis a, and Lewis b. Inactivation of the bacterial genes encoding Lewis x and Lewis y results in an inability of *H. pylori* to colonize mice *in vitro*; preincubation of *H. pylori* with anti-Lewis x monoclonal antibodies inhibits bacterial binding to gastric epithelial cells, suggesting that *H.*

pylori Lewis antigens may also mediate adhesion. Studies that have focused on adherence of *H. pylori* to gastric epithelium have also provided insights into the topographic distribution of this organism within the stomach (Kuster *et al*., 2006).

2.4.2 Virulence factors of *Helicobacter pylori*

The role of *H. pylori* in gastroduodenal diseases has become firmly established. Intense research into *H. pylori* has lead to the discovery of virulence factors such as the *vacA* and *cagA* and some other proteins like *iceA* (Ge and Taylor, 1998; van Doorn *et al*., 1998; Tanih *et al.,* 2010c). Proteins have revealed many aspects of the relationships between this bacterium, the gastric mucosal surface, and the induction of disease (van Doorn *et al*., 1998; Smith *et al.,* 2002). Disease outcome is the result of the intricate, ongoing interplay between environmental, bacterial, and host factors. Strain-to-strain genetic variability in bacterial virulence factors do not only affect the ability of the organism to colonize and cause disease but also affect inflammation and gastric acid output (Figueiredo *et al*., 2002; Kuster *et al*., 2006).

Vacuolating cytotoxin *vacA*, produced by *H. pylori* isolates is a protein complex which is present in about 50% of all *H. pylori* strains (Haas, 2002; Tanih *et al.* 2010c). *VacA* is a protein complex of 500 to 600 kDa, consisting of 87 kDa subunits (Haas, 2002). By cloning and genetic characterization of *vacA* genes, it has been shown that initially a precursor protein is synthesized, which is then actively secreted (Ge and Taylor, 1998). *vacA* leads to the formation of acidic vacuoles in epithelial cells and consequently to cell injury or may lead to their death following infection and colonization of an *H. pylori* strain carrying the gene. The cell injury which may lead to cell death is believed to result from induction of apoptosis. *vacA* gene comprises two variable regions, the s region and the m region (van Doorn *et al*.,

1998; Wang *et al*., 2003; Kuster *et al*., 2006). *vacA* alleles vary between toxigenic (Tox+) and non toxigenic (Tox-) strains, the differences being most marked in the region encoding the signal sequence (s region) and mid region gene (m region) (Letley *et al.*,1999). The mosaic combination of s- and m-region allelic types determines the production of the cytotoxin and thereby associated with pathogenicity of the bacterium (Ge and Taylor, 1998; Asrat *et al.,* 2004).

s1m1 strains produce a large amount of toxin and are strongly associated with a higher degree of inflammation and epithelial damage in the gastric mucosa (Kuster *et al*., 2006). *s1m2* strains produce moderate amount of toxins while s*2m1* strains produce very little or no toxin (van Doorn *et al*., 1998; Tanih *et al.,* 2010c). There is global variation in the distribution of *vacA* alleles in different ethnic populations and in different geographical regions (Wang *et al.,* 2003). Four families of the *vacA* alleles have been so far identified based on the signal encoding peptide, encoded by the s region (s1a, s1b, s1c, and s2); and three families based on the middle (m) region (m1, m2a, and m2b) (Ge and Taylor, 1998; van Doorn *et al*., 1998; Wang *et al*., 2003). Geographic differences exist in *H. pylori* genotypes, based either on virulence associated genes such as *vacA* and *cagA* or "housekeeping genes" (Bravo *et al*., 2002; Falush *et al*., 2003). For example *vacA*, s1a is prevalent in North Europe (Datta *et al*., 2003) while s1b is prevalent in South Africa, Portugal, Spain, Central and South America (Bravo *et al*., 2002; Wang *et al*., 2003).

The well-characterized virulence determinant in *H. pylori* strains is *a* 40-kb pathogenicity island for which the *cagA* protein is a marker (Ally *et al.,* 1999; Bravo *et al.,* 2002; Andreson *et al.,* 2002). This gene cluster was probably inherited by horizontal transfer from an unknown microorganism. It is present in about 60-70% of all persons infected with *H. pylori*

(Kidd *et al.,* 1999). It is highly immunogenic and associated with severe clinical symptoms; although all *H. pylori* strains induce gastritis, *cagA*+ strains significantly augment the risk for severe gastritis, peptic ulcer disease, and distal gastric cancer compared to that incurred by *cagA*− strains (van Doorn *et al*., 1998; Kidd *et al.,* 1999). A combination of these genotypes is responsible for a prolong and severe risk of disease associated with *H. pylori* infection (Asrat *et al*., 2004).

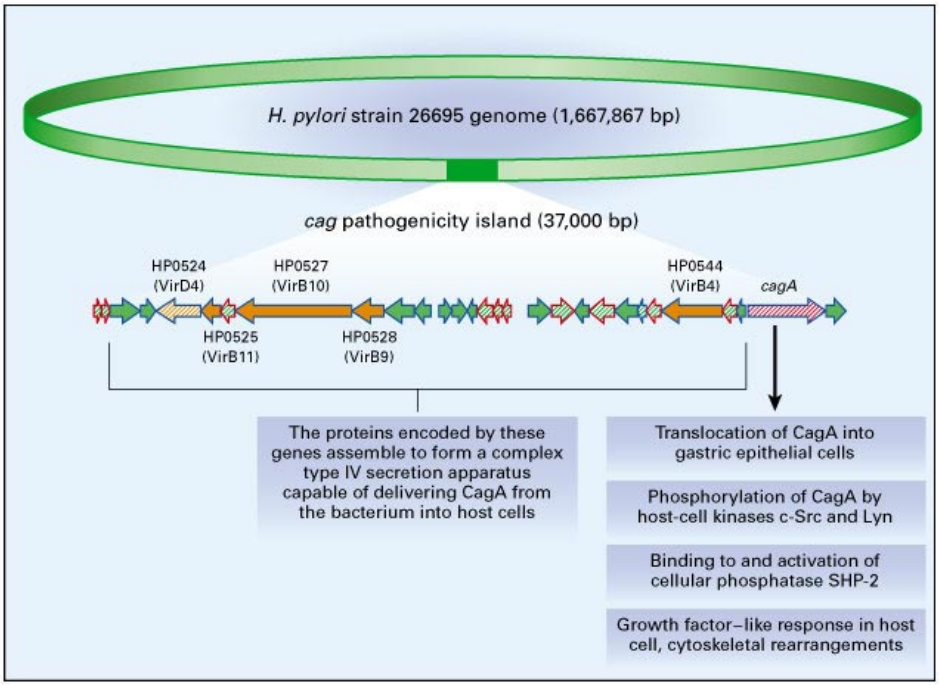

Fig 2.1: The cag Pathogenicity Island (www.lsge.org/pathogenesis of *H. pylori* infection).

A novel gene that has also been discovered is designated *iceA* (induced by contact with epithelium). There are two main allelic variants of the gene*: iceA1* and *iceA2* (van Doorn *et al*., 1998; Smith *et al.,* 2002; Wong *et al.,* 2001). The function of *iceA* is not yet clear but there is significant homology to a type II restriction endonuclease. The expression of *iceA1* is up-regulated on contact between *H. pylori* and human epithelial cells and may be associated

with peptic ulcer disease and other gastric related diseases (van Doorn *et al.,* 1998; Arents *et al*., 2001). Analysis of *iceA2* has demonstrated that five different variants of the gene exist (A-E) based on the size (amino acids) and/or sequence (Kidd *et al.,* 2001).

Table 2.1: Virulence factors of *H. pylori* (Adapted & modified, Haas, 2002).

Virulence Factor	**Function**	**Genes**
Cytotoxin	Induction of vacuoles, damage of epithelial cells, inhibition of MHC class 2 antigen processing	*vacA*
Proteins of cag Pathogenicity islands	IL-8 induction,tyrosine dephosphorylation of host proteins, cytoskeleton rearrangements, type IVsecretion apparatus, cell scattering	*cagA,* hp0520-hp0546
	induced by contact to with epithelium	*iceA*
Urease	Protection against acidification, cleavage of urea	*ureAB* (structure genes), *ureE-I*(accessory and regulatory genes)
Flagella (flagellin)	Motility, chemotaxis	*FlaA*, *FlaB*
Adhesin	Adherence to the mucosal surface	*alpAB,babA2*(Lewis^{b} receptor
Superoxide dismutase	Neutralization of metabolic toxic products	*Sod*
Catalase	Neutralization of metabolic toxic products	*katA*
Phospholipase(s)	Damage of membrane cells	
Protease(s)	Degradation of proteins, mucin respectively IgA	
Housekeeping genes	May not be directly associated with virulence of the bacterium	atpA, efp, mutY, ppa, trpC, ureI and yphC.

2.5 Immune pathogenesis in *H. pylori* infection

If a bacterial species is to persistently colonize its host, its most formidable challenge is to evade immune clearance. *H. pylori* has evolved strategies to avoid global activation of this system (Permin and Andersen, 2005). *H. pylori* evade immune clearance and elicit systemic and mucosal immune responses including the harsh environment in the gastric mucosa, which, however, is unable to clear the infection (Suarez *et al*., 2006). Multiple lines of evidence suggest that the immune response contributes to the pathogenesis associated with the infection (Bodger and Crabtree, 1998; Suarez *et al*., 2006; Tanih *et al.,* 2010a).

Instead of killing the colonizing bacteria, the immune response may lead to destruction of epithelial cells and thinning of the mucosal lining leading to increased mucosal contact with luminal acid (Bodger and Crabtree, 1998; Fan *et al*., 1998). This process is first associated with up-regulation of various genes that are associated with the innate immune system including various Toll-like receptors; complement factor C3, lactoferrin, and bactericidal/permeability-increasing protein. The Toll-like receptor induction in particular occurs through the bacterial LPS; signaling pathways utilized by these receptors all appear to eventuate in NF-κB activation and proinflammatory gene expression (Peek, 2005). Another mechanism through which *H. pylori* may persist is by limiting the bactericidal effects of proinflammatory molecules, such as nitric oxide (Permin and Andersen, 2005; Peek, 2005; Ernst *et al*., 2006).

The bacteria invade the host cells and induce a host immune response, but persistence of the infection suggests that the response is not effective in eliminating the infection (Bodger and Crabtree, 1998). Both natural and acquired specific immune responses to *H. pylori* are elicited at gastric mucosal level. The immune response to the gastro duodenal infection by *H.*

pylori is characterized by mucosal infiltration of lymphocytes, plasma cells, neutrophils and monocytes (Bodger and Crabtree, 1998; Kuipers and Michetti, 2005).

Persons infected with *H. pylori* have been reported to have elevated titres of IgG and IgA antibodies directed at membrane proteins (MP), flagelin, urease, LPS and *H. pylori* adhesin A (HpaA). These results suggest that the infection induces a large recruitment of immune cells into the gastric mucosa, particularly IgA-producing cells (Mattsson *et al*., 1998; Johansson *et al.,* 2004). The inflammatory process is further characterized into various cytokines such as IL-2, IL-3, IL-12, as well as IFN-γ (Bodger and Crabtree, 1998; Kuipers and Michetti, 2005).

H. pylori induces the recruitment of Th1 and Th2 cells into the gastric mucosa, but there appears to be preferential activation of CD4+ cells rather than CD8+ cells (Lundgren *et al.*, 2003). Several studies have noted that the T helper cell response to *H. pylori* is polarized, since CD4+ T cells in the gastric mucosa of infected individuals produce the Th1 cytokines, interleukin (IL)-12, interferon (IFN)-γ and TNF-α, whereas IL-4, a Th2 cytokine production by these T cells is absent (Yamasaki *et al*., 2004; Fritz *et al.,* 2006; Suarez *et al*., 2006). Cytokines, particularly IL-8, are produced by both gastric epithelium and activated macrophages **(**Suarez *et al*., 2006; Rasmus *et al*., 2007). Besides presenting *H. pylori* antigens to the specific T cells recruited into the gastric antrum, antigen presenting cells release several cytokines, such as IL-1, IL-6, TNF-α and IL-12, whose local concentration strongly influence the developing specific T-cell response (Yamasaki *et al*., 2004). Mucosal T cells specific for *H. pylori* represent the real director of the specific immune response tightly regulating both cell-mediated and humoral responses, as well as the rate of local B-cell activation and proliferation (Yamasaki *et al*., 2004).

Data suggest that host gastric immune response to *H. pylori* can influence the clinical picture and that gastroduodenal disease may be an immunopathological consequence of a Th1-polarized response to some *H. pylori* antigens (Bodger and Crabtree, 1998), whereas exhaustive and deregulated *H. pylori* -induced T cell-dependent B-cell activation may support the onset of low-grade gastric B-cell lymphoma (De Jong *et al.,* 2008). *H. pylori* infection is thought to cause gastric cancer by eliciting vigorous T-helper (Th1) pro-inflammatory cellular immune responses in gastric mucosa and the resulting mucosal injury (Bodger and Crabtree, 1998). In uncomplicated chronic gastritis and gastric MALTomas most of gastric *H. pylori*-specific T cells showed combined secretion of both Th1- and Th2-type cytokines (Yamasaki *et al*., 2004; Rasmus *et al*., 2007).

Some infection with *H. pylori* elicits Th2 instead of Th1-dominant immune responses to thwart their elimination and could plausibly modulate *H. pylori*-induced immune response towards one less damaging to the gastric mucosa (protective). This immune response results in the elaboration of pro-inflammatory cytokines (IL-4, IL-5, IL-10) (Rasmus *et al*., 2007). Reports have indicated that Th2 response may provide protection against gastric cancer (Segal *et al*., 2001). It is has been suggested that persons living in high *H. pylori*-prevalence areas with low gastric-cancer incidence like in Africa might have Th2-type dominant *H. pylori*-specific responses (Mbulaiteye *et al.,* 2006).

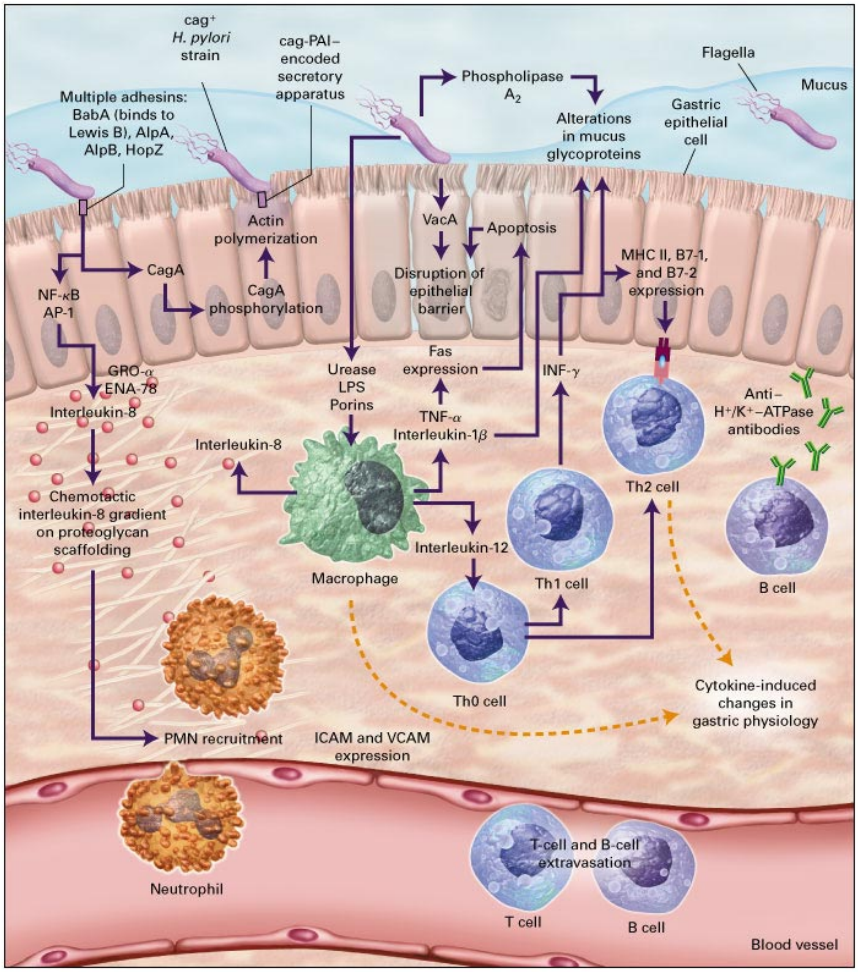

Fig. 2.2: Pathogen-host interactions in the pathogenesis of *H. pylori* infection **(**www.lsge.org/pathogenesis of *H. pylori* infection).

2.6 Gastric mucosal barrier and ulcer development

The stomach is protected by a mucosal barrier that prevents gastric secretions and other destructive agents from injuring the epithelial and deeper layers of the stomach wall. The integrity of the mucosal layer is maintained by tight cellular junctions and the presence of protective mucus layer (Porth, 2002). Two of the major causes of gastric irritation and ulcer formation are aspirin or non-steroidal anti-inflammatory drugs (NSAIDs) and infection with *H. pylori* (Bodger and Crabtree, 1998; Kuster *et al.,* 2006). Aspirin and NSAIDs exert their

destructive effects by irritating the gastric mucosa and inhibiting prostaglandin synthesis (Porth, 2002). *H. pylori* is an infectious agent that thrives in the acid environment of the stomach and disrupts the mucosal barrier that protects the stomach from the harmful effects of its digestive enzymes. This disruption might lead to one or more gastroduodenal disease conditions. The risk of development of these disorders depends on a variety of bacterial, host and environmental factors that mostly relate to the pattern and severity of gastritis (Kuster *et al.,* 2006).

2.6.1 Gastritis

Gastritis refers to inflammation of the gastric mucosa. There are many causes and types of gastritis, most of which can be grouped as acute or chronic gastritis **(http://en.wikipedia.org/wiki/Gastritis)**.

2.6.1.1 Acute gastritis

Acute gastritis refers to the transient inflammation of the gastric mucosa (either proximal and distal stomach mucosa or pangastritis) (Kuster *et al.,* 2006). It is most commonly associated with local irritants such as bacterial endotoxins, caffeine, alcohol, and aspirin. Depending on the severity of the disorder, the mucosal response may vary from moderate oedema and hyperaemia to haemorrhagic erosion of the gastric mucosa (Porth, 2002). Clinical manifestation of acute gastritis varies. Aspirin-related symptom includes heartburn or sour stomach. Gastritis associated with excessive alcohol consumption is a different situation; it often causes transient gastric distress, which may lead to vomiting and, in more severe situations, to bleeding and hematemesis (http//www.tjclarko.com/d_ulcers.htm). Gastritis caused by toxins of infectious organisms, such as staphylococcal enterotoxins, usually has an abrupt and violent onset, with gastritis distress and vomiting ensuing approximately 5 hours

after the ingestion of a contaminated food source. Acute gastritis usually is a self-limiting disorder; complete regeneration and healing usually occur within several days (Porth, 2002).

2.6.1.2 Chronic gastritis

When colonisation becomes more persistent, a close correlation exists between the level of acid secretion and the distribution of gastritis which might eventually lead to chronic gastristis (Kuster *et al.,* 2006). Chronic gastritis is characterised by the absence of grossly visible erosions and the presence of chronic inflammatory changes leading eventually to atrophy of the glandular epithelium of the stomach. The changes may become dysplastic and possibly transform into carcinoma. Factors such as chronic alcohol abuse, cigarette smoking, and chronic use of NSAIDs may contribute to the development of the disease (Palmer *et al.*, 2002; Furuta and Delchier*,* 2008). There are four major types of chronic gastritis: autoimmune gastritis, multifocal atrophic gastritis, *Helicobacter pylori* gastritis and chemical gastritis (Porth, 2002; Furuta and Delchier*,* 2008). *H. pylori* gastritis is a chronic inflammatory disease of the antrum and body of the stomach. It is the most common type of chronic non-erosive gastritis in the United States. Chronic infection with *H. pylori* can lead to gastric atrophy and intestinal metaplasia. *H. pylori* can also cause peptic ulcer and has been linked to the development of gastric adenocarcinoma (Kuster *et al.,* 2006).

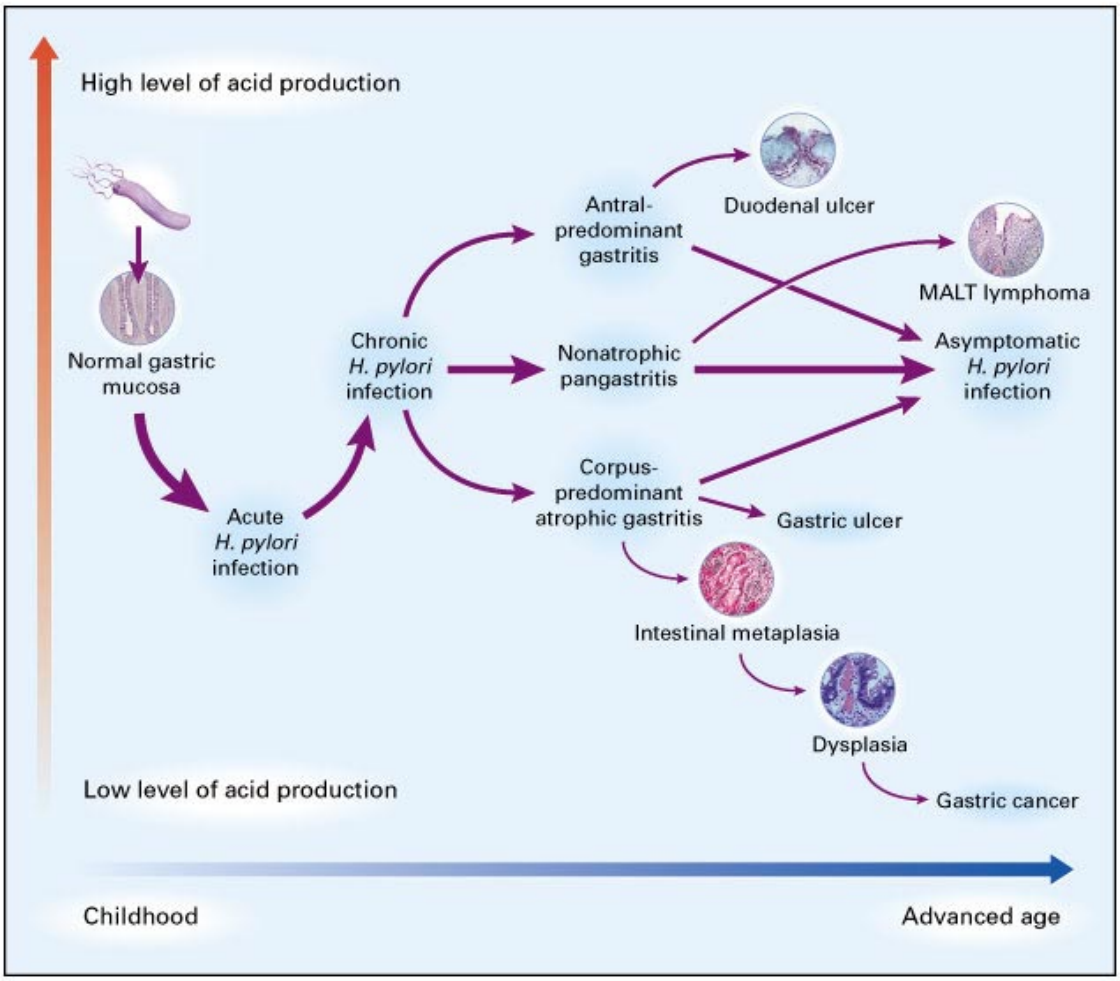

Fig 2.3. Natural history of *Helicobacter pylori* infection **(**www.lsge.org/pathogenesis of *H. pylori* infection).

2.7 Peptic ulcer disease.

Peptic ulcer disease is a term used to describe a group of ulcerative disorders that occurs in areas of the gastrointestinal tract that are exposed to acid-pepsin secretions (http//www.tjclarko.com/d_ulcers.htm). The most common form of peptic ulcer are duodenal and gastric ulcers (Calam, 1998; Kuster *et al*., 2006). Gastric ulcers mostly occur along the lesser curvature of the stomach while duodenal ulcers occur in the duodenal bulb. Most cases of peptic ulcer are caused by *H. pylori* infection (van Doorn *et al*., 1998; Smith *et al*., 2002;

Porth, 2002; Ndip *et al.,* 2008; Tanih *et al*., 2010c). It has been reported that virtually all persons with duodenal ulcer and 70% of persons with gastric ulcer have *H. pylori* infection (van Doorn *et al.,* 1998; Figueiredo *et al*., 2002). Two other forms of gastric ulcers, Zollinger-Ellison syndrome and stress ulcers have different causes. Peptic ulcer disease, with its remissions and exacerbations, represent a public health problem (Smith *et al.*, 2002). Approximately 10% of the population have or will develop peptic ulcer. Duodenal ulcers occur five times more commonly than gastric ulcers. Ulcers in the duodenum occur at any age and are frequently seen in early adulthood. Gastric ulcers tend to affect the older age group, with a peak incidence between 55 and 70 years of age. Both types of ulcers affect men three to four times more frequently than women (Porth, 2002).

A peptic ulcer can affect one or all layers of the stomach or duodenum. The ulcer may penetrate only the mucosal surface, or it may extend into the smooth muscle layers. Occasionally, an ulcer penetrates the outer wall of the stomach or duodenum (Calam, 1998). Spontaneous remissions and exacerbations are common (Porth, 2002; Furuta and Delchier*,* 2008). Healing of the muscularis layer involves replacement with a scar tissue; although the mucosal layer that covers the scarred muscle layer regenerate, the regeneration is often less perfect, which contributes to the repeated episodes of ulceration. Since the early 1980s, there has been a radical shift in thinking regarding the cause of peptic ulcer (Calam, 1998). No longer is peptic ulcer thought to result from a genetic predisposition, stress, or dietary indiscretions. Most cases of peptic ulcer are caused by *H. pylori* infection (Calam, 1998; Porth, 2002). The second most common cause of peptic ulcer is NSAID and aspirin use (Porth, 2002). Aspirin appears to be the most ulcerogenic of NSAIDs (Calam, 1998). Ulcer development in NSAID user is dose dependent, but some risk occurs even with aspirin doses of 325mg/day (http//www.tjclarko.com/d_ulcers.htm; McQuaid, 2001; Furuta and Delchier*,*

2008). Much of the familial aggregation of peptic ulcer that formerly was credited to genetic factors is probably due to intrafamilial infection with *H. pylori* rather than genetic susceptibilities (Calam, 1998).

Since its identification in 1982, *H. pylori* has generated world wide interest. Eradication of the organism can result in resolution of gastritis, with subsequent ulcer healing. There is a 10% to 20% prevalence of gastric ulcers and 2% to 5% prevalence of duodenal ulcers among chronic NSAID users. The pathogenesis of NSAID-induced ulcers is thought to involve mucosal injury and inhibition of prostaglandin synthesis. In contrast to peptic ulcer from other causes, NSAID-induced gastric injury is often without symptoms, and life-threatening complications can occur (Calam, 1998).

2.7.1 Clinical manifestations of peptic ulcer.

The clinical manifestations of uncomplicated peptic ulcer focus on discomfort and pain. The pain, which is described as burning, gnawing, or cramp-like, usually is rhythmic and frequently occurs when the stomach is empty - between meals and at 1 or 2 o'clock in the morning **(**Furuta and Delchier*,* 2008**).** The pain is usually located over a small area near the midline of the epigastrium near the xiphoid, and may radiate below the costal margins, into the back or rarely to the right shoulder (Furuta and Delchier*,* 2008). Superficial and deep epigastric tenderness and voluntary muscle guarding may occur with more extensive lesions. An additional characteristic of ulcer pain is periodicity. The pains tend to recur at intervals of weeks or months. During an exacerbation, it occurs daily for a period of several weeks and then remits until the next recurrence. Characteristically, the pain is relieved by food or antacids (Porth, 2002).

2.7.2 Complications of peptic ulcer.

The complications of peptic ulcers include haemorrhage, obstruction, and perforation. Haemorrhage is caused by bleeding from granulation tissue or from erosion of an ulcer into an artery or vein. It occurs in up to 10% to 20% of persons with peptic ulcer (McQuaid, 2001). Evidence of bleeding may consist of hematemesis or melena. Bleeding may be sudden, severe, and without warning, or it may be insidious, producing only occult blood in stool. Up to 20% of persons with bleeding ulcers have no antecedent symptoms of pain; this is particularly true with person's receiving NSAIDs. Acute haemorrhage is evidenced by the sudden onset of weakness, dizziness, thirst, cold and moist skin, the desire to defecate, and the passage of loose, tarry, or even red stools and coffee-ground emesis. Signs of circulatory shock develop depending on the amount of blood lost (McQuaid, 2001).

There is a feeling of epigastric fullness and heaviness after meals. With severe obstruction, there is vomiting of undigested food. Perforation occurs when an ulcer erodes through all the layers of the stomach or duodenum wall. Perforation develops in approximately 5% of persons with peptic ulcers usually from ulcers on the anterior wall of the stomach or duodenum (McQuaid, 2001). With perforation, gastrointestinal contents enter the peritoneum and cause peritonitis, or penetrate adjacent structures such as the pancreas. Radiation of pain into the back, severe night distress, and inadequate pain relief from eating foods or taking antacids in persons with a long history of peptic ulcer may signify perforation (McQuaid, 2001).

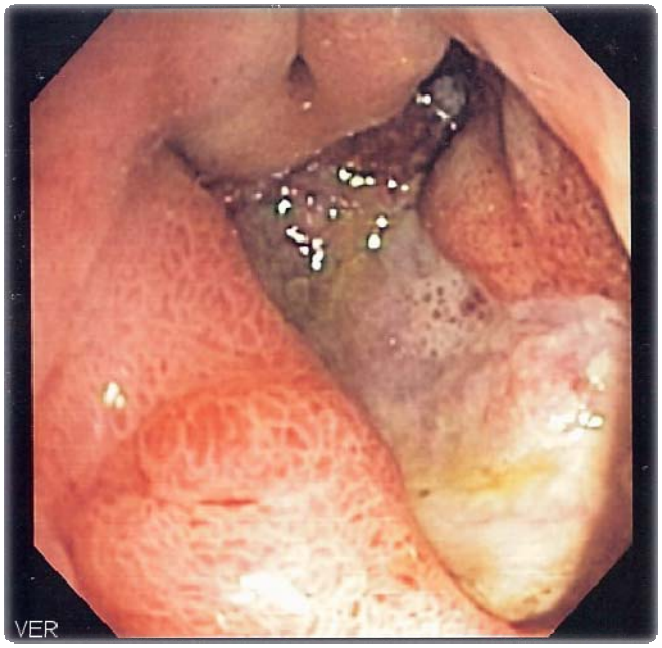

Fig 2.4: Endoscopic image of deep gastric ulcer in the gastric antrum **(http://en.wikipedia.org/wiki/Gastritis**).

2.8 Tumours of the stomach

2.8.1 Gastric carcinoma

Infection with *H. pylori* appears to serve as a cofactor in some types of gastric carcinomas (Wotherspoon, 1998; Kuster *et al.,* 2006). Although its incidence has decreased during the past 50 years, stomach cancer is the seventh most frequent cause of cancer mortality in the United States. In 2001, it was estimated that approximately 21,700 Americans were diagnosed with stomach cancer and 12,800 died of the disease (Porth, 2002). The disease is much more common in other countries and regions, principally Japan, Central Europe, the Scandinavian countries, South and Central America, Soviet Union, China, and Korea and is the major cause of cancer death worldwide (Porth, 2002). Among factors that increase the risk of gastric cancer is genetic predisposition, carcinogenic factors in diet (e.g., *N*-nitroso compounds and benzopyrene found in smoked and preserved foods), autoimmune gastritis,

and gastric adenomas or polyps (Ernst *et al*., 2006; Furuta and Delchier, 2008). The incidence of stomach cancer in the United States has decreased fourfold since 1930, presumably because of improved storage of food with decreased consumption of salted, smoked, and preserved foods (Palmer *et al*., 2002). Infection with *H. pylori* appears to serve as a cofactor in some types of gastric carcinomas.

Virtually all tumours are adenocarcinomas arising from mucus-secreting cells in the base of the gastric crypts. Most develop upon a background of chronic atrophic gastritis with intestinal metaplasia and dysplasia. Between 50% and 60% of gastric cancers occur in the pyloric region or adjacent to the antrum (Furuta and Delchier, 2008). Compared with a benign ulcer, which has smooth margins and is concentrically shaped, gastric cancers tend to be larger, irregularly shaped, and have irregular margins.

2.8.2 Gastric lymphoma

A close link between *H. pylori* infection and the development of gastric (MALT) has been established in some studies. *H. pylori* has been confirmed in most cases of MALT lymphoma (Wotherspoon, 1998; Tanih *et al.,* 2009). Primary gastric lymphoma accounts for less than 5% of all the gastric malignancies (Wotherspoon, 1998). The stomach is however, the most common site for extranodal non-Hodgkin's lymphoma and 60% of all primary gastrointestinal lymphomas occur at this site. Lymphoid tissue is not found in the normal stomach but lymphoid aggregates develop in the presence of *H. pylori* infection. *H. pylori* is closely associated with the development of a low-grade lymphoma (MALToma). Superficial MALTomas may be cured by *H. pylori* eradication (Palmer *et al*., 2002). The clinical presentation is similar to that of gastric cancer and endoscopically the tumour appears as a polypoid or ulcerating mass. The prognosis depends on the stage of diagnosis. Low grade

MALT lymphomas often regress following the eradication of *H. pylori* and they relapse with re- infection with *H. pylori* (Yamasaki *et al*., 2004).

2.9 Epidemiology and anticipated modes of transmission of *Helicobacter pylori*

H. pylori, the principal species of the genus *Helicobacter,* is a common human pathogen that is responsible for a variety of gastro–duodenal pathologies in the developed and developing world, where the prevalence of human infection is generally high (Abdulrasheed *et al.,* 2005; Kuster *et al*., 2006; Tay *et al*., 2009). The prevalence of infection varies but is falling in most developed countries (Segal *et al*., 2001); with rates from 25–50% in developed countries to 70–90% in the second and third world countries (Asrat *et al.,* 2004; Ndip *et al.*, 2004; 2008; Tay *et al*., 2009; Dube *et al*., 2009b). Despite improved treatment modalities, *H. pylori* related gastrointestinal pathology is common and remains a major burden on Western health systems. The prevalence is increased in African American, Hispanic, Asian and Native American population and infection rates are similar in males and females **(**Dehesa *et al*., 1991**)**.

The prevalence of *H. pylori* infection has also been reported to vary widely by geographic area, age, race, and socioeconomic status (Malcolm *et al*., 2004). Although there is geographical and socio–demographic variation in the prevalence of human infection*,* prevalence does not parallel the incidence of morbidity caused by the infection (Mukhopadhyay *et al*., 2000; Ndip *et al.,* 2004; Asrat *et al*., 2004). In Africa, for example, the prevalence of infection is very high but the incidence of gastric carcinoma and other *H. pylori*-associated morbidities is relatively low. This apparent anomaly has been termed the 'African enigma' (Holcombe, 1992; Tanih *et al.,* 2009). Coinfection with other organisms is known to modulate the *H. pylori* immune reaction and has been proposed to explain the

"African enigma" (Fritz *et al,* 2006). Studies conducted in various parts of Africa have revealed very high seroprevalences (61%–100%) of *H. pylori* infection (Holcombe, 1992; Ndip *et al*., 2004; Dube *at al.,* 2009b). Seroprevalence varies both among countries and between the different racial groups present within each country.

In the West, seroprevalence with this organism has been depicted though the rates are not as high as those elaborated in Africa. For example, Mourad-Baars *et al*. (2007) reported a seroprevalence of 1.2% in their study population in the Netherlands. Most individuals harbour specific antibodies for most of their lives (Holcombe, 1992) especially in Africa. It is not possible to ascertain when infection occurs clinically hence most of the information on the rates of *H. pylori* in geographically and demographically diverse populations comes from seroprevalence studies or ^{13}C urea breath tests.

Retrospective seroepidemiological studies have shown a cohort effect consistent with the hypothesis that infection is mainly acquired in early childhood (Logan and Walker, 2002; Thomas *et al*., 2004). In a rural village of Linqu Country, Shandong Province, China, a study of 98 children found that nearly 70 percent of those aged 5-6 years were infected with *H. pylori* , a rate equivalent to that reported for adults in that area, suggesting that most infection takes place early in childhood (Thomas *et al*., 2004). Generally, 50% of all children are infected by the age of 10 years, with prevalence rising to 80% in adults (Segal *et al*., 2001). In their study of Kenyan school children, Nabwera *et al.* (2000) observed high prevalence among their subjects who were only aged 3–5 years, indicating that most children in the study area were infected before they reached their third birthday (Table 2.2).

The generally high prevalences of human infection with *H. pylori* seen in Africa are an indication that effective public-health interventions need to be developed (Dube *et al*., 2009b). The variations seen in the prevalence of infection between and among populations indicate that parameters such as age, cultural background, genetic predisposition, socio–economic status and environmental factors all play a role in the acquisition and transmission of the organism (Segal *et al*., 2001; Dube *et al.,* 2009b).

A number of authors have emphasized the role of other factors such as smoking, alcohol consumption, occupational exposure, waterborne exposures, hygienic practices, density/crowding, social factors, family history of gastric disease and diet (Ogihara *et al*., 2000; Brown, 2000; Iso *et al*., 2005). Within countries, there may be a similarly wide variation in prevalence between the more affluent urban populations and the resource-poor rural populations. A lack of proper sensitization, good drinking water and basic hygiene, as well as poor diet and overcrowding, all seem to play a role in the high prevalence of infection in the developing world (Ndip *et al.,* 2004; Dube *et al*., 2009b).

Table 2.2: Prevalence of human infection with *Helicobacter pylori:* examples in some representative countries in the world.

Country	Prevalence of infection	References
Cameroon, Nigeria and the Gambia	50% among children aged < 5years.	Segal *et al.*(2001); Ndip *et al*., (2004)
Cote d'Ivoire	55% among children aged <10years.	Segal *et al.,* (2001)
Democratic Republic of Congo	62.4% seroprevalence among study participants.	Longo-Mbenza *et al*. (2007)
Egypt	62.2% seroprevalence among dyspeptic patients, 11% among asymptomatic controls.	Said *et al*. (2008)
Ethiopia	93% among patients with peptic ulcer disease.	Henriksen *et al.* (1999)
Kenya	45.6% among children aged < 3years.	Langat *et al*. (2006)
Senegal	82.8% among asymptomatic individuals	Mbengue *et al*. (1997)
South Africa	50.6% in Venda, in the North of the country	Samie *et al*. (2007)
Tunisia	21% among children aged < 5years, 69% among older children	Maherzi *et al*. (2003)
Malaysia	Low seropositive of 14.2% was found in Northern Peninsular of Malaysia	Sasidharan and Uyub, (2000)
China	High seroprevalence was found among children in China	Dong-hong *et al*.(2009)
Netherlands	Low seroprevalence of 1.2% of *H. pylori* infection	Mourad-Baars *et al*. (2007)
Finland	Seroprevalence declined from 38 to 12%.	Rehnberg-Laiho *et al*. (2001)
England and Wales	Prevalence between 4-3% to 30% in the subjects enrolled.	Vyse *et al*. (2002)
Canada	23.1% seroprevalence in patients in Ontario.	Naja *et al*.(2007)
Peru	High prevalence of 47.8% was detected in Peruvians.	Ramírez-Ramos *et al*. (2005)

There appears to be a substantial reservoir of *H. pylori* aside from the human stomach. Animals, e.g. cats, monkeys etc. harbour organisms that resemble *H. pylori* but under particular circumstances (Fox *et al*., 1995). These animals could be reservoirs for human infection. Although possibly important in some circumstances, neither a zoonotic reservoir nor food appears to be significantly involved in acquisition of *H. pylori*. Thus the major question of transmission is how *H. pylori* move from the stomach of one person to that of another (Kuster *et al.,* 2006).

2.9.1 Modes of Transmission

Until recently, however, it has been difficult to assess accurately the incidence (or route) of infection because of the inaccuracy and cost of detecting (non-invasively) *H. pylori*. Primary acquisition in adults, or re-infection after successful eradication, does occur but is less common, with an annual incidence of 0.3-0.7% in developed countries and 6-14% in developing countries (Logan and Walker, 2002). Although epidemiologic studies have addressed a variety of factors such as bacterial, host genetic and environmental factors to delineate the causative links of *H. pylori* infection, knowledge of reservoirs and transmission modes remain poor (Thomas *et al*., 2004; Asrat *et al*., 2004; Ndip *et al.,* 2004). However, some routes have been described (Tanih *et al.,* 2008; Tanih *et al.,* 2010c).

2.9.1.1 Gastro- oral routes

Since humans are the only known reservoir of infection, it is likely that in developed countries *H. pylori* is picked up from siblings, other children, or parents predominantly via the gastro-oral route (Brown*,* 2000). The organism has been recovered from vomitus after specific culture based approaches on selective media (Leung *et al.,* 1999; Ndip *et al.,* 2003).

2.9.1.2 Iatrogenic transmission

Iatrogenic transmission is that in which tubes, endoscopes or specimens in contact with the gastric mucosa from one person are introduced to another person (Akamatsu *et al*., 1996). Adequate sterilization and disinfection of endoscopes has reduced the incidence of transmission (Tytgat, 1995). Interestingly, endoscopists, especially those who do not wear gloves during procedures, are at risk of becoming infected. Occupationally acquired infections have also been reported in gastroenterologists (Kikuchi and Dore, 2005). This is however the least common form of transmission.

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2.9.1.3 Faecal–oral transmission

Faecal–oral transmission is perhaps the most important. Although *H. pylori* has been isolated from the faeces of young children infected with the organism (Thomas *et al.,* 2004), faecal isolation is not common; this could indicate that shedding is intermittent. Faecally contaminated water may be a source of infection (Brown, 2000; Kuster *et al*., 2006) but the organism has proved difficult to be isolated from water. Food-borne transmission and unclean hands have also not been substantiated (Kersulyte *et al*., 1999).

2.9.1.4 Oral–oral transmission

Oral–oral transmission has been identified in the case of African women who premasticate the foods that they give to their infants (Me´graud, 1995). Premastication of food was common in Burkino Faso families with both mother and child sero-positive for *H. pylori* compared to frequencies in families with a sero-positive mother and a sero-negative child (Aditya *et al*., 2009). There is evidence of intrafamilial transmission and of poor living conditions increasing the risk of infection (Aguemon *et al*., 2005). Although dental plaque has been proposed to be a possible route of transmission (Desai *et al.,* 1991**)** this has failed in

other studies **(**Bernander *et al.,* 2003). In a recent study in South Africa, it was deduced that the oral cavity is unlikely to contribute to the spread of this organism as oral cavities were found not to favour prolonged colonization by the organism (Olivier *et al.,* 2006; Dube *et al*., 2009a).

2.9.1.5 Sexual Transmission

There is no identified association of infection with sexual transmission (Perez- Perez *et al*., 1991) and such transmission, if it occurs, must be uncommon.

2.10 Conventional methods for detection and isolation of *Helicobacter pylori*

A number of diagnostic tests are available for *H. pylori*. They may be divided into tests that indirectly determine the presence of the microorganism (antibody tests in blood, urine, or saliva) or direct tests that detect the intact organism (histology and culture), antigens shed from the organism (stool antigen test), or metabolic functions of the organism (rapid urease test and urea breath test) (Tanih *et al.,* 2008). Several methods, both invasive and non-invasive, are available for the detection of *H. pylori* infection**.**

2.10.1 Invasive tests

H. pylori can be detected at endoscopy by histology, culture, urease test, or PCR, each with inherent advantages and disadvantages. All these biopsy based methods for detecting the organism are liable to sample error because infection is patchy **(**Tanih *et al.,* 2008). Consensus guidelines therefore recommend multiple biopsies be taken from the antrum and corpus for histology and one other method (either culture or urease testing) (Krogfelt *et al*., 2005).

2.10.1.1 Histology

H. pylori can be recognized on tissue sections stained with haematoxylin and eosin. Supplemented stains such as Giemsa, Genta, Gimenez, Warthin-Starry silver, and Creosyl violet are needed to detect low levels of infection and to show the characteristic morphology. Histologic examination allows evaluation of the type and degree of inflammation and direct observation of bacteria, if present (Krogfelt *et al*., 2005). Histology test has an important advantage in that, in addition to the historical record provided, sections from biopsies can be examined at any time, and that gastritis, atrophy or intestinal metaplasia can be assessed (Logan and Walker, 2002).

2.10.1.2 Culture

Microbiological isolation is the theoretical 'gold standard' for identifying any bacterial infection, but culture of *H. pylori* can be unreliable because of risk of overgrowth or contamination (Logan and Walker, 2002). The prevalence of multi-resistant strains makes it increasing likely that culture and antibiotic sensitivity testing may become a prerequisite for patients with persistent infection after initial or repeated treatment failure (Krogfelt *et al.,* 2005). Isolation of the organism is technically demanding and requires proper specimen transport, specific medium (use of selective supplements, horse blood and specific medium following the manufacturer's recommendations) and micro-aerobic growth conditions (5–6% O_2, 10% CO_2, 80–85% N_2) (Ndip *et al*., 2003).

2.10.1.3 Biochemical Tests

A batch of three biochemical tests have tremendously been employed in the identification of *H. pylori* especially in resource limited laboratories which are particularly common in the developing countries (Ndip *et al*., 2008; Tanih *et al.,* 2010b). These tests include: the urease,

oxidase and catalase test. The urease test is simple and quick whose principle rests on the powerful urease activity of the organism and its exclusive ecological niche in the gastric mucosa. Urease activity in the sample is detected by the rise in pH, which follows the production of ammonium ions from urea. The rise in pH induces a colour change of phenol red indicator in the culture medium from yellow to pink or red (Cheesbrough, 1998). Catalase test makes use of hydrogen peroxide (H_2O_2) and a positive test is indicated by effervescence on the slide when the organism is emulsified in H_2O_2, while for a negative test no effervescence is recorded (Cheesbrough, 1998). For the oxidase test, strips formulated with the use of oxidase reagent (N, N-dimethyl-p-phenylenediamine) are used. Test organism is smeared on the strips. Observation in colour change is read as positive or negative depending on the manufacturer of the strip's instruction (Cheesbrough, 1998).

2.10.2 Non-invasive tests

Non-invasive methods include the urea breath test, serology and stool antigen test. Unlike the invasive approach, the non-invasive tests do not require highly specialized equipment. In addition, they may be more appropriate for use in paediatric patients, where techniques such as serology are insensitive (Chisholm *et al*., 2004) and invasive methods are undesirable. Furthermore, it may be used for the follow-up of treatment.

2.10.2.1 Serology

H. pylori elicit a local mucosal and systemic antibody response. Circulating IgG antibodies to the organism can be detected by enzyme linked immunosorbent assay (ELISA) or latex agglutination tests. These tests are generally simple, reproducible, inexpensive, and can be done on stored samples. They have been used widely in epidemiological studies, including retrospective studies to determine the prevalence or incidence of infection (Krogfelt *et al*.,

2005). The main disadvantage is that guidelines are not established for follow-up of antimicrobial therapy. Serological diagnosis has been widely used but a recent consensus statement on *H. pylori* infection indicates that gastrointestinal endoscopy with a biopsy is the preferred method of investigating for patients with digestive symptoms suggestive of disease (Logan and Walker, 2002).

2.10.2.2 Urea breath test

This test makes use of the metabolic functions of the organism. Non invasive detection of *H. pylori* by the ^{13}C-urea breath test is based on the fact that a solution of urea labelled with carbon-13 will be rapidly hydrolysed by the urease enzyme of *H. pylori* (Brown, 2000). The resulting CO_2 is absorbed across the gastric mucosa and hence, via the systemic circulation, excreted as $^{13}CO_2$ in the expired breath. The ^{13}C-urea breath test detects current infection and is not radioactive. It can be used as a screening test to assess eradication and to detect infection in children. The similar but radioactive ^{14}C-urea breath test cannot be performed in primary care (Logan and Walker, 2002).

2.10.2.3 Faecal antigen test

In the stool antigen test, a simple sandwich ELISA is used to detect the presence of *H. pylori* antigens shed in faeces. The stool antigen test has been studied widely and has been reported to have high sensitivity and specificity in adults and children (Chisholm *et al*., 2004) compared with other test such as the urea breath test. These studies and more about the efficiency of the stool antigen test was emphasised in studies in the United States, United Kingdom and other parts of the world (Chisholm *et al*., 2004). The main advantage of the test is that it can be used in large scale epidemiological studies of acquisition of *H. pylori* in children **(**Logan and Walker, 2002**).**

2.10.2.4 Molecular diagnostic methods for identification of *Helicobacter pylori.*

Molecular microbiologists have used a number of molecular based techniques to diagnose pathogenic microorganisms (Wong *et al.,* 2001; Bolek *et al.,* 2007). Modern biology techniques are currently providing a number of solutions to diagnostic problems. These are based on the assumption that each species of microorganism has some unique genetic makeup. These methods have been categorized into nucleic acid sequence based approach as well as polymerase chain reaction (PCR) based methods. A number of these techniques have been used in the identification and characterization of *H. pylori* as described below.

2.10.2.4.1 Polymerase chain reaction

The Polymerase Chain Reaction (PCR) is a technique for isolating and exponentially amplifying a fragment of DNA via enzymatic replication (Pavlov *et al*., 2004). It is used to amplify specific regions of a DNA strand. This can be a single gene, part of a gene, or a non-coding sequence. The PCR is able to replicate *H. pylori* DNA in biological material (van Doorn *et al*., 1998; Datta *et al.,* 2003). PCR based tests are extremely sensitive and, since they use DNA, they do not require the living micro-organisms to detect infection being sufficient for recognition.

PCR techniques have been used for research into genetic variation between different strains of *H. pylori*. Several loci have been identified in the genome, including the genes for urease subunits, the locus encoding the 16S ribosomal RNA, and the gene for the major flagellin and several other genes. PCR techniques have been applied to gastric biopsies, saliva and gastric juice samples and they have been used to detect *H. pylori* DNA in stool samples (Mackay *et al*., 2003; Dube *et al.,* 2009a). Their disadvantages are that a positive finding does not imply the presence of a viable strain, and therefore an infective bacterial product; and great care

must be taken to avoid contamination with DNA from other patients (Logan and Walker, 2002). Several PCR based techniques have been employed for the diagnosis of *H. pylori,* these include Reverse transcriptase PCR (RT–PCR), conventional PCR, real time- PCR, multiplex PCR and nested PCR (Wong *et al.,* 2001; Bolek *et al.,* 2007).

Reverse transcriptase PCR (RT–PCR) is a two-step process for converting mRNA to complementary DNA (cDNA) and the subsequent amplification of the reversely transcribed DNA. In the first step, there is the conversion of mRNA to cDNA using reverse transcriptase while in the second step there is amplification of cDNA with the aid of a thermo stable DNA polymerase, upstream and downstream DNA primers, dNTP's to generate million copies of DNA **(**Yamaoka *et al*., 1996**).**

Diagnosis of *H. pylori* can also be done using conventional, multiplex or Real-Time PCR. These different forms of PCR are improvements of the conventional PCR. Multiplex-PCR is a widely used, fast and accurate method for detecting *H. pylori* having virulence-associated genes such as *vacA, cagA* and *iceA* (Smith *et al*., 2002). Recently, Real-time PCR also known as quantitative PCR (qPCR) is gradually replacing conventional PCR (Glocker *et al*., 2005; Diouf *et al*., 2009) due to the advantages attached to this form of PCR; it offers excellent sensitivity and specificity, low risks of contamination, ease of performance and speed (Espy *et al.,* 2006).

Real-time PCR also simultaneously quantify and amplify specific parts of a given DNA molecule. It is used to determine whether or not a specific sequence is present in the sample; and if so, the number of copies in the sample (He *et al*., 2002). This method requires extensive optimisation of the number of PCR cycles to obtain results during logarithmic

DNA amplification (He *et al.*, 2002). This form of PCR is increasingly being used for clinical diagnostic purposes as well as to identify microorganisms in food and environmental samples. In any PCR-based detection of *H. pylori*, false-positive results may be due to residual *H. pylori* DNA on fibre-optic endoscopes (following inadequate cleaning and disinfection) or from cross-contamination during the processing of specimens in the laboratory. Great care must be taken to avoid contamination with DNA from other patients (Tanih *et al.,* 2009). Detection of *H. pylori* using gene capture and PCR has been evaluated (Mackay *et al*., 2003; Cherie *et al.,* 2005).

Nested PCR – Nested polymerase chain reaction is a modification of polymerase chain reaction intended to reduce contamination in products due to the amplification of unexpected primer binding sites. Nested PCR involves two sets of primers, used in two successive runs of PCR, the second set intended to amplify a secondary target within the first run product. Maciorkowska *et al*. (2007) used nested and semi nested PCR methods in their study for genotyping signal region s1/s2 and midregion m1/m2 alleles of *H. pylori.*

PCR-LiPA: This reverse hybridization assay consist of a nitrocellulose strip that contains a number of oligonucleotide probes used for type specific detection of *H. pylori* genotypes, immobilized as parallel lines (van Doorn *et al.,* 1998). This assay makes use of biotinylated PCR products normally placed in a plastic trough and a DNA denaturation solution (e.g ethylenediaminetetraacetic acid) is added unto the PCR product to denature it. A preheated hybridization buffer (e.g 2X standard saline citrate, Tris –HCl, sodium dodecyl sulphate) is added and a LiPA strip is submerged in the solution followed by incubation in a shaking water bath. Strips are washed with standard saline citrate and sodium dodecyl sulphate and subsequently rinsed in phosphate buffer and conjugate (streptavidin/alkaline phoshatase) is

added. After rinsing the strip a substrate is added. Hybrids are visible as purple-coloured probe lines and hybridization patterns are interpreted visually.This technique has been widely used for detection of *H. pylori* genotypes (van Doorn *et al.,* 1998; Debets-Ossenkopp *et al.,* 2003).

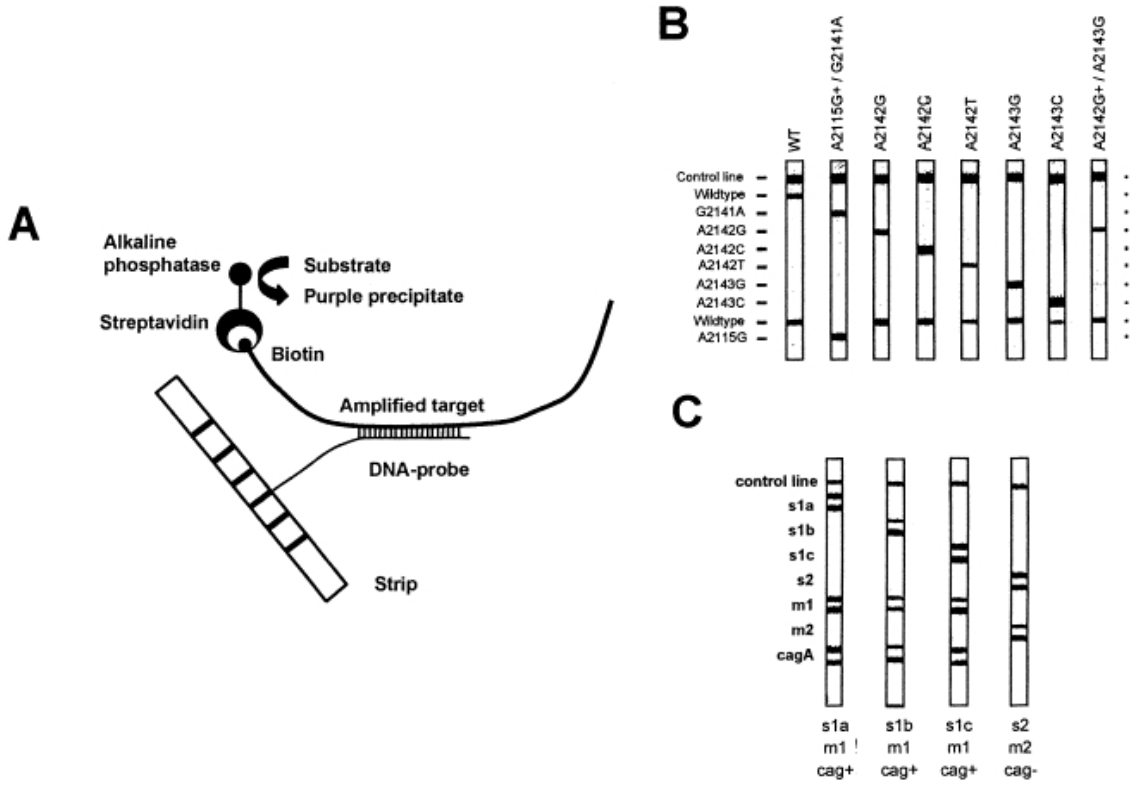

Fig 2.5

Genotyping of *H. pylori* isolates using reverse hybridization. Panel A shows the principle of the reverse hybridization line probe assay (LiPA). Specific oligonucleotide probes are tailed and immobilized onto membrane strips in parallel lines. Biotinylated PCR products are denatured and hybridized to the probes at highly stringent conditions. Hybrids are detected by a streptavidin-alkaline phosphatase conjugate and substrate, resulting in a purple precipitate. Panel B shows the LiPA for detection of specific mutations in the 23S rDNA, which are associated with macrolide resistance of *H. pylori*. Panel C shows the *H. pylori* virulence LiPA, permitting simultaneous typing of virulence-associated *vacA* and *cagA* genes in a single reverse hybridization step after multiplex PCR (www.ncbi.nlm.nih.gov/bookshelf/picrender.fcgi).

2.10.2.4.2 Nucleic acid sequence based approaches

Several nucleic acid sequence based approaches including Southern and Northern blotting, restriction fragment length polymorphism (RFLP), DNA sequencing, multilocus sequence typing (MLST), DNA microarrays and pulse field gel electrophoresis (PFGE) have been used for the identification of *H. pylori* (Tonokastsu *et al.,* 1994; Fujimoto *et al.,* 1994; Mukhopadhyay *et al.*, 2000; Maeda *et al*., 2001; Smith *et al.,* 2003; Devi *et al.,* 2006). For example, direct sequencing has been employed by Mukhopadhyay *et al*. (2000) to determine the sequences of *cagA, vacA* and *iceA in H. pylori* strains from Calcutta, India. Also, MLST has been employed in research on *H. pylori* and a number of allelic diverse strains have been determined based on the seven housekeeping genes: *atpA, efp, mutY, ppa, trpC, ureI* and *yphC* (Devi *et al*., 2006).

Southern blotting is a method routinely used to check for the presence of a DNA sequence in a sample of specimen. This technique along with Northern blotting has been used in the detection of *H. pylori* in different sample types for example, stool, gastric biopsy and serum etc (Tonokatsu *et al.,* 1994). Automated immunomagnetic separation coupled with PCR amplification and microarray hybridization has been applied to detect *H. pylori* from both clinical and environment samples (Han *et al.* 2007). A number of investigators working with *H. pylori* have used a DNA microarray for rapid identification of *H. pylori* pathotypes by virulence gene detection (Maeda *et al.,* 2001). Furthermore, Smith *et al.* (2003) successfully used PFGE to compare clinical isolates of *H. pylori* from Lagos and Ife in Nigeria. They found PFGE a useful typing tool in their environment except for the limitation of non digestion of some *H. pylori* DNA by the restriction endonucleases NotI/NruI (Smith *et al.,* 2003). Other typing methods that have provided useful insights include ribotyping, random

amplified polymorphic DNA (RAPD) analysis and other types of high-resolution typing methods (Akopyanz *et al*., 1992).

2.11 Test methods used for determining antibiogram of *H. pylori.*

There are multiple test methodologies to determine the antibiogram of an organism. Standardized susceptibility test methods are based upon rapidly growing, aerobic micro organisms in which overnight incubation results in definitive endpoints. *In vitro* susceptibility testing for fastidious organisms (such as *H. pylori*) that require complex media for growth, incubation in atmosphere other than ambient air, and grow slowly are problematic and in general are not standardized. The Clinical and Laboratory Standards Institute (CLSI) approved the agar dilution method as the test of choice for testing *H. pylori* (Osato, 2000). Breakpoints are minimum inhibitory concentrations (MICs) of a drug at which an organism is deemed either susceptible or resistant to the antibiotic using standard dosing regimens containing that drug (Osato, 2000).

The usual methods of phenotypic detection of resistance are still widely used. The recent European standardisation (Glupczynski *et al*., 2002) has led to recommendation of a similar protocol to that of the US CLSI. Given the important difference observed in MICs between clarithromycin susceptible and resistant strains, the simple and cheap disk diffusion method has been validated (Grignon, 2002). New methods have been developed, which are essentially molecular given that point mutations are the unique mechanism of resistance in *H. pylori*, and PCR based methods are most often used. Other methods include broth microdilution, Epsilometer or Etest. The Etest and the disk diffusion test methods have the advantage of allowing the visualization of resistant sub-populations of bacteria within zones of inhibition (Alarcon *et al*., 1998; Megraud *et al*., 1999).

2.12 Treatment

Antibiotics are essential for the prevention and treatment of bacterial infections in humans, domestic animals and livestock. *H. pylori* infection presents a unique therapeutic challenge. Determining the optimum treatment of infection is difficult because the organism lives in an environment not easily accessible to many medications. Recommended regimens pose a number of difficulties to patients such as poor compliance; specifically, having to take a large number of pills at least twice daily and coping with unpleasant adverse effects do little to encourage patient cooperation (Hardin and Wright, 2002; Tanih *et al.,* 2010a). Apart from patient non-compliance, antibiotic resistance is the major cause of treatment failure (Tanih *et al*., 2010a).

Antibiotic resistance has become a global concern (Westh *et al.,* 2004). In recent years, there has been an increasing incidence of multiple resistance in many microorganisms that are human pathogens, largely as a result of the indiscriminate use of the antimicrobial drugs that are commonly employed in the treatment of infectious diseases. Since drug control is much tighter in some areas than others, the prevalence of antimicrobial resistance tends to vary with geographical region (Debets-Ossenkopp *et al.,* 1999; Ndip *et al.,* 2008). Data on antibiotic susceptibility and resistance in *H. pylori* highlighted regional differences in resistance patterns for clarithromycin and metronidazole (Me´graud, 2004). These differences are not trivial, as they have a major effect on the success of eradication therapy. Treating *H. pylori* infection is therefore a concern. Although several antibiotic agents are being used world-wide for the treatment of *H. pylori* infection, drug resistance to most of them is already well established (Kalach *et al.,* 2001; Kwon *et al*., 2001; Gerrits *et al*., 2002; Tanih *et al*., 2009; Tanih *et al.,* 2010c).

In recent years, several different treatment regimens have been proposed for the eradication of *H. pylori* from a patient. The only conditions, for which such treatment is strongly recommended, on the basis of unequivocal supporting evidence, are, however, peptic ulcer disease and low-grade gastric MALT lymphoma (Knigge, 2001). The success of any antimicrobial regimen for *H. pylori* eradication depends on patient compliance and lack of antimicrobial resistance (primary and secondary). Complicated regimens and/or those associated with adverse effects may result in non-compliance and treatment failure (Lesbros-Pantoflickova *et al*., 2007; Ndip *et al*., 2007b).

Primary resistance is associated with innate resistance of the organism, (i.e., pre-existing resistance) (Kim *et al.,* 2001). Primary resistance in *H. pylori* can develop in nature by acquisition of resistance factors through genetic transformation. *H. pylori* are genetically "competent" cells that allow uptake of foreign bacterial DNA, sometimes containing antibiotic resistance markers, and incorporation of the DNA fragment into the bacterial genome. Primary resistance can be detected by pre-treatment susceptibility testing and this allows prescription of treatment regimen tailored specifically to eradicate the infection (Kim *et al.,* 2001). In patients harbouring resistant isolates, standard multidrug eradication therapies show significant reductions in efficacy (Osato, 2000). Thus pre-treatment susceptibility testing not only enhances the probability of a successful treatment outcome, but also reduces the cost of treatment because expenses related to evaluation of the patient and additional drugs would not be incurred (Osato, 2000).

Secondary resistance is defined as resistance that emerges following sub-optimal or unsuccessful treatment (Kim *et al.,* 2001). Treatment usually does not produce resistant organisms, but rather selects for a resistant sub-population of organisms. Within each

population of organisms, there often exist sub-populations of bacteria that are more resistant to specific antibiotics than the general population of bacteria (Osato, 2000). Anti-infective treatment selects for these resistant bacteria by killing those bacteria that are susceptible, yet leaving the resistant bacterial population unaffected. The resistant bacteria then re-populate in the stomach (Osato, 2000).

Theoretically, current combination regimens should demonstrate 85%–95% efficacies. Single-agent therapy should not be used because of the unacceptably low frequency of eradication. Clinical trials have shown that the triple-drug regimens offer the most effective treatment, with *H. pylori* cleared from up to 96% of treated patients (Kwon *et al*., 2001; Aboderin *et al*., 2007). The current regimen of choice is a combination of either an antisecretory drug such as a proton-pump inhibitor, which may include omeprazole, lanoprazole, rabeprazole, or pantoprazole or ranitidine bismuth citrate with two antibiotics in this group (clarithromycin, amoxicillin, metronidazole, tetracycline) given for 14 days (Knigge, 2001; Ndip *et al.,* 2008). Such combinations have superior efficacy to, and are better tolerated than, the standard bismuth-based triple combination (i.e. bismuth subsalicylate, metronidazole and tetracycline hydrochloride) (Knigge, 2001; Meurer and Bower, 2002). Eradication of the organism has been shown to result in ulcer healing, prevention of peptic ulcer recurrence and may also reduce the prevalence of gastric cancer in high risk populations (Sepulvedo and Coelho, 2002; Tanih *et al.,* 2009).

According to a (controversial) theory, pretreatment with a proton-pump inhibitor may reduce the success of eradication therapy, by inducing the transition of the target bacteria into a dormant state (Bytzer and O'Morain, 2005). In adults, the current standard ('Maastricht') triple therapy is a combination of a proton-pump inhibitor with amoxicillin and

clarithromycin. In the event of eradication failure, a quadruple combination, containing a proton-pump inhibitor, bismuth, metronidazole and tetracyline, has been suggested (Triantafyllopoulou, 2006). A combination of levofloxacin, tinidazole and a proton-pump inhibitor (rabeproizole) has recently been shown to be superior to the 'Maastricht' regimen as second-line therapy (Triantafyllopoulou, 2006). When Loffeld and Fijen (2003) compared first-line treatments for adults, they found 2 weeks' treatment including a combination of a proton pump inhibitor with clarithromycin and metronidazole to be less effective than a similar treatment with a proton-pump inhibitor plus clarithromycin and amoxicillin. Unfortunately, many patients originating from Africa and Middle East and other parts of the world are infected with *H. pylori* isolates that appear resistant to clarithromycin (Loffeld and Fijen, 2003) and, in addition, clarithromycin is not acid stable and may cause dyspepsia (Hardin and Wright, 2002).

In Egypt, Sherif *et al.* (2004) reported universal high-level primary resistance to metronidazole in children infected with *H. pylori*, although there was less resistance to other selected antibiotics. Similarly poor responses to metronidazole have been reported elsewhere in the World (Al-Quarashi *et al.,* 2001; Ndip *et al*., 2008; Tanih *et al.,* 2010a). Very high frequencies of resistance to amoxicillin and metronidazole have been reported in Nigerian and Cameroonian patients (Abdulrasheed *et al*., 2005; Ndip *et al*., 2008). Treatment failures in children are mostly attributable to noncompliance (resulting from adverse effects) and/or resistance to metronidazole and clarithromycin. If treatment fails, knowledge of the antimicrobial sensitivities of the infecting *H. pylori* can clearly be helpful in selecting suitable antibiotics for the second line treatments. In children with documented *H. pylori* infection, however, all regimens should continue to be prescribed for 7–14 days, until the effectiveness

of short-course treatments in this group of patients can be thoroughly determined (Ables *et al*., 2007).

Metronidazole and clarithromycin are the main representative antibiotics used in *H. pylori* eradication (Kobayashi *et al*., 2001). Further to this, Smith *et al*. (2001) reported that metronidazole and amoxicillin were the main drugs used in the treatment of infections in Nigeria. However, metronidazole-containing regimens have been shown to limit effectiveness in African and Asian countries because of increasing prevalence of resistance to this drug. High resistance rates have been reported in some areas of South Africa (Jeong *et al.,* 2001, Tanih *et al.*, 2010a).

Amoxicillin and tetracycline which are drugs commonly prescribed for patients presenting with *H. pylori*- related pathologies was shown by Sherif *et al*. (2004) to have as low as 2% resistance. In contrast, very high resistance rates to these drugs have been reported in Nigerian patients (Abdulrasheed *et al*., 2005). In Kenya, most of the isolates studied by Lwai-Lume *et al*. (2005) were susceptible to clarithromycin (93.6%), amoxicillin (95.4%) and tetracycline (98.1%), and all of those investigated by Henriksen *et al*. (1999) remained sensitive to ampicillin. In Nigeria, Abdulrasheed *et al*. (2005) reported that all the isolates they investigated was susceptible to ciprofloxacin, and, in consequence, recommended the incorporation of ciprofloxacin into the existing empiric regimen for the treatment of *H. pylori* infections in Nigeria. Administration of amoxicillin requires the co-administration of a proton pump inhibitor and an adjunctive agent since its activity is pH dependent. This could increase chances of patient non-compliance coupled with already stated resistance problems. Tetracycline has the advantage of low cost, but has been found to cause teeth discoloration in children and photosensitivity reactions (Hardin and Wright, 2002).

A study by Ndip *et al.* (2008) in Cameroonian patients showed marked *in-vitro* multiple antimicrobial resistances to the commonly used eradication agents, which is in line with other reports worldwide (Pilotto *et al.,* 2000; Kalach *et al.,* 2001; Mégraud, 2004; Tanih *et al.,* 2009). In Ethiopia, however, all the isolates investigated by Asrat *et al*. (2004) were found to be sensitive to clarithromycin, erythromycin and tetracycline, while 76% and 6% of the isolates were resistant to metronidazole and amoxicillin, respectively. Such regional variation in resistance patterns probably reflects geographical variation in local antibiotic-prescription practices and antibiotic use and abuse. This high resistance rates to commonly used antimicrobial drugs in *H. pylori* treatment posses serious public health problems and is therefore necessary that new drug regimens be examined.

2.13 Properties of antibiotics used in the treatment of *H. pylori* infection

Amoxicillin is an acid stable aminopenicillin that is well absorbed in the gastrointestinal (GI) tract. It inhibits the final step in the biosynthesis of the bacterial cell wall (transpeptidation), but other effects have yet to be elucidated (Van-Zwet *et al.,* 1999). Metronidazole is a nitroimidazole drug. It is a pro-drug that requires activation; and its absorption in the GI is good (Kwon *et al*., 2001; Debets-Ossenkopp *et al.,* 1999; Chisholm *et al.,* 2004). Immediate products generated by intracellular reduction of the nitro group or free radicals damage DNA and other molecules. Clarithromycin is a semi-synthetic macrolide. Its solubility increases in acid conditions. It inhibits protein synthesis by penetrating the cell wall and binding with the 50S ribosomal subunits, inhibiting translocation of aminoacyl-tRNA and inhibiting polypeptide synthesis (Gerrits *et al.,* 2002; Glocker *et al.,* 2005). Tetracycline binds 30S ribosomal subunit at a position that blocks the binding of the aminoacyl-tRNA to the acceptor site on the mRNA-ribosome complex thereby inhibiting protein synthesis (Osato, 2000).

2.14 Resistance mechanisms

H. pylori like a few other bacteria, is able to acquire resistance to most of the antibiotics used in the treatment regimens (Mégraud, 2004; Glocker *et al.,* 2005; Tanih *et al.,* 2010b). The mechanism rarely involves plasmids. However, resistance could increase progressively due to the selection pressure (Njume *et al*., 2009). As in many bacteria, drug efflux proteins can contribute to natural insensitivity to antibiotics and to emerging antibiotic resistance (Bina *et al.,* 2000).

2.14.1 Metronidazole resistance mechanisms

Metronidazole (Mtz)-containing regimens have recently been shown to have limited effectiveness because of the increasing prevalence of resistance to this drug (Al-Quarashi *et al*., 2001; Buta *et al*., 2010). The resistance mechanisms in anaerobic organisms and *H. pylori* vary. Mtz, a synthetic nitroimidazole is a prodrug and becomes active when reduced in the cytosol of the microorganism to a toxic metabolite. Unstable Mtz radicals react rapidly with proteins, RNA and DNA, eventually resulting in cell death (Jeong *et al*., 2001; Njume *et al.,* 2009; Buta *et al*., 2010). Under the conditions of low-redox potential in anaerobic organisms, drug activation can be catalyzed by nitroreductases such as pyruvateflavodoxin reductase by means of a single electron transfer event. *H. pylori* possesses this enzyme, but owing to its microaerophilic nature, molecular oxygen is also present and can compete with the Mtz radical for electrons in a futile cycle that restores the prodrug along with superoxide. Instead, a separate mechanism seems to account for most Mtz sensitivity in *H. pylori.* A non-oxygen-sensitive NADPH nitroreductase encoded by the *rdxA* gene reduces Mtz by a two-electron transfer step into a toxic metabolite that cannot be retransformed to its parent by molecular oxygen (Kwon *et al.,* 2001, Buta *et al.,* 2010).

The vast majority of clinically isolated (Tankovic *et al.,* 2000) or experimentally induced (Jenks *et al.,* 1999) Mtz-resistant clones contain a mutation somewhere in the *rdxA* coding sequence. However, there have been reports that mutation of a second reductase NAD (P)H-flavin oxidoreductase encoded by *frxA* could also confer low-level Mtz sensitivity in some strains (Kwon *et al.,* 2001) and a role for oxygen sensitive reductases has not been formally excluded. Such resistance has been linked mostly to genetic mutations in the *rdxA* and *frxA* genes of the bacterium (Jeong *et al*., 2001).

Sequencing candidate genes, such as the reductases (mentioned above), in sensitive and resistant isolates has provided support for the idea of these genes playing a role in resistance, but reports also show that frameshift mutations in *frxA* occur with similar frequencies in sensitive and resistant strains (Chisholm and Owen, 2004). Jeong *et al.* (2001) concluded that most Mtz resistance in *H. pylori* depend on *rdxA* inactivation, of which mutations in *frxA* can enhance resistance, and that genes conferring Mtz resistance without *rdxA* inactivation are rare or nonexistent in *H. pylori* populations. Although null mutations in a *rdxA* gene that encodes oxygen-insensitive NAD(P) H nitroreductase was reported in Mtz-resistant *H. pylori,* an intact *rdxA* gene has also been reported in Mtz-resistant *H. pylori,* suggesting that additional Mtz resistance mechanisms exist in *H. pylori* (Kwon *et al*., 2001).

2.14.2 Clarithromycin resistance mechanisms

Clarithromycin, an intracellularly active antibiotic, is one of the cornerstones in present ulcer therapy and in eradication of *H. pylori* in general. With increasing use there is increased risk of bacterial resistance. The high frequency of clarithromycin resistance among African isolates of *H. pylori* is thought to be the result of treatment with less expensive macrolides and the subsequent development of clarithromycin cross-resistance (Loffeld and Fijen, 2003).

Clarithromycin acts by binding to the peptidyl transferase region of *23SrRNA* and inhibits bacterial protein synthesis. Clarithromycin resistance has been linked to mutation in 23S rRNA (Ahmad *et al,* 2009). Several reports have demonstrated that more than 90% of macrolide resistance in *H. pylori* is mediated by either of two transition mutations Adenine to Guanine (A→G) at adjacent positions 2142 and 2143 in the bacterium's 23SrRNA gene (Ahmad *et al,* 2009).

A transversion mutation (A→C) at position 2143 has been reported to be the cause of resistance in 7% of the resistant isolates. The mutations are stable, and resistance to one macrolide leads to cross-resistance to other members of this group of antibiotics (Ahmad *et al,* 2009). Other mutations that have been observed in clarithromycin resistant *H. pylori* isolates are A2515G and T2717C, A2116G, G2141A, A2144T, T2182C, G2224A, C2245T etc.

2.14.3 Amoxicillin resistance mechanisms

H. pylori has been considered to seldom become resistant to amoxicillin in recent years. Reports are available concluding that amoxicillin resistance is a cause of unsuccessful eradication **(**Rimbara *et al.,* 2007**).** Amoxicillin resistance in *H. pylori* is thought to develop because of amino acid substitutions in the penicillin binding proteins leading to structural alterations in the protein (Deloney and Schiller, 2000) or changes in the proteins involved in the bacterium's cell-wall synthesis. This is consistent with earlier reports made by Van-Zwet *et al.* (1999) in which it was indicated that amoxicillin acts by interfering with peptidoglycan synthesis, especially by blocking transporters named penicillin binding proteins (PBP). They further indicated that amoxicillin-resistant *H. pylori* strains harbour mutations on the pbp-1a

gene with amino acid substitution Ser-414→Arg appears to be involved, leading to a blockage of penicillin transport.

Resistance to β- lactamase in Gram negative bacteria may also be associated with mutation of PBPs, changes in drug permeability and multiple-drug efflux mechanisms. Resistance to amoxicillin may also result from the production of β -lactamases by the bacterium (Rimbara *et al.,* 2007). Colonization of the stomach with β -lactam- resistant bacteria of other species may lead to the transfer of amoxicillin resistance to *H. pylori* (Ndip *et al.,* 2008).

2.14.4 Tetracycline resistance mechanisms

Tetracycline is a major component of quadruple therapy for *H. pylori* infection. Generally, *H. pylori* tetracycline resistance is rare (Mégraud, 2004; Cameron *et al*., 2004; Dzierzanowska *et al.,* 2005). Tetracycline is a protein synthesis inhibitor active against gram-positive and negative bacteria, chlamydiae, mycoplasmas, rickettsia, and some protozoan parasites.

Tetracycline inhibits bacteria growth by disrupting codon-anticodon interactions at the ribosome, specifically, by binding to the subunit of 30S, preventing attachment of aminoacyl-tRNA to the acceptor site. Tetracycline resistance has been attributed to mutations in the 16SrRNA-encoding genes that affect the binding site of tetracycline (Wu *et al.,* 2005). The change in a nucleotide triplet (AGA-926 to 928→TTC), cognate of the positions 965 to 967 in *Escherichia coli*, has been associated with resistance to these compounds probably because of a lack of binding to the h1 loop, which is the binding site of tetracyclines. Single or dual mutations at these positions lead to intermediary MICs (Wu *et al.,* 2005). Tetracycline-resistant strains with no mutation in position 926 to 928 have also been described (Mégraud,

2004; Cameron *et al*., 2004; Dzierzanowska *et al.,* 2005). Studies of isolates from the Netherlands and Australia showed that high level tetracycline resistance was associated with triple mutations **(**Cameron *et al*., 2004**).** The need to have three mutational events can explain the rarity of tetracycline resistance (Cameron *et al*., 2004).

2.14.5 Plasmids and resistance

Extra chromosomal plasmid DNA is present in approximately half of all the strains although the type strain (NCTC 11637) is plasmid free (Dharmalingam *et al.*, 2003). The number and size of plasmids can vary considerably from strain to strain but many strains have a single plasmid with size 1.8-63 kbp. *H. pylori* plasmids have also been associated with drug resistance (Kuster *et al.,* 2006)**.** Plasmid mediated resistance mechanism involves horizontal transmission of the mobile factor. Several investigators have worked on the presence and significance of plasmids in *H. pylori*. Owen *et al.* (1993) examined biotypes, ribopatterns, whole cell protein patterns and plasmid profiles of paired *H. pylori* clinical isolates from 17 patients. Their results indicated that emergence of resistance to metronidazole in *H. pylori* was unlikely to be attributed to plasmid coded determinants because there was no direct association between emergence of resistance and acquisition of a plasmid (Owen *et al.,* 1993). Strains of *H. pylori* may become resistant by mutations in the chromosomal genes, by acquisition of exogenous DNA, or by transformation. There is a theoretical possibility of foreign DNA acquisition by plasmids, transposons or integrons (Dharmalingam *et al.*, 2003).

2.14.6 Efflux mechanism and resistance in *Helicobacter pylori*

Efflux of compounds is a phenomenon commonly observed in bacteria of which *H. pylori* is not an exemption (Borges-Walmsley and Walmsley, 2001). In this process, organisms are protected from possible toxic effects of metabolite accumulation or external compounds, and compound efflux results in a decreased susceptibility for a variety of antibiotics. Efflux can be mediated through specific pumps or through pumps that transfer a broad range of substrates (including antibiotics, detergents, and dyes) (Kutschke and Boudewijn, 2005).

Five families of multidrug efflux transporters have been described: small multidrug resistance (SMR) proteins, multidrug and toxic compound extrusion (MATE) proteins, the major facilitator superfamily (MFS), the ATP-binding cassette (ABC) superfamilies, and the resistance-nodulation-cell division (RND) family (hefF, hefC, and hefI) (van Amsterdam *et al.*, 2005). Generally, in gram-negative bacteria, the last three efflux pumps or translocases are located in the inner membrane and are therefore also called inner membrane efflux proteins (IEPs), which act with two other components, a periplasmic efflux protein (PEP), which facilitates the interaction with the other component, and an outer membrane efflux protein (OEP), which is TolC or a TolC homolog (van Amsterdam *et al.*, 2005).

Bina *et al*. (2000) evaluated the relevance of three putative efflux systems in *H. pylori* resistance to antibiotics and concluded that, in contrast to what is usually described for gram-negative bacteria such as *Escherichia coli* or *Pseudomonas aeruginosa*, efflux systems did not play a role in the intrinsic resistance to antibiotics. In contrast, Kutschke and Boudewijn (2005) and van Amsterdam *et al*. (2005) in their studies showed that similar to other gram negative organisms, *H. pylori* contains an active multidrug efflux mechanism and therefore

compound efflux needs to be taken into account when determining resistance mechanisms in this organism.

2.14.7 Natural resistance in *H. pylori*

H. pylori is intrinsically resistant to the polymyxins, trimethoprim, sulphonamides and vancomycin some of which are used as selective agents in isolation media. Most strains, but not all are also resistant to cefsulodin, nalidixic acid and antifungal compounds (Megraud, 1997). They are susceptible as a species to the penicillins, most cephalosporins, macrolides, tetracyclines, nitroimidazoles, nitrofurans and quinolones (Alarco´n *et al*., 1999). Exposure to suboptimal concentrations of these drugs gives the bacteria a chance to develop resistance against the drugs (Cowan, 1999). Wild-type strains are susceptible to β-lactams (except cefsulodin), fosfomycin, macrolides, aminoglycosides, tetracyclines, chloramphenicol, rifampins, fluoroquinolones, 5-nitroimidazoles, and nitrofurans (Megraud, 1998). With the exception of chloramphenicol (because of toxicity) and aminoglycosides (because of a lack of diffusion), they have all been used in *H. pylori* eradication regimens (Choung *et al*., 2006; Tanih *et al*., 2008).

2.15 Prevention and control

In general, it is wise for persons to wash their hands thoroughly, eat food that has been properly prepared, and drink water from a safe clean source. The Centres for Disease Control (CDC) is currently studying the routes of transmission and possible prevention measures against *H. pylori* (CDC, 2005). *H. pylori* could be a prime target for vaccine therapy, considering that the organism is difficult and expensive to eradicate, and also responsible for significant morbidity and mortality. Studies in the early 1990's provided evidence of possible vaccine development based on murin models but it was later learned that the key mechanism

of protective immunity occurred via stimulation of T-helper type cells and not by antibody production; making vaccine development procedures inconclusive (Hardin and Wright, 2002).

2.15.1 Vaccination

Effective vaccines could provide long-term solutions to many important infectious diseases with *H. pylori* inclusive; however, vaccine development has been hampered by the slow identification of protective antigens. With the rapidly rising antibiotic resistance, and substantial adverse effects experienced by patients in both developing and developed world, it is obvious that chemotherapy alone will be insufficient to control this important pathogen (Sutton *et al*., 2000).

Vaccination is generally considered to be the most effective method against bacterial infections with *H. pylori* infection and *H. pylori*-associated diseases inclusive. Thus, the vaccine can either be prophylactic, i.e. given to persons before they are infected with *H. pylori*; or therapeutic, where the vaccine is given after the person has been infected with the organism (Sutton *et al.,* 2000). Therapeutic vaccination is aimed at decreasing the amount of bacteria or even to eradicate the infection (Sutton *et al.,* 2000). Vaccination can be given as an oral or parenteral immunization. In most experiments with oral immunization, the antigen consists of *H. pylori* fractions administered together with a specific adjuvant. Intranasal immunization using peptide or nucleotide adjuvants have been used successfully (Moschos *et al*., 2005) as well as *H. pylori* lysate. Parenteral immunization has often been a genetic immunization with expression plasmid vectors, which encode antigen proteins and adjuvants in protection against *H. pylori* infection (Dzwonek *et al*., 2004). Johansson *et al*. (2004) compared the intranasal, rectal, intrajejunal and oral route of immunization and found the oral

route to be optimal for inducing antigen-specific IgA antigen response in the stomach. Most of the examples of vaccination against *H. pylori* in animal models reported in the literature concern the use of either whole cell preparation or single purified antigens administered mucosally (Permin and Andreson, 2005).

Because of the difficulty to culture *H. pylori*, genetic engineering has been used for developing *H. pylori* vaccines. Genetically engineered vaccines have many advantages but their immunoprotective effect is usually poor because of the narrow specificity of single antigen components. Using an adjuvant may improve the immune effect. Lactose is now being used as an adjuvant (Mao and Yan, 2004). Recombinant attenuated *Salmonella typhimurium* DNA vaccine carrying *H. pylori hpaA* gene may be a candidate (Xu *et al*., 2005) and recombinant *Salmonella enterica serovar* typhi Ty21a vaccine expressing *H. pylori* urease A and B has been successfully used in humans (Metzger *et al.,* 2004; Xu *et al*., 2005). Also recombinant *H. pylori* urease has been used as a vaccine candidate (Fujii *et al*., 2004). To produce a vaccine generally effective against different strains of the organism, a stable, conserved, and strong antigen is necessary. Attention has been paid to catalase, HSP, *iceA*, and *BabA* of *H. pylori*, but some of these antigens cross react with other similar proteins of the bacteria. Yang *et al*. (2005) revealed that *vacA* is not a suitable antigen for vaccine development.

Antibiotic resistance is an ever increasing problem with the treatment of most microbial infections including *H. pylori* infection (Thyagarajan *et al.,* 2003). New antimicrobial agents are therefore being developed to overcome the problem of antibiotic resistance in bacterial pathogens, such as combination of antibiotics with plant extract and other natural products that possess antimicrobial activity (Ndip *et al.,* 2007b; Tanih *et al*., 2009). Combinations of

drugs have often been used for the treatment of drug resistant infections as this takes advantage of different mechanisms of action.

2.16 Alternative approaches to circumvent the problem of resistance

2.16.1 Medicinal Plants

Phytomedicines have shown great promise in the treatment of several intractable infectious diseases, including opportunistic AIDS infections. Plants are natural blueprints for the development of new drugs (Iwu *et al.,* 1999, Tanih *et al.,* 2009; Njume *et al*., 2009). Of a total of 422,000 flowering plants reported in the world (Govaerts, 2001), more than 50,000 are used for medicinal purposes (Schippmann *et al.*, 2002). The use of medicinal plants all over the world predates the introduction of antibiotics and other modern drugs into Africa. Herbal medicine has been widely used, and still forms an integral part of healthcare' in Ethiopia (Desta, 1993), and Argentina (Anesini and Perez, 1993). The World Health Organization estimates that 3.5 million people in developing countries rely on plant-based medicine for their primary healthcare (WHO, 1987).

In African countries, traditionally used medicinal plants are sold in market places or prescribed by traditional healers in their homes (Fyhrquist *et al*., 2002). Although herbal medicine is well established in many African cultures and traditions, and is a normal part of life for almost 80% of the people in Africa (Cotton, 1996), relatively little information on it is available in the scientific literature. Many plants, belonging to various families, have been screened in the search for new antibacterial agents, and several herbs have been identified as potential sources of drugs (Tabuti *et al*., 2003).

The screening of plant extracts and plant products for antimicrobial activity has shown that higher plants represent a potential source of several novel and potentially important antibiotic prototypes (Tshikalange *et al*., 2005). Plants are rich in a wide variety of secondary metabolites, such as tannins, terpenoids, alkaloids, and flavonoids, which have been found *in vitro* to have antimicrobial properties (Cowan, 1999). Antimicrobial phytochemicals can be divided into several categories (phenolics, terpenoids, essential oils, alkaloids, lectins, polypeptides and polyacetylenes) (Cowan, 1999).

Plant based substances might provide a suitable basis for new anti-*H. pylori* therapies because they possess well-established antimicrobial actions (Eloff *et al.*, 2008); the chemical complexity of these substances and the broad-spectrum effectiveness of some of them suggest that acquired antibiotic resistance would be unlikely (O'Gara *et al*., 2000). Anti-*H. pylori* activity has been recorded in several indigenous medicinal plants in different parts of the world. In some parts of Africa, members of the family Compositae are used in the treatment of gastro–intestinal disorders, lumbago and stomach ache (Akinyemi *et al.,* 2005).

In Cameroon, Ndip *et al*. (2007b) reported that extracts of *Ageratum conyzoides*, *Lycopodium cernua*, *Scleria striatinux*, *Emilia coccinea, Eryngium foetidium*, *Aulatandra kamerunensis*, *Tapeinachilus ananassae*, *Euphorbia hirta*, *Acanthus montanus* and *Scleria verrucosa* were active against *H. pylori* isolates. A abersonine chloride alkaloid from the fruits of *Voacanga africana* and a *protoberberine alkaloid* (7, 8-dihydro-8-hydroxy-palmatine) are two plant-derived molecules with proven cytoprotective, ulcer-healing and *in-vitro anti-Helicobacter* properties (Boda *et al.,* 2006). Extracts of the East African medicinal plants *Entada abyssinica* (stem bark), *Terminalia spinosa* (young branches), *Harrisonia abyssinica* (roots), *Ximenia caffra* (roots), *Azadirachta indica* (leaves and stem bark) and *Spilanthes mauritiana*

(roots and flowers) have all been reported to be active against strains of *H. pylori* (Fabry *et al.,* 1996). Garlic oil and garlic powder have also been shown to have high levels of anti- *H. pylori* activity *in vitro*, and chronic *H. pylori* disease appears to be reduced by eating Allium as vegetables (O'Gara *et al.,* 2000).

Garlic-derived materials might provide a suitable basis for new anti-*H. pylori* therapies because they possess well-established antimicrobial actions and the chemical complexity of such materials and their broad-spectrum effectiveness indicate that acquired resistance to them would be unlikely to develop (O'Gara *et al.,* 2000). It is important, however, to study the safety and efficacy of these and similar materials before their wide-spread use can be promoted.

2.16.2 Honey as a potential lead to new drugs.

Honeys from New Zealand and Saudi Arabia have been shown to inhibit the growth of *H. pylori in vitro* when used at concentrations approximating 20% (v/v); Medihoney^{TM} and Manuka honeys have equally been documented to posses *in-vivo* activity against ulcers, infected wounds and burns (Davis, 2005). These encouraging observations have motivated scientists to investigate the activities of honeys further. Recently, the clinical use of honey has received increasing interest (George and Cutting, 2007). Ndip *et al*. (2007a) investigated various honeys and found that Eco honey, Mountain, Manuka and Capillano honeys all exhibited inhibitory activity against *H. pylori* isolates *in vitro*, when used at a concentration of 10% (v/v). The possibility that these honeys contain compounds with useful therapeutic potential against *H. pylori* is the subject of current research (Manyi –Loh *et al*., 2010). Honey has been found to be effective in the treatment of gastro-enteritis. It consists not only of sugars but also an abundance of minerals, vitamins, enzymes and amino acids (Saridaki-

Papakonstadinou *et al.,* 2006). Previous reports and investigations have confirmed the beneficial effects of honey when used as an antiseptic for wounds, burns and ulcers, improving the assimilation of calcium and magnesium and decreasing acidity (Cushnie and Lamb, 2005).

Honey's impact on the healing of wounds is probably linked to the stimulation of inflammatory- cytokine production by monocytes (Tonks *et al.,* 2003); and its antimicrobial activity varies between types (Basualdo *et al*., 2007). The osmotic effect of honey, its naturally low pH, and the presence of hydrogen peroxide, phenolic acids, lysosomes and flavanoids are all thought to help inhibit bacterial growth when applied to a wound (Cushnie and Lamb, 2005; Mohammad and Mohammad, 2007). Its prolonged use is not likely to lead to the development of 'drug' resistance as its action is not mediated via a single mechanism (Mohammad and Mohammad, 2007). It works differently from other antibiotics that attack a bacterium's cell wall or inhibit intracellular metabolic pathway. Its hygroscopic nature allows it to draw moisture out of the environment and dehydrates any nearby bacteria (Simon *et al*., 2009). The possibility therefore of using honey orally, for the treatment of *H. pylori* infections, seems worthy of some research attention.

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CHAPTER THREE

Helicobacter pylori prevalence in dyspeptic patients in the Eastern Cape Province of South Africa: ethnicity and disease status.

ABSTRACT

Helicobacter pylori is an important cause of gastritis and a number of studies have suggested that it may be important in the genesis of duodenal ulcer recurrence. The organism chronically infects billions of people worldwide, and prevalence has been reported to vary between and even within countries. This study examined *H. pylori* infection in patients who were referred to Livingstone Hospital, Port Elizabeth with gastric related morbidities and determined the prevalence of infection with respect to age, sex, endoscopic diagnosis, ethnic background and lifestyle**.** Gastric biopsies were collected from 254 consecutive patients and *H. pylori* isolated on Columbia agar base (Oxoid LTD, Basingstoke, Hampshire, England) supplemented with 7% sheep's blood (Oxoid, UK) and Skirrow's supplement (Oxoid, UK) containing trimethoprim (2.5 mg), vancomycin (5 mg), cefsulodin (2.5 mg). Amphotericin (2.5 mg) was added to the medium. Recovered isolates were identified following standard microbiology and biochemical techniques. Presumptive isolates were further confirmed by PCR targeting the *glmM* gene. The 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 <0.05 were required for significance. The overall prevalence of *H. pylori* was 66.14% (168/254). Prevalence was highest (100%) amongst patients with duodenitis (1/1), gastric cancer (GC) (4/4) and gastric erosion (5/5) as they were all positive for the organism. However, patients with gastritis (75%; 6/8), gastric ulcer (GU) (70.8%; 17/24), duodenal ulcer (DU) (65%; 26/40), non-ulcer dyspepsia (NUD) (64.7%%; 55/85), and gastro- oesophageal reflux disease (GERD) (64%; 29/45) also had high

prevalence rates of the organism. The organism was not isolated (0%) from patients with gastro-duodenitis and atypical oesophageal reflux disease respectively. The prevalence of infection was highest amongst the coloured (66.92%; 87/130) and lowest in whites (59.52%; 25/ 42). No significant difference was however observed between the ethnic backgrounds and between males and females ($p > 0.05$). Prevalence increased with age and peaked at around 45-54yrs. *H. pylori* infection may be a common cause of gastric related morbidities in the Eastern Cape Province of South Africa and therefore calls for more elaborate studies involving molecular and genotyping methods to delineate the situation.

3.1 INTRODUCTION

Helicobacter pylori is the principal species of the genus *Helicobacter*; it is a Gram negative curved rod that inhabits the gastric mucosa of the human stomach. It chronically infects billions of people worldwide (Asrat *et al*., 2004; Ndip *et al*., 2008; Carilho *et al.,* 2009); and is responsible for one of the most frequent chronic bacterial infections involving more than 50% of the world's population (Go, 2002; Pounder and Ng, 1995). *H. pylori* is one of the most genetically diverse of bacterial species, and is a major cause of at least 90% of duodenal ulcers, 70% of gastric ulcers, NUD and GERD (Parsonnet *et al.,* 1991; NIH, 1994; Henriksen, 2001; Figueiredo *et al*., 2005). It plays a role in adenocarcinoma of the distal stomach; mucosa associated lymphoid tissue lymphoma (MALT) and primary gastric non-Hodgkin's lymphoma in many societies (Kersulyte *et al.,* 1999; Kidd *et al.,* 2001; Matsuhisa *et al*., 2003; Permin & Anderson, 2005; Carrilho *et al*., 2009). Their ability to adhere, invade, evade host defences and cause tissue damage is largely due to their production of colonization and virulence factors (Horiuchi *et al*., 2001).

Epidemiological studies have demonstrated that the prevalence of *H. pylori* infection increases with advancing age and is higher in developing countries and among low socioeconomic level populations, probably due to conditions that favour the acquisition of infection such as precarious hygiene, crowded living conditions, and absence or deficiency of sanitation (Graham *et al.,* 1991; Louw *et al.,* 1993a; Ndip *et al*., 2004; Asrat *et al*., 2004).

Although there is geographical and socio–demographic variation in the prevalence of human infection with *H. pylori* (Ndip *et al*., 2004; Asrat *et al*., 2004; Aguemon *et al*., 2005), prevalence does not parallel the incidence of morbidity caused by the infection. In Africa, for example, *H. pylori* infection is relatively common (Louw *et al*., 1993a; Asrat *et al*., 2004;

Ndip *et al*., 2004; 2008) and the organism is the main cause of at least 90% of duodenal ulcers and 70% of gastric ulcers on the continent (Louw *et al*., 1993a; Ndip *et al*., 2008). Palmer *et al*. (1994) and Ndip *et al.* (2008) documented the involvement of *H. pylori* in gastric patients in Cameroon, revealing a similar pattern to that recorded by Baako and Darko (1996), among Ghanian patients with dyspeptic symptoms and Louw *et al.* (1993b) in South African patients with NUD. Albeit a very high prevalence of *H. pylori* infection on the continent, the incidence of gastric carcinoma and other *H. pylori* associated morbidities is relatively low. This apparent anomaly has been termed the 'African enigma' (Holcombe, 1992; Kidd *et al.,* 1999).

The prevalence of *H. pylori* infection differs among ethnic groups, races and economic conditions (Iso *et al*., 2005). *H. pylori* infection seems to be common in South Africa, as expected in developing countries. Pelser *et al.* (1997) documented a high prevalence (67 - 84%) of *H. pylori* antibodies in children in Bloemfontein, while Mosane *et al*. (2004) also documented *H. pylori* IgG antibodies in South African mothers and their children. Although these studies and a few others reported the prevalence of the organism in the country, most of them adopted a sero-prevalence approach, which does not provide more information other than the presence of antibodies which are poor makers of active infection. Notwithstanding Louw *et al*. (1993b) documented a prevalence of 63% of the pathogen in NUD patients in Cape Town based on histology, while O'keefe *et al*. (2000) reported a prevalence of 81% in NUD patients.

Reports on *H. pylori* prevalence in South African populations are therefore cursory (McNulty *et al*., 2004; Samie *et al.,* 2007). Published data on the prevalence of the organism in dyspeptic patients in the Eastern Cape Province which is predominantly rural with poor

sanitation and household hygiene, factors which favours spread of the organism are lacking. Therefore a profound investigation of the prevalence of the organism in the Eastern Cape Province based on ethnicity, disease profile, and socio-economic status emerges as an imperative rationale that would form the basis of future clinical and epidemiological studies.

3.2 MATERIALS AND METHODS

3.2.1 Study subjects

Two hundred and fifty-four consecutive patients referred for endoscopy at the Livingstone Hospital, Port Elizabeth, South Africa between May and December 2008 were evaluated. The nature and purpose of the study was explained to the patients until fully understood. Only patients who gave informed consent were enrolled, who had not received treatment with broad spectrum antibiotics, non-steroidal anti-inflammatory drugs or proton pump inhibitors in the previous 3 months, and who did not have a history of dysphagia, gastric surgery or upper gastrointestinal bleeding. The study was approved by the institutional review board of the University of Fort Hare and the Eastern Cape Department of Health (Protocol number EcDoH-Res 0002).

3.2.2. Endoscopy, Questionnaire and culture of *H. pylori*

All patients underwent a complete physical examination and history taken by a resident gastroenterologist. Ethnic background, smoking and alcohol intake as well as demographic data were recorded. Some of the patients had previously undergone *H. pylori* eradication therapy.

Two antral and corpus biopsy specimens each were obtained from the patients at endoscopy for a total of 1016 specimens. The biopsies were immediately placed in sterile bijou bottles containing 0.2g/L of cysteine and 20% glycerol in Brain heart infusion (BHI) broth and transported in ice to the laboratory within 2hrs of collection for culture. Biopsies were homogenised under aseptic conditions in 0.2g/L of cysteine and 20% glycerol in BHI broth and a loop full plated primarily on freshly prepared Columbia agar base (Oxoid LTD, Basingstoke, Hampshire, England) supplemented with 7% sheep's blood (Oxoid, UK) and

Skirrow's supplement (Oxoid, UK): trimethoprim (2.5mg), vancomycin (5mg), cefsulodin (2.5mg). Amphotericin (2.5mg) was also added to the medium. All plates were incubated at 37 ºC for 3 – 5 days under microaerophilic conditions (5–6% O_2, 10% CO_2, 80–85% N_2) (Anaerocult Basingstoke, Hampshire, England). Isolates were identified based on colony morphology and positive oxidase, urease and catalase tests as previously reported (Ndip *et al*., 2003; Kullavanijaya *et al*., 2004). Presumptive isolates were further confirmed by PCR targeting the *glmM* gene as previously reported (Burucoa *et al*., 1999). A reference strain of *H. pylori* (NCTC 11638) was included as a positive control. Confirmed isolates were suspended in 20% glycerol and stored at -80 ºC (Sanyo, Japan) for future experiments.

3.3 Statistical Analysis

An SPSS program was used to determine Pearson's chi square test and Fisher's exact test. The 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 (Centers for Disease Control and Prevention, Atlanta, GA, USA). P-values <0.05 were required for significance.

3.4 RESULTS

3.4.1 Patient characteristics and endoscopic findings

A total of 254 subjects were enrolled in the study. Their mean age was 44.5 ± 15.7 years (range = 5–93 years), with 83.07% (211/254) being older than 35 years. There were 90 males and 164 female subjects, for an overall male: female ratio of 1:1, 8.

Endoscopy was performed on all the 254 patients enrolled for the study. Of the abnormal findings; NUD was the most common (33.85%; 86/254) followed in descending order by GERD (17.32%; 45/254), DU (15.35%; 40/254), GU (9.44%; 24/254) (Table 3.1). Some of the subjects had had dyspeptic symptoms for <12 months, others had been afflicted for >20 years, but most (>65%) had dyspeptic symptoms for 1-10 years.

3.4.2 *H. pylori* prevalence in the study population

One hundred and sixty-eight of the 254 subjects had *H. pylori* positive culture giving an overall prevalence of 66.14%. A total of 296 *H. pylori* strains were obtained from the 1016 biopsy specimens (508 antral and 508 corpus). The mean incubation time was 4 days. The percentage positivity for antrum was 61.02% (310/508), and for corpus 55.51% (282/508).

3.4.3 *H. pylori* culture positivity and endoscopic findings

Of the 168 positive subjects; *H. pylori* prevalence was highest in patients with NUD (32.73%; 55/168), and lowest (0%; 0/168) in those with atypical oesophageal reflux disease and gastroduodenitis respectively (Table 3.1). Also, of the 254 subjects enrolled in the study; the prevalence of *H. pylori* with respect to endoscopic findings was highest in patients with duodenitis (1/1), GC (4/4) and gastric erosion (5/5) as they were all positive for the organism. However, patients with gastritis (6/8), GU (17/24), DU (26/40), NUD (55/85), and GERD (29/45) also had high prevalence rates of the organism. The organism was not isolated (0/0)

from patients with gastro-duodenitis and atypical oesophageal reflux disease respectively (Table 3.1)

Table 3.1: Gastro duodenal pathologies and prevalence of *H. pylori* infection in 254 patients referred for endoscopy.

Endoscopic diagnosis	**Number of cases (%)**	**No. positive for *H. pylori***	**% Positive**
Non ulcer dyspepsia	85(33.46)	55	32.73
Gastro-oesophageal reflux disease	45(17.72)	29	17.26
Duodenal ulcer	40(15.75)	26	15.48
Gastric ulcer	24(9.45)	17	10.11
Gastritis	8(3.15)	6	3.57
Duodenitis	1(0.39)	1	0.59
Gastroduodenitis	2(0.78)	0	0
Gastric cancer	4(1.57)	4	2.38
Gastric erosion	5(1.97)	5	2.96
Antral gastritis	2(0.79)	1	0.59
Atypical-oesophageal reflux disease.	1(0.39)	0	0
Others	37(14.57)	24	14.28
Total	254	168	100%

3.4.4 Prevalence of *H. pylori* infection and ethnic background

Of the 254 patients enrolled for the study, 82 (32.28%) were blacks, 130 (51.18%) were coloured and 42 (16.53%) were whites. *H. pylori* prevalence was highest among the coloured (66.92%; 87/ 130), followed by blacks (65.85%; 54 /82) and whites (59.52%; 25/ 42). The prevalence of the organism was also determined by gender in the different ethnic groups. The highest rate of isolation was recorded among black females (71.69%; 38/53), followed by whites (68.18%; 15/22) and coloured (67.77%; 61/90). For the males, the prevalence was highest among the coloured (70%; 28/40), followed by blacks (55.17%; 16/29) and whites (50%; 10/20). (Table 3.2). The difference between the ethnic groups, sex and age were however not statistically significant (P>0.05).

Table 3.2: Ethnic background and prevalence of *H. pylori* infection.

Gender		*H. pylori*			
		+	-	Total	% positive
F Ethnic background	B	38	15	53	71.69%
	C	61	29	90	67.77%
	W	15	7	22	68.18%
Sub total		114	51	165	
M Ethnic background	B	16	13	29	55.17%
	C	28	12	40	70%
	W	10	10	20	50%
Sub total		54	35	89	
Grand Total		168	86	254	

F= Female; M= Male; B= Black; W= White; C= Coloured

3.4.5 Demographic profiles and *H. pylori* infection

Analysis by age groups showed that the prevalence of *H. pylori* infection increased with age and peaked at 45-54yrs (72.85%) (Table 3.3). *H. pylori* prevalence was also analysed with respect to sex. Of the 165 females enrolled for the study, 114 (69.09%) were positive, while for the males 54 of 89 (60.67%) were positive. This difference was however not statistically significant (P>0.05) (Fig 3.1).

The prevalence of *H. pylori* with respect to alcohol consumption and smoking were also investigated. Of the 168 subjects positive for *H. pylori*, 71 (42.26%) were smokers against 46/86 (53.5%) non smokers. On the other hand, 60 of 168 (35.71%) positive subjects were alcohol consumers, while 28/86 (32.55%) did not consume alcohol. These differences did not reach statistical significance (p>0.05) (Table 3.3).

Table 3.3: Association between epidemiological risk factors and *H. pylori* infection (univariate analysis).

Variables and categories Age (yrs)	Number of subjects (%)	*H. pylori* +ve (%)	*H. pylori* –ve (%)	OR (95%CI)	χ^2 test	P-value
15-24	10(4)	8 (80)	2 (20)	1.00(0.06-17.08)	0.31	1.0
25-34	33(13)	21 (63.6)	12(36.4)	0.44(0.04-2.78)	0.94	0.5
35-44	40(16)	25 (62.5)	15 (37.5)	0.42 (0.04-2.65)	0.45	0.5
45-54	70(28)	51 (73)	19 (27)	0.67(0.06-3.82)	0.01	1.0
55-64	47(18)	35 (74.5)	12 (25.5)	0.73 (0.07-4.45)	0.00	1.0
> 65	54(21)	28 (51.8)	26 (48.2)	0.27 (0.03-1.55)	1.69	0.2
Gender						
Male	89(35.4)	54 (60)	36 (40)	1		
Female	165(65.6)	114 (69.5)	50 (30.5)	1.45 (0.82-2.57)	1.47	0.2
Antibiotic treatment						
Yes	16 (6.3)	14 (87.5)	2 (12.5)	3.82 (0.84-35.27)	2.53	0.11
No	238(93.7)	154 (64.7)	84 (35.3)	1		
Smoking						
Yes	168 (66)	71 (42.3)	97 (57.7)	0.64 (0.36-1.11)	2.45	0.1
No	86 (34)	46 (53.5)	40 (46.5)	1		
Alcohol consumption						
Yes	168 (66)	60 (35.7)	108 (64.3)	1.15 (0.64-2.07)	0.13	0.7
No	86(34)	28 (32.6)	58 (67.4)	1		

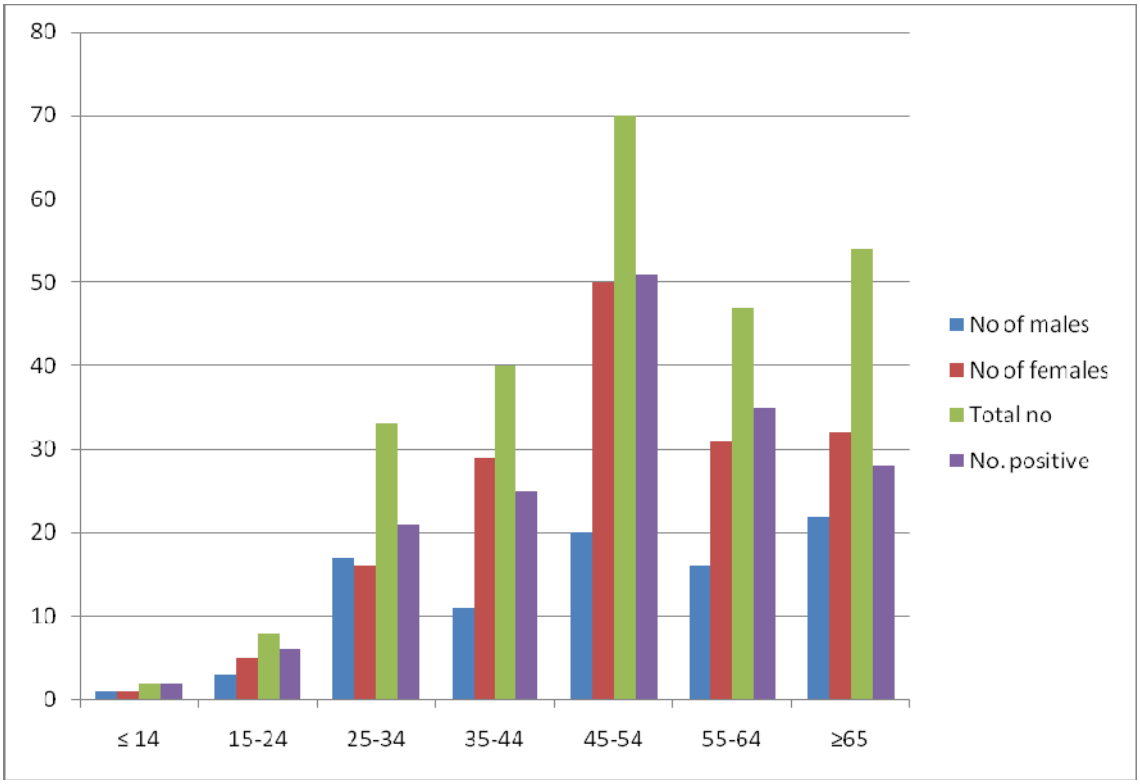

Fig 3.1: Prevalence of *H. pylori* infection with respect to age and gender

3.5 DISCUSSION AND CONCLUSION

The generally high prevalence of human infection with *H. pylori* seen in Africa is an indication that effective public-health interventions need to be developed. The variations seen in the prevalence of infection between and among populations could indicate that factors including age, cultural background, genetic predisposition, socio–economic status and environmental conditions all play a role in the acquisition and transmission of *H. pylori* (Ndip *et al*., 2004; Aguenon *et al*., 2005). Within countries, there may be a similarly wide variation in prevalence between the more affluent urban populations and the resource-poor rural populations (Ndip *et al*., 2004; Aguenon *et al*., 2005; Ahmed *et al.,* 2007).

All the dyspeptic patients investigated in the present study were those who presented with signs of gastric related morbidities at the endoscopic unit of Livingstone Hospital in Port Elizabeth, Eastern Cape Province of South Africa. Culture was used to investigate the presence of *H. pylori* infection among patients and also to determine the prevalence of infection among ethnic groups and races as related to other demographic characteristics. An overall prevalence of 66.14% (168/254) was found. The prevalence rate is similar to other studies documented in Cote d'Ivoire, Egypt, Ghana, Kenya, Malawi, Nigeria, Cameroon, Zaire, Zimbabwe and parts of Korea [(60–94%) Kidd *et al*., 1999; Ndip *et al.,* 2008)]. Kidd *et al*. (2001) had also reported that in Africa endoscopic studies in dyspeptic individuals revealed the presence of the organism in most of the subjects. Previous studies conducted in South Africa (Louw *et al*., 1993a, b; O' keefe *et al*., 2000) had equally incriminated this organism in cases of duodenal and gastric ulcers.

Prevalence was highest (100%) amongst patients with duodenitis (1/1), GC (4/4) and gastric erosion (5/5) as they were all positive for the organism. The numbers of patients recruited for

this study with duodenitis, gastroduodenitis, gastric cancer, gastric erosion, antral gastritis, atypical-oesophageal reflux disease were respectively very low (1, 2, 4, 5, 2 and 1); however patients with most of these disease conditions turn out to be positive and thereby present with high percentage prevalence. However, patients with gastritis (75%; 6/8), GU (70.8%; 17/24), DU (65%; 26/40), NUD (64.7%%; 55/85), and GERD (64%; 29/45) also had high prevalence rates of the organism. This corroborates the findings of other investigators (Kidd *et al*., 1999; Matsuhisa *et al*., 2003; Said *et al*., 2008; Onyekwere *et al*., 2008) who reported either one or two of the above clinical conditions as the most common endoscopic findings in *H. pylori* infection. It has been suggested that up to 95% of duodenal and 70% of gastric ulcers are attributable to infection by this pathogen and most cases occur in middle aged subjects (Louw *et al*., 1993a; Rothenbacher, 2007; Ndip *et al*., 2008).

More importantly, these results are in harmony with previous studies reported in South Africa (Louw *et al*., 1993b; O' keefe *et al*., 2000). Louw *et al*. (1993b) for example, documented an overall incidence of *H. pylori* infection in 80% of gastric and 95% of duodenal ulcer patients. On their part, O' keefe *et al.* (2000) reported an overall *H. pylori* prevalence of 80%; with prevalence rates of 78%, 81%, and 81% in patients with GC, peptic ulcer (PU) and NUD respectively.

In the present study, of the 168 patients who were positive for *H. pylori* by culture, 4 had gastric cancer, of which they were all positive for the organism (100%; 4/4). This corroborates the findings of O'keefe *et al.* (2000) who also reported a prevalence of 78% in cancer patients, but in disagreement with the findings of Uemura *et al.* (2001) who reported gastric cancers in 2.9% of their *H. pylori* infected and none of their uninfected patients. In this study, values on GC are high when compared to other studies conducted in Africa and

propounded as the 'African enigma'. However, it has also been suggested that 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). It is thought that variants of IFNGR1 are more prevalent in Africans and appear to play a significant role in the infection of human host contributing to a high prevalence even-though there is relatively low pathogenecity in Africa (Thye *et al*., 2003; Ndip *et al*., 2004). The possible reason advanced for this could be linked to the different sample sizes used in these studies. Just 4 out of 254 patients had GC. Also, South Africa has a heterogeneous population structure including whites with ancestral background in the Western world were GC is high (Thye *et al*., 2003**)**; and the 4 patients with GC in this study were all whites. Gastric cancer is a terminal condition for *H. pylori* positive patients hence once a patient is identified as having gastric cancer; the person will invariably have the infection. Also, gastric cancer develops in persons with *H. pylori* but not in uninfected persons (Uemura *et al.*, 2001).

Multiple biopsy specimens were cultured. This is in line with the consensus guideline, which recommends that multiple biopsies be taken from the antrum and corpus because of the patchy nature of *H. pylori* infection (Krogfelt *et al*., 2005). *H. pylori* was isolated from both the antrum and the corpus. The percentage positivity for antrum was 61.02% (310/508), and for corpus 55.51% (282/508). This result is in accordance with the findings of Carrilho *et al.* (2009) who reported a higher prevalence of *H. pylori* in the antrum compared to the corpus. Louw *et al.* (1993b) also reported the antrum to be more colonised than the corpus. Very often the infection starts in the antrum and spread to the corpus after extensive mucosal damage (Kersulyte *et al*., 1999).

The prevalence of *H. pylori* infection was highest among the coloured 66.92% (87/130) and blacks 65.85% (54/ 82) than in whites 59.52% (25/42). The findings of this study are in harmony with the study of other investigators (Graham *et al*., 1991; Louw *et al*., 1993a). In a similar study in the Western Cape Province of South Africa, Louw *et al.* (1993a) reported a prevalence of 40% in whites as against 71% in the coloured. The high prevalence of *H. pylori* infection among the coloured and black South Africans compared to the white can partially be explained by other known risk factors for *H. pylori*, particularly socioeconomic determinants such as lower income, lower educational level, and greater household crowding as previously suggested (Louw *et al*., 1993a).

Females, particularly blacks, had a higher prevalence (71.69%) than males (55.17%). A few other studies have found a higher prevalence among women but numerous studies have found no sex difference (Replogle *et al*., 1995; Malaty *et al*., 2002; Chong *et al*., 2008). It is possible that the risk of acquisition is not different between the sexes. Prevalence was probably higher in females than males because more females were recruited in the study than males; it is also speculated that females are more likely to have infections (such as genitourinary infections).

H. pylori infection increased with age and peaked at around 45-54yrs. This is similar to the results reported by Chong *et al.* (2008) in which the prevalence of infection increased with age and was highest between the age ranges 20 – 25 years. The organism is ubiquitous with acquisition in childhood being the rule. People usually get infected with *H. pylori* early in life but however, rarely develop clinically significant disease but do come down with disease when they get of age (Fig.3.1). Seropositivity has been reported to increase with age at a rate of 0.3-1% per year (Graham *et al*., 1991; Segal *et al*., 2001). Serological studies conducted in

different parts in Africa have shown that the majority of subjects are infected with the organism (61-100%), having antibodies for the most of their lives (Holcombe, 1992).

This results also indicated that alcohol consumption and smoking were not significantly associated with *H .pylori* infection (P> 0.05); although the OR (OR= 1.15; 95% CI: 0.64 - 2.07) indicates that alcohol consumption may be a risk factor for *H. pylori* infection. However, a previous study had reported that *H. pylori* infection was slightly lower in smokers than in non smokers (Iso *et al*., 2005); and this has been attributed to the promotion of acid secretion by smoking and resultant generation of an environment not conducive to *H. pylori* survival (Ogihara *et al*., 2000). *H. pylori* prevalence among alcohol consumers has been suggested to be lower than in non alcohol consumers because alcohol may kill the organism in the stomach, however, this remain a matter of speculation (Iso *et al*., 2005).

In conclusion, *H. pylori* infection may be a common cause of gastric related morbidities in the Eastern Cape Province of South Africa and therefore calls for more elaborate studies involving molecular and genetic typing methods.

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CHAPTER FOUR

Marked susceptibility of South African *Helicobacter pylori* strains to ciprofloxacin and amoxicillin: clinical implications.

ABSTRACT.

Helicobacter pylori eradication using the two antibiotics regimen and a proton pump inhibitor often fails due to increasing drug resistance, making it imperative to find substitutes. In this study, 200 *H. pylori* isolates obtained from gastric biopsies of patients presenting with gastric related morbidities attending Livingstone hospital in Port Elizabeth located in the Eastern Cape Province of South Africa were evaluated for their susceptibility to seven antibiotics. *H. pylori* was isolated following standard microbiology procedures and susceptibility determined using the Kirby-Bauer disc diffusion and agar dilution methods. Comparisons of antimicrobial resistance rates with respect to sex of the patients were determined using chi-square test and a P value of < 0.05 was considered significant. Marked susceptibility was observed for ciprofloxacin (100%) and amoxicillin (97.5%). Isolates also demonstrated good activity to clarithromycin (80%) and gentamycin (72.5%). However, marked resistance (95.5%) was observed for metronidazole. The MIC ranged from 0.0625– 8 μg/mL. The lowest MIC with a range of 0.0625–1μg/mL was recorded for ciprofloxacin while the highest (5–8μg/mL) was noted for gentamycin. Multidrug resistance was a common phenomenom encountered in this study and therefore calls for urgent studies involving newer and broad spectrum antibiotics to address the problem. Inclusion of ciprofloxacin and amoxicillin in the treatment regimen of *H. pylori* infection in this study environment is advocated.

4.1 INTRODUCTION

H. pylori infection presents a unique therapeutic challenge. Determining the optimum treatment of infection is difficult because the organism lives in an environment not easily accessible to many medications. The recommended treatment regimens pose a number of difficulties to patients such as poor compliance and unpleasant adverse effects (Hardin and Wright, 2002; Tanih *et al.,* 2010a). Apart from patient non-compliance, antibiotic resistance is the major cause of treatment failure (Tanih *et al*., 2010a). Although several antibiotic agents are being used worldwide for the treatment of *H. pylori* infection, drug resistance to most of them is already well established (Kalachi *et al*., 2001; Kwon *et al.*, 2001; Gerritis *et al*., 2002; Tanih *et al*., 2010b) and has become a global concern (Westh *et al.,* 2004). In recent years, there has been an increasing incidence of multiple resistances in many microorganisms that are human pathogens, largely as a result of the indiscriminate use of the antimicrobial drugs that are commonly employed in the treatment of infectious diseases (Debets-Ossenkopp *et al*., 1999; Ndip *et al*., 2008).

The reliable treatment of *Helicobacter pylori* infection has been difficult, and successful regimens generally require two or more antimicrobial drugs coupled with an acid inhibitor (Hung *et al*., 2009). In recent years, several different treatment regimens have been proposed for the eradication of *H. pylori* from a patient. The only conditions, for which such treatment is strongly recommended, on the basis of unequivocal supporting evidence, are, however, peptic ulcer disease and low-grade gastric MALT lymphoma (Knigge, 2001). Eradication of the organism is the first therapeutic approach that constitutes a reliable long-term prophylaxis of peptic ulcer relapse, accelerating ulcer healing and reducing the rate of ulcer complications (Yuen *et al*., 2005). However, the prevalence of multi-drug resistant strains, especially in developing countries (Smith *et al*., 2001; Poon *et al*., 2002) makes it increasingly likely that

culture and antibiotic sensitivity testing may become a pre-requisite for patients with persistent infection after initial or repeated treatment failure (Krogfelt *et al.,* 2005).

Antibiotic resistance has been increasingly recognised as a major cause of treatment failure. Primary resistance against clarithromycin and metronidazole is common in many countries (Pilotto *et al.,* 2000; Kim *et al*., 2001; Kato *et al*., 2002; Meyer *et al*., 2002; Poon *et al*., 2002; Wolle *et al*., 2002). Resistance to different antibiotics used as first-line treatment varies between countries and communities, and may also change with time and geographic location (Me´graud *et al*., 1999; Ndip *et al*., 2005). Given that the organism has been shown to be associated with substantial morbidity and mortality (Sherif *et al*., 2004) coupled with the increase in treatment failure due to emerging resistance to currently used antibiotics, it will be valuable to determine the current susceptibility/resistance patterns of *H. pylori* strains circulating in the Eastern Cape Province of South Africa for empiric chemotherapy and prevention of the emergence of further resistance.

Recently, *H. pylori* isolates were identified from patients presented with gastric related morbidities in the Eastern Cape of South Africa (Tanih *et al.,* 2010c). Studies on antimicrobial susceptibility/ resistance patterns of *H. pylori* are lacking in South Africa (Wong *et al*., 2000). This study investigated the susceptibility and resistance patterns of these *H. pylori* isolates to seven different antibiotics.

4.2 MATERIALS AND METHODS

4.2.1 Clinical specimens.

Study samples were gastric antrum and corpus biopsies. Biopsies were taken after informed consent was given. The biopsies were immediately placed in sterile bijou bottles containing 0.2g/L of cysteine and 20% glycerol in Brain heart infusion broth, stored in a cold thermos flask (using ice) then immediately transported to the microbiology laboratory, Faculty of Science and Agriculture, Fort Hare University.

4.2.2 Bacterial strains

Briefly, *H. pylori* strains used in this study were isolated from gastric biopsies of patients with gastric related morbidities following the previously reported scheme in chapter 3 (Tanih *et al.,* 2010c).

4.2.3 Antibiotics susceptibility testing

Susceptibility testing was carried out by the disk diffusion (Kirby-Bauer) technique, which conforms to the recommended standard of the Clinical and Laboratory Standard Institute (CLSI\NCCLS, 2005) (Ndip *et al*., 2008). Antibiotics usually indicated for triple therapy, including metronidazole (5 μg), clarithromycin (15 μg), tetracycline (10 μg), amoxicillin (10 μg), gentamicin (10 μg), ciprofloxacin (5 μg) and erythromycin (10 μg) (Mast Diagnostics UK) were used. This selection was based on the current treatment regimen used in South Africa. Brain heart infusion (BHI) agar (Oxoid, England) containing 7% horse blood and *Helicobacter pylori* selective supplement (Oxoid, England) were used. The bacteria inoculums were prepared from subcultures and 4-5 colonies of the isolate were emulsified in 3ml sterile normal saline and the turbidity adjusted to $1.5x10^8$ CFU/mL (corresponding to 0.5 McFarland standards). A sterile cotton swab dipped into the standardized bacteria suspension

was used to evenly inoculate BHI agar plates and allowed to dry for 10-15 minutes. Thereafter, all the disks for this study were placed on the plates and pressed gently to ensure complete contact with agar. A distance of at least 15mm was maintained from the edges of the plates to prevent overlapping of inhibition zones. The plates were incubated at 37^{O}C for 2-5 days. They were then examined and the diameter of the zone of inhibition measured. *Helicobacter pylori* control strain NCTC 11638 was included in all the experiments to determine susceptibility or resistance (Ndip *et al*., 2008).

4.2.4 Determination of Minimum Inhibitory Concentration (MIC)

The MIC was determined by the agar dilution method (EUCAST, 2000; Osato *et al*., 2001; Ndip *et al*., 2008). The following antibiotics were used: clarithromycin, tetracycline, amoxicillin, metronidazole, gentamicin, ciprofloxacin, and erythromycin. A stock of each antibiotic was prepared by dissolving the powder in 10mL of sterile phosphate buffered saline (PBS) (Sigma-Aldrich, Dorset, UK) solution of pH 7.2. Two-fold serial dilution of each antibiotic was carried out in PBS. The range of antibiotic concentrations obtained for clarithromycin was (0.06-1.0µg/mL), while for amoxicillin, tetracycline, ciprofloxacin, gentamicin, erythromycin and metronidazole it was (0.625-10µg/mL). A volume of 13.5mL of the base medium (Brain Hearth Infusion Agar) enriched with 7% horse blood and selective supplement was prepared and 1.5mL of the serially diluted antibiotic was added to the medium (EUCAST, 2000) to obtain the desired concentration. This was poured into sterile petri dishes and then allowed to solidify. Fresh pure isolates were grown for 3 days and the inoculums prepared as described above (0.5 McFarland). The inocula were plated and incubated under microaerophilic condition for 3 to 5 days at 37^{o}C. After incubation, the MIC value was read as the lowest concentration of the antibiotic that inhibited bacteria growth (no visible growth). A plate free of antibiotic was included as a negative control in every MIC

determination. The resistant breakpoint used for metronidazole, amoxicillin, gentamicin and erythromycin were >8 μg/mL, ≥ 2 μg/mL for tetracycline, >1.0 μg/mL for clarithromycin and ≥ 1.0 μg/mL for ciprofloxacin (Osato, 2000; Kim *et al.,* 2001; Ndip *et al*., 2008).

4.2.5 Statistical analysis

The Epi Info 2000 software package (Center for Disease Control and Prevention, Atlanta, GA, USA) was used for the statistical analysis. Comparisons of antimicrobial resistance rates with respect to sex of the patients were determined using chi-square (X^2) test and a P value of < 0.05 was considered significant.

4.3 RESULTS

4.3.1 Antimicrobial patterns

Of the 200 isolates subjected to antimicrobials, 100% susceptibility was recorded for ciprofloxacin and 97.5% for amoxicillin. Marked resistance were noted for metronidazole (95.5%) (Table 4.1). The antimicrobial resistance patterns exhibited by isolates are shown in Table 4.2. A total of 19 antibiotypes were noted. Of the 200 strains, 6 (3%) showed no resistance to all the antibiotics. The predominant resistant pattern MET^R was observed in 49 (26.06%) of isolates. Thirty-two (17.02%) showed multidrug-resistance to metronidazole and erythromycin (MET^RERT^R). The least resistance pattern were exhibited by ERT^R (0.53%) and $CLA^RTET^RAMX^RMET^RGEN^RERT^R$ (0.53%).

Table 4.1: Antibiotic sensitivity results of *H. pylori* strains isolated from gastric biopsy specimen

Antibiotics	Antrum No. sus (%)	Corpus No. sus (%)	Antrum No. res (%)	Corpus No res (%)	Overall Sus (%)	Overall Res (%)	MIC ug/ml
Clarithromycin	87(82.07)	73(77.65)	19(17.92)	21(22.34)	160(80)	40(20)	0.125 -1.0
Tetracycline	69(66.34)	66(68.75)	35(33.65)	30(31.25)	135(67.5)	65(32.5)	1.25-2.0
Amoxicillin	103(98.09)	92 (96.84)	2(1.90)	3(3.15)	195(97.5)	5(2.5)	2.5–5.0
Metronidazole	6 (5.94)	3 (3.03)	95(94.05)	96(96.96)	9(4.5)	191(95.5)	-
Gentamicin	84(75.67)	61(68.53)	27(24.32)	23(31.46)	145(72.5)	55(27.5)	5-8.0
Erythromycin	69(64.48)	42(45.16)	38(35.51)	51(54.83)	111(55.5)	89(44.5)	2.5-5.0
Ciprofloxacin	107(100)	93(100)	00(0)	00(0)	200(100)	00(0)	0.0625-1.0

4.3.2 Antibiotic Resistance by Sex

Of the 200 strains tested for susceptibility, 67 were from males and 133 from females. The prevalence of metronidazole resistance in females and males was 65.44% and 34.55% while in gentamicin it was 69.09% and 30.90% respectively (Fig 4.1) In general there was a higher prevalence of resistant isolates in females when compared with male patients. However this did not reach statistical significance (P>0.05)

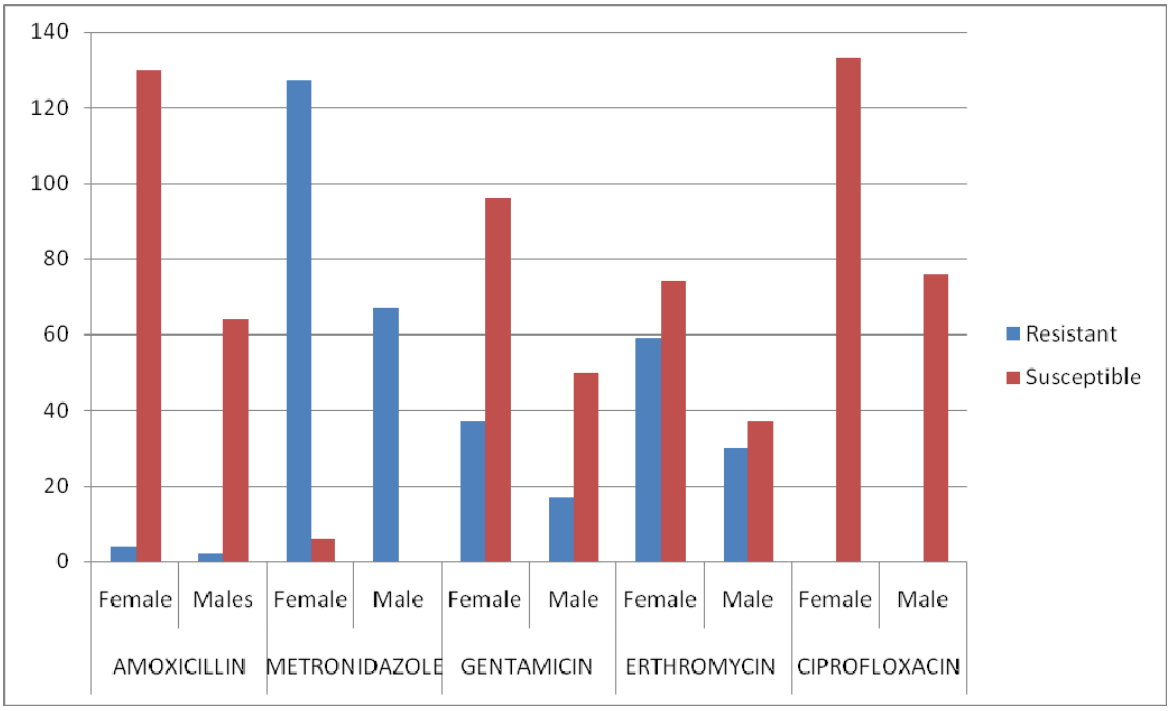

Fig. 4.1: Prevalence of antibiotic resistant isolates in males and females.

4.3.3 MIC determination

Of the seven antibiotics, metronidazole showed no MIC within the susceptible breakpoint range. MIC values for the other antibiotics ranged from 0.125 -1.0µg/mL for clarithromycin; 1.25–2.0µg/mL for tetracycline, 2.5–5µg/mL for amoxicillin, gentamicin 5–8.0µg/mL, erythromycin 2.5–5.0µg/mL, and ciprofloxacin 0.0625–1.0µg/mL (Table 4.1).

Table 4.2: Antimicrobial resistance patterns of *H. pylori*

No.	Antibiotypes	Number of strains showing pattern (%)
A1	MET^R	49(25.93)
A2	ERT^R	1(0.53)
A3	MET^RGEN^R	12(6.35)
A4	MET^RERT^R	32(16.93)
A5	CLA^RAMX^R	2(1.06)
A6	CLA^RMET^R	2(1.06)
A7	TET^RMET^R	11(5.85)
A8	$MET^RGEN^RERT^R$	13(6.91)
A9	$TET^RMET^RGEN^R$	10(5.3)
A10	$TET^RMET^RERT^R$	16(8.51)
A11	$CLA^RTET^RMET^R$	9(4.78)
A12	$CLA^RMET^RGEN^R$	5(2.65)
A13	$CLA^RMET^RERT^R$	6(3.19)
A14	$AMX^RMET^RGEN^RERT^R$	2(1.06)
A15	$CLA^RTET^RMET^RGEN^R$	2(1.06)
A16	$CLA^RTET^RMET^RERT^R$	8(4.25)
A17	$TET^RMET^RGEN^RERT^R$	5(2.65)
A18	$CLA^RTET^RMET^RGEN^RERT^R$	3(1.59)
A19	$CLA^RTET^RAMX^RMET^RGEN^RERT^R$	1(0.53)

CLA, clarithromycin; TET, tetracycline; AMX, amoxicillin, MET, metronidazole; GEN, gentamicin; ERT, erythromycin; CIP, ciprofloxacin.

4.4 DISCUSSION AND CONCLUSION

The resistance of *H. pylori* to currently available antibiotic treatment regimen is a growing problem (Nahar *et al.,* 2004). Despite the fact that culture is the "gold standard" detection method for most pathogenic bacteria, the specific requirements of *H. pylori* in terms of transport and growth render it difficult to culture. In contrast to what has been previously published and recommended, culture which is a prerequisite for antimicrobial susceptibility testing, was also found to be an accurate way to diagnose *H. pylori* infection (Megraud *et al*., 1999).

These results revealed marked antimicrobial susceptible rates of 100% for ciprofloxacin, and 97.5% for amoxicillin. These findings corroborate those of other investigators (Adamek *et al.,* 1998; Kalach *et al*., 2001; Kato *et al.,* 2002; Eltahawy *et al*., 2002; Mirza *et al*., 2007) who also reported marked susceptibility (100%) to amoxicillin or ciprofloxacin in their studies. Eighty percent of the isolates in this study were susceptible to clarithromycin, 72.5% to gentamicin, 67.5% to tetracycline, 55.5% to erythromycin and 4.5% to metronidazole.

Metronidazole resistance has been reported in 10–50% of all adult patients infected with *H. pylori* in developed countries (Adamek *et al*., 1998); virtually all strains have been found to be resistant to the agent in the developing countries (Me´graud 1998; Kumala, 2006). Smith *et al*. (2001) in Nigeria reported 100% resistance to metronidazole. Females were observed to be more resistant to metronidazole than males. The higher resistance observed with metronidazole in females may be because of the use of the drug in the treatment of trichomoniasis and bacterial vaginosis, which is common in this environment. This data however showed no significance ($P < 0.05$) of a higher prevalence of resistant strains to the antibiotics in females than males. This confirms the results of other studies (Parsons *et al*.,

2001; Meyer *et al.,* 2002; Suerbaum and Michetti, 2002; Nahar *et al*., 2004). In general, the high prevalence of metronidazole resistance in developing countries is probably because of the frequent use of nitroimidazole derivatives for the treatment of protozoa infections and gynaecological problems (Loffeld & Fijen, 2003; Nahar *et al*., 2004). This resistance has been linked mostly to genetic mutations in the *rdxA* and *frxA* genes (Debets-Ossenkopp *et al*., 1999; Kwon *et al*., 2000).

Although the rates of amoxicillin resistance are relatively low, and resistance is absent in ciprofloxacin, studies in different parts of the world have demonstrated resistance for both drugs (Quintana-Guzmán *et al*., 1998; Agel *et al*., 2000; Eltahawy *et al.,* 2002; Mishra *et al*., 2006). High resistance rates have been reported for amoxicillin in other studies (Smith *et al.,* 2001; Hu *et al*., 2007). Smith *et al*. (2001) in their study in Western Nigeria documented a resistance rate of 100% for amoxicillin. Amoxicillin resistance is thought to develop because of the structural alterations in the penicillin binding protein (Deloney and Schiller, 2000) or changes in proteins involved in cell wall synthesis (Wosten *et al.,* 1997). Generally, the possibility of bacterial strains acquiring resistance to amoxicillin is therefore difficult. Colonisation of the stomach with β-lactam-resistant bacteria may lead to the transfer of amoxicillin resistance to *H. pylori*. *H. pylori* have also been reported to lose the resistant phenotype because of freezing and storage (Nahar *et al*., 2004); this may also help to explain the observed differences. However, it is speculated these differences could also be accounted for by the local antibiotic prescription practices and usage in the community since drug control is much tighter in some areas than others (Debets-Ossenkopp *et al*., 1999; Ndip *et al*., 2008).

Twenty percent of the isolates in this study were resistant to clarithromycin. These findings are in line with other reports (Gotoh *et al.,* 1997; Adamek *et al.,* 1998; Debets-Ossenkopp *et al*., 1999; Iovene *et al*., 1999; Me´graud *et al*., 1999; Cabrita *et al*., 2000; Toracchio & Merzio, 2003; Nahar *et al.,* 2004) that documented high rates of clarithromycin resistance. The high prevalence of clarithromycin resistance (20%) observed in this study maybe partly because of the use of other less expensive macrolides linked to cross-resistance with clarithromycin as suggested earlier (McMahon *et al*., 2003; Nahar *et al*., 2004); clarithromycin is an expensive drug, and hence less abused. However, both clarithromycin-susceptible and resistant strains have been isolated from some patients with no history of exposure to macrolides (Matsuoka *et al*., 1999). This may suggest that administration of clarithromycin may select for the resistant strains. In line with the result of this study, Loffeld and Fijen (2003) also documented a high clarithromycin resistance rate in ethnic Turkish patients and in those originating from Africa and the Middle East. This study also revealed a very high resistance rate of the isolates to erythromycin (44.5%) and gentamicin (24.5%). Clarithromycin resistance might have been provoked by the use of erythromycin in pregnancy or other macrolides for chlamydial or non-gonococcal urethritis/cervicitis with subsequent cross-transfer of resistance as suggested earlier (Nahar *et al*., 2004).

The resistance rate of 32.5% observed for tetracycline in this study is low compared with the 100% reported in Western Nigeria (Smith *et al*., 2001). As with other antibiotics, resistance to tetracycline increases because of selection pressure with the use of the drugs. This corroborates earlier report on the misuse of tetracycline in Buea, Cameroon thus contributing to the emergence of resistance (Ndip *et al.,* 2005). Resistance rates of 5–59% have been reported in Asia (Nahar *et al*., 2004). However, other studies have reported very low rates of

resistance to this drug. Cases have been reported in Spain (0.7%), the UK (0.5%), Hong Kong (0.5%) and Korea (5.3%) (Me´graud, 2004).

Antimicrobial resistance revealed a total of 19 patterns of which the most prevalent displayed pattern was metronidazole (MET^R) accounting for 49(26.06%) of the isolates. This was closely followed by metronidazole and erythromycin (MET^RERT^R) with a percentage of 17.06%. The least resistance patterns were noted for erythromycin (ERT^R) and clarithromycin, tetracycline, amoxicillin, metronidazole, gentamicin and erythromycin ($CLA^RTET^RAMX^RMET^RGEN^RERT^R$) accounting for 0.53% of the isolates, respectively. Approximately, 73.40% (138/189) of the isolates were resistant to two or more antibiotics of which 42.55% (80/189) were resistant to three or four antibiotics. Multidrug resistance found amongst isolates in this study may be because of the indiscriminate use of these drugs for the treatment of other infections, and may serve as a major selective pressure for antibiotic resistant bacteria (Ndip *et al*., 2005).

In the present study, MIC values for the five antibiotics that demonstrated susceptibility to isolates ranged from 0.125–1.0µg/mL for clarithromycin, 1.25–2.0µg/mL for tetracycline, 2.5–5.0µg/mL for amoxicillin, 5–8.0µg/mL for gentamicin, 2.5–5.0µg/mL for erythromycin and 0.625–1.0µg/mL for ciprofloxacin. These values are similar to other studies reported in Canada (Nahar *et al.,* 2004) and Nigeria (Abdulrasheed *et al*., 2005) and in agreement with the Clinical and Laboratory Standard Institute approved quality control ranges for *H. pylori* (Osato, 2000).

This study revealed low rates of susceptibility of the isolates enrolled in this study to currently recommended treatment regimen used in South Africa. This gives an indication of

the need to establish baseline susceptibility data for empiric treatment of cases as well as conducting studies involving newer and broad spectrum antibiotics to address resistance.

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CHAPTER FIVE

Prevalence of *Helicobacter pylori vacA, cagA and iceA* genotypes in South African Patients with upper gastrointestinal diseases.

ABSTRACT

Helicobacter pylori (H. pylori) colonisation is associated with a number of gastroduodenal diseases but their expression varies. Clinical response to *H. pylori* infection may be determined by specific virulence–associated bacterial genotypes. The distribution of different bacterial genotypes varies geographically. The aim of this study was to investigate the diversity of putative virulence markers of *H. pylori*: *cagA, vacA and iceA* in the Eastern Cape Province of South Africa. Gastric biopsies were obtained from one hundred dyspeptic patients; (30 with peptic ulcer disease (PUD), 10 gastro-oesopharyneal reflux disease (GERD), 40 non-ulcer dyspepsia (NUD), 8 gastritis (GS) and 12 (others). *H. pylori* was cultured and strains were studied. Bacterial genotypes *cagA, vacA* (s and m subtypes) and *iceA* were analyzed by polymerase chain reaction (PCR) using specific primer sequences. *CagA* was identified in 90% of the strains investigated. Fifty-eight of the 100 strains had the *vacA* signal sequence genotype s1 and 26 had subtype s2. Combined *vacA* s1/s2 was detected in 16 of the strains. *vacA* middle region analysis showed that 8(8%) strains were m1 while 50 were m2. Combined *vacA* m1/m2 was detected in 36 of the strains. s1m2 (20%) and s2m2 (20%) genotypes were the most common allelic combinations of the *vacA* gene among the strains. Multiple *vacA* genotypes were detected in this study, amongst which the most prevalent was s1m1m2 (61%) 28/46. *IceA1* was present in 2 (2%) of the strains while *iceA2* was present in 58 of all the samples analysed. Twenty-six percent of the strains identified had both *iceA1* and *iceA2*. All the strains tested positive for the *ureC (glmM)* gene. This study revealed a high prevalence of *vacA, cagA* and *iceA2,* suggesting that these genotypes might

be associated with gastroduodenal pathologies of patients in the Eastern Cape Province of South Africa.

5.1 INTRODUCTION

H. pylori is a Gram-negative curved rod that inhabits the gastric mucosa of the human stomach. It chronically infects billions of people worldwide, is one of the most genetically diverse of bacterial species, and is a major cause of peptic ulcer disease and gastric cancer in many populations (Rudi *et al*., 1999; Andreson *et al.,* 2002; Cover, 2005; Ndip *et al.,* 2008)**.** Several potential markers (*vacA, cagA*) related to risk of gastroduodenal diseases with *H. pylori* infection and several other 'housekeeping genes' such as *ureA* and *ureC* (van Doorn *et al.,* 1998; Letley *et al.,* 1999; Smith *et al*., 2002) which might not be directly linked to virulence of the strain have been identified.

The vacuolating cytotoxin (*vacA*) gene is present in virtually all *H. pylori* strains and contains two variable regions; the signal sequence (s) region, which encodes the signal peptide, and the middle (m) region (Arents *et al*., 2001; Smith *et al.,* 2002). The s region has two subtypes, s1 and s2 alleles. The s1 exists as an s1a, s1b and s1c. The m region also has two subtypes, m1 and m2 which occurs as, m2a, or m2b (Bravo *et al.,* 2002; Datta *et al.,* 2003; Wang *et al.,* 2003; Asrat *et al*., 2004).

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 (van Doorn *et al*., 1998; Letley *et al.,* 1999; Figueiredo *et al.,* 2002). The mosaic combination of s- and m-region allelic types determines the production of the cytotoxin and thereby associated with pathogenicity of the bacterium (Asrat *et al*., 2004). The *vacA* s1m1 strains produce large amounts of toxin and are strongly associated with a higher degree of inflammation and epithelial damage in the gastric mucosa; s1m2 strains produce moderate

amounts of toxin while the s2m1 strains produce very little or no toxin (van Doorn *et al.,* 1998; Figueiredo *et al*., 2002; Kuster *et al*., 2006; Ko *et al.,* 2008).

The cytotoxin-associated gene (*cagA*) is a marker for a genomic pathogenicity island of 40 kb (Akopyanz *et al.,* 1992). Several genes of this cag island, such as *picB,* encode proteins that enhance the virulence of the strains, by increasing the production of interleukin 8 (IL-8) by gastric epithelial cells amongst others (van Doorn *et al.,* 1998). The *cagA* gene is present in 60-70% of *H. pylori* strains and encodes a high molecular weight antigenic protein (120-140kDa) (Kidd *et al.,* 1999).

Another virulence associated gene designated *iceA* (induced by contact with epithelium) has been identified. There are two main allelic variants of the gene: *iceA1* and *iceA2*. The function of *iceA1* is not yet clear but there is significant homology to a type 11 restriction endonuclease. The expression of *iceA1* is upregulated on contact between *H. pylori* and human epithelial cells and maybe associated with peptic ulcer disease (van Doorn *et al*., 1998) and enhanced acute neutrophilic infiltration. However, linkage between the *iceA1* genotype and ulcer disease is not universal (Kidd *et al*., 2001). Only *iceA1* RNA is induced following adherence *in vitro* (Kidd *et al.,* 2001).

Sequence typing has indicated that different *H. pylori* genotypes predominate in different human populations. In particular, African strains seem to be distinct from those of other continents (Falush *et al.,* 2003). Many studies have shown geographic differences in predominant *H. pylori* genotypes, based either on virulence associated genes such as *vacA* and *cagA* or "housekeeping genes" that are present in all *H. pylori* strains and not particularly linked to virulence (van Doorn *et al.,* 1998; Bravo *et al*., 2002).

H. pylori colonisation is very common in South Africa, as in other developing countries (Pelser *et al*., 1997; Mosane *et al.,* 2004; Samie *et al*., 2007; Tanih *et al*., 2010b). In a Sowetan study of asymptomatic children aged 6-15 years, 86.5% were infected with a *vacA* positive strain and 87% with a *cagA*-positive strain. The majority of *cagA*-positive strains carried *vacA* s1 allele while most *cagA*-negatives carried the *vacA* s2 allele (Ally *et al.,* 1999). In a separate study, *vacA* diversity was demonstrated among South African *H. pylori* strains; interestingly, no strains with the *vacA* s1a genotype were found among the isolates from black or mixed-race South Africans (Letley *et al.,* 1999). In another study by Kidd *et al.* (1999); *vacA* s1 genotype and a fragment length of the 3' region of *cagA* were identified and were associated with significant clinical disease. The aim of this study was to examine the genotypes of *H. pylori* strains, *vacA, cagA* and *iceA,* using PCR based methods, in patients from the Eastern Cape Province of South Africa, which is a predominantly rural region, a predisposing factor for *H. pylori* acquisition. Furthermore, this study is expected to provide baseline information for future clinical-epidemiological studies of this population.

5. 2 MATERIALS AND METHODS

5.2.1 Study subjects.

Two hundred and fifty-four consecutive patients referred for endoscopy at the Livingstone Hospital, Port Elizabeth, South Africa between May and December 2008 were evaluated. The nature and purpose of the study was explained to the patients until fully understood. Only patients who gave informed consent were enrolled. The study was approved by the institutional review board of the University of Fort Hare and the Eastern Cape Department of Health (Protocol number EcDoH-Res 0002).

5.2.2 Culture and identification.

Antral and corpus gastric mucosal biopsy samples were taken from each dyspeptic patient. The biopsies were immediately placed in sterile bijou bottles containing 0.2g/L of cysteine and 20% glycerol in Brain heart infusion (BHI) broth and transported in ice to the laboratory within 2 hr of collection for culture. *H. pylori* was cultured using standard method and isolates were identified based on colony morphology, Gram staining, oxidase, catalase, and urease tests (Ndip *et al*., 2008). Isolates were further confirmed by amplification of the *glmM* gene. Confirmed isolates were suspended in 20% glycerol and BHI and stored at -80 ºC (Sanyo, Japan) until genotyping was performed. *H. pylori* reference strain NCTC 11638 was included in all experiments.

5.2.3 Genomic DNA extraction.

DNA was extracted from one hundred *H. pylori* strains. Isolates were centrifuged at 10,000 x g for 5 min, and DNA extracted from the pellets by use of the QIAamp DNA kit (Qiagen DNA extraction kit, SA) according to the manufacturer's recommendations and stored at -20°C until analysis. DNA extraction negative controls were performed in parallel by

including sterile tubes without samples to check for contamination of the DNA extraction reagents.

5.2.4 Polymerase Chain Reaction (PCR).

PCR analysis of the targeted genes was performed using Thermo-stat Taq DNA polymerase (ABgene, UK) and manufacturer-provided reaction buffer. Five microlitre of DNA were added to 50µl of reaction mixture containing 1×PCR buffer, 1.5mM $MgCl_2$, 0.2mM (each) dNTPs (ABgene, UK) and 0.5µM of respective oligonucleotide primers. 1.25U/µl thermo-stat Taq DNA polymerase (ABgene, UK) was added to each tube. PCR was performed with a thermal cycler (MJ Research, USA). The amplification cycles consisted of an initial denaturation of target DNA at 95°C for 15min and then denaturation at 94°C for 1 min, primer annealing at 60°C, 56°C, and 50°C for *cagA*, (*vacA* m1, m2, s1/s2 and *iceA*), and *glmM* respectively for 1 min and extension at 72°C for 1 min. All reactions were performed through (35 cycles) except *glmM* (40cycles). The final cycle included an extension step for 5 min.

The primers used to amplify the targeted genes are summarised in Table 5.1. Negative controls were added to each PCR run including all reagents except template DNA which was substituted with ultra pure water (Sigma-Aldrich, UK). Amplification of DNA was analysed by agarose gel electrophoresis using standard procedures (van Doorn *et al.,* 1998). Briefly, aliquots of amplified samples (5µl) were electrophoresed on 2% high-resolution agarose gel in TAE buffer. The gel was stained with ethidium bromide 0.5ug/ml. The amplified bands were visualised under ultraviolet light and photographed.

Table 5.1: **PCR primers for amplification of *cagA, vacA, iceA* and *glmM* sequences.**

Gene	Primer sequence	PCR product (bp)	Reference
cagA	5'-TTGACCAACAACCACAAACCGAAG-3' 3'- CTTCCCTTAATTGCGAGATTCC-5'	183	Smith *et al.,* 2002
vacA s1/s2	5'-ATGGAAATACAACAAACACAC-3' 3'-CTGCTTGAATGCGCCAAAC-5'	259/286	Smith *et al.,* 2002
vacA m1	5'-GTCAAAATGCGGTCATGG-3' 3'- CCATTGGTACCTGTAGAAAC-5'	290	Smith *et al.,* 2002
vacA m2	5'-GGAGCCCCAGGAAACATTG-3' 3'- CATAACTAGCGCCTTGCAC-5'	352	Smith *et al.,* 2002
IceA1	5'-GTTGGGTAAGCGTTACAGAATTT-3' 3'- CATTGTATATCCTATCATTAC-5'	567	Smith *et al*., 2002
IceA2	5'- GTTGGGTATATCACAATTTAT-3' 3'- TTRCCCTATTTTCTAGTAGGT-5'	229 or 334	Smith *et al.,* 2002
glmM	5'- TTTGGGACTGATGGCGTGAGGGGTAA-3' 3'-GGACATTCAAATTCACCAGGTTTTGAG-5'	1142	Burucoa *et al*., 1999

5.2.5 **Statistical Analysis**.

Epi info version 2000 (Center for Disease Control and Prevention, Atlanta, Ga.) was used for statistical analysis. Chi square or Fischer exact test was applied to test whether differences in prevalence between values of the various genotypes or disease conditions were significant at P-value < 0.05.

5.3. RESULTS

5.3.1 Distribution of *vacA*, *cagA* and *iceA* genotypes

H. pylori DNA was successfully extracted from 100 strains. DNA integrity and specificity was confirmed by *glmM* amplification, which rendered the expected band size of all isolates (data not shown). All the samples were positive for the *glmM* (100/100).

Most of the samples were positive for *vacA* at least for one of the alleles (the s-region or the m-region). The most virulent *vacA*s1 allele reported in most studies was predominantly present in these strains 58/100 (58%), and was visualised as a band of 259bp on agarose gel electrophoresis (Fig 5.1), whereas 26/100 (26%) of the isolates had the *vacA*s2 genotype (Table 5.2). Combined *vacA* s1/s2 was detected in 16 (16%) of the strains. The middle region of the *vacA* gene was detected in 94/100 (94%) of the strains. *vacA* m1 was detected in 8 (8%) strains while m2 was found in 50 (50%) of the strains (Fig 5.4). Combined *vacA* m1/m2 was detected in 36 (36%) of the strains. The s genotypes were more equally distributed than m1 and m2 genotypes. Genotyping of the middle region failed in 6/100 (6%) of the strains. s1m2 and s2m2 genotype were the most common allelic combinations of the *vacA* gene among the strains. Eight percent of the strains harboured s1m1 genotype. The genotype s2m1 was not identified in this study.

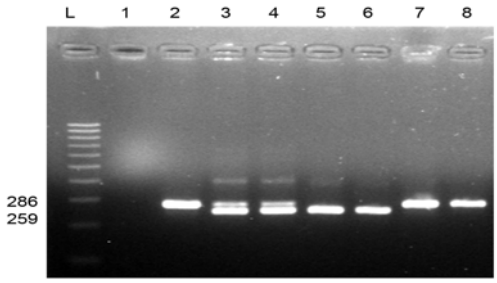

Fig. 5.1. Detection of *s1* and *s2* alleles of the *vacA* gene.

Lane L, marker; lane1 negative control; lanes 2, 7 and 8 s2 allele; lanes 5 and 6 s1 allele; lanes 3 and 4 s1/s2 alleles. Numbers on the left indicate molecular size (in base pairs).

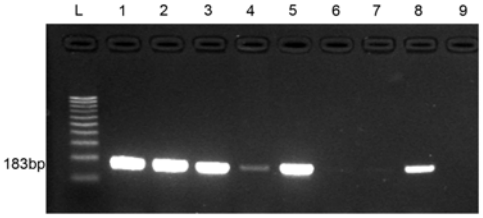

Fig. 5.2. Agarose (2%) gel electrophoresis of PCR products for *cag A* detection.

Lanes 1, 2, 3, 4, 5 and 8, *cagA* +; lanes 6, 7 and 9, *cagA*-; lane l, marker.

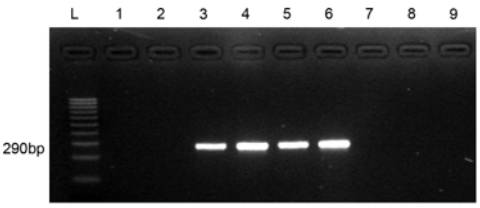

Fig. 5.3. Detection of m1 allele of the *vacA* gene.

Lane L, marker; lanes 1, 2, 7, 8 and 9 m1 absent; lanes 3, 4, 5 and 6 m1 present.

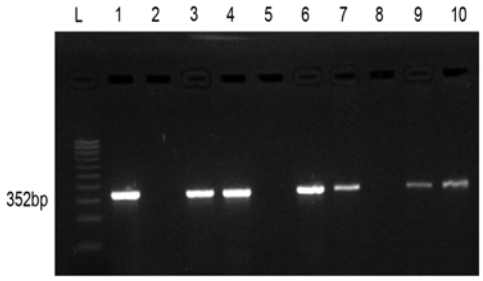

Fig. 5.4 *vacAm2* detection.
Lane L, marker; lanes 1, 3, 4, 6, 7, 9 and 10 *m2* positive; lanes 2, 5 and 8 *m2* negative

Multiple *vacA* genotypes were detected in this study 46/100 (46%) with *vacA* s1m1m2 genotype being the most prevalent allelic combination 28(28%) (Table 5.3) indicating the presence of mixed infection. Multiple *vacA* genotypes appeared to be more prevalent in patients with non ulcer dyspepsia (20/40, 50%) than in those with the other disease conditions enrolled in this study, although the difference was not statistically significant ($p>0.05$).

Amplification of the *cagA* gene was visualised as a band of 183bp (Fig 5.2) and was present in 90% of the strains (Table 5.3). *IceA1* was detected in 2 (2%) and *iceA2* was found in 58 (58%) cases. Mixed *iceA (iceA1+iceA2)* genotypes were found in 26(26%) of the strains. *IceA* genotype was completely absent in 14 (14%) of the strains (Table 5.3).

5.3.2 Association amongst the genotypes

In this investigation, *vacA* (s and m region) strains had a higher prevalence for both *cagA* positive and *iceA2* positive than with the negatives. Fifty-six (96.6%) of the *vacA*s1 strains were *cagA* positive. The majority of the *vacA*s1 strains were *iceA2* positive, 52 (73.3%) while 12 (22.2%) of *vacA*s1 were *iceA1* positive. Four strains which were positive for *vacA*s1 and s2 respectively were negative for the *iceA* gene (Table 5.2).

Most of the s1m2 carried a *cagA* positive (90%) and *iceA2*-positive (57.1%) allele while s2m2 represented 90% of the *cagA* positive strains and 60% *iceA2*–positive. Examination of the various combinations (s1m1, s1m2, s2m2) revealed a higher prevalence of *iceA2* than *iceA1* (Table 5.2).

Table 5.2: Association of *vacA* with *cagA* and *iceA* genotypes

vacA	*cagA+ (%)*	*cagA- (%)*	*iceA1 (%)*	*iceA2 (%)*	*iceA- (%)*
s-region					
s1	56(96.6)	2(3.4)	12(22.2)	52(73.3)	4(4.4)
s2	24(92.3)	2(7.7)	8(23.5)	22(64.7)	4(11.7)
s1s2	12(75)	4(25)	2(14.3)	12(85.7)	4(14.3)
m-region					
m1	8(100)	0(0)	4(8.7)	42(91.3)	0(0)
m2	46(92)	4(8)	24(21.4)	80(71.4)	10(11.1)
m1m2	34(94.4)	2(5.6)	8(18.18)	36(81.81)	0(0)
s\m region					
s1m1	8(100)	0(0)	2(20)	8(80)	0(0)
s1m2	18(90)	2(10)	10(35.7)	16(57.1)	2(7.1)
s2m2	18(90)	2(10)	4(20)	12(60)	4(20)
Multiple vac	40(86.9)	6(13.1)	10(17.9)	44(78.6)	2(3.8)
Incomplete *vac*	6(100)	0(0)	2(33.3)	2(33.3)	2(33.3)

5.3.3 Relationship between genotypes and gastroduodenal diseases

Of the 100 strains studied, 40 were from patients diagnosed with non ulcer dyspepsia (NUD), 30 with peptic ulcer disease (duodenal ulcer and gastric ulcer), 10 gastro-oesophageal reflux disease (GERD), 8 gastritis (GS) and 12 had signs and symptoms of gastroduodenal disease and not a particular disease condition per se (Table 5.3). *vacA* s1m2 genotype was detected in all the disease conditions mentioned above but at a higher frequency in strains from patients with GS (4/8, 50%) and peptic ulcer disease (8/30, 27%). s2m2 was completely absent in patients presenting with gastritis but had the highest prevalence in patients with NUD (8/40, 20%). Percentage prevalence of s1m1 in GERD (20%) is higher than the percentage in NUD (15%). Also, s2m2 has same prevalence 20% in PUD, GERD and NUD. Most of the strains with the combination s1m1 were from patients with NUD. Multiple *vacA* genotypes were detected in this study with s1m1m2 being the most prevalent with the highest frequency in patients with NUD (16/40, 40%); however, there was no statistical correlation of the combinations of *vacA* genotypes with the various disease status ($P > 0.05$) (Table 5.3).

Table 5.3. *vacA*, *cagA*, and *iceA* status of *Helicobacter pylori* from 100 strains

Genotype status	PUD n=30 (%)	GERD n=10 (%)	NUD n=40 (%)	GS n=8 (%)	OTHERS n=12 (%)	Total n=100 (%)
vacA						
s1m1	0(0)	2(20)	6(15)	0(0)	0(0)	8(8)
s1m2	8(26.7)	2(20)	2(5)	4(50)	4(33.3)	20(20)
s2m2	6(20)	2(20)	8(20)	0(0)	4(33.3)	20(20)
s1m1m2	6(20)	2(20)	16(40)	2(25)	2(16.6)	28(28)
s2mIm2	0(0)	2(20)	0(0)	0(0)	2 (16.6)	4(4)
s1 s2m2	4(13.3)	0(0)	4(10)	2(25)	0(0)	10(10)
s1s2m1m 2	4(13.3)	0(0)	0(0)	0(0)	0(0)	4(4)
Incomplete *vac s or m*	2(6.7)	0(0)	4(10)	0(0)	0(0)	6(6)
cagA						
cagA+	24(80)	10(100)	36(90)	8(100)	12(100)	90(90)
cagA-	6(20)	0 (0)	4(10)	0(0)	0(0)	10(10)
IceA						
IceA1	0(0)	0(0)	2(5)	0(0)	0(0)	2(2)
IceA2	18(60)	8(80)	22(55)	6(75)	4(33.3)	58(58)
IceA-	10(33.3)	0(0)	4(10)	0(0)	0(0)	14(14)
*IceA*1+ *IceA2*	4(13.3)	2(20)	10(25)	2(25)	8(66.6)	26(26)

5.4 DISCUSSION AND CONCLUSION

H. pylori colonisation is associated with a spectrum of gastroduodenal pathologies. Although infection is universally associated with gastritis, the development of clinically significant disease seems to depend on a number of factors, including the virulence of the infecting strain, the susceptibility of the host and environment cofactors. Several studies have shown that the incidence and/or severity of gastroduodenal disease related to *H. pylori* vary geographically. This phenomenom is partly due to a difference in distribution of pathogenic markers in circulating strains of the microorganism.

Molecular studies on *H. pylori* have resulted in the identification of a number of non-conserved candidate virulence markers and their association with the clinical outcome of disease. Kidd *et al.* (2001) and some other authors (Ally *et al.,* 1999; Letley *et al.,* 1999) have carried out a number of studies in South Africa to determine the genotype of *H. pylori* circulating in their study area (predominantly affluence), but the present study is the first in the Eastern Cape Province (predominantly rural with deprived living conditions) aimed at investigating the prevalence of various virulence factors (*vacA, cagA* and *iceA*) using PCR based methods and their relationship with the clinical outcome of disease.

Vacuolating cytotoxin has been implicated in the pathogenesis of peptic ulceration and a number of gastroduodenal pathologies. *vacA* signal type s1a are often associated with peptic ulceration, while *vacA*s2 strains are usually found in patients with no ulcers, *vacA*slb strains seem to be intermediately ulcerogenic. The *vacA*s1 and s2 leader sequences are different in a small insert, totalling 27bp, carried by the *vacA*s2 allele which has a reduced capacity to secrete *vacA* toxin. South African *H. pylori* isolates were previously characterised by the

universal presence of *cagA* but have differences in vacuolating cytotoxin gene (*vacA*) alleles (Kidd *et al*., 2001).

According to the results of this study, the most virulent *vacA*s1 allele was predominant in this study population 58(58%) (Table 5.2) a finding which has also been observed in other studies in South Africa and the world (Letley *et al*., 1999; Bravo *et al*., 2002; Smith *et al*., 2002). Ally *et al*. (1999) reported a high prevalence of *vacA*s1 *H. pylori* strains in their study in South Africa. Asrat *et al*. (2004) and Smith *et al*. (2002) also demonstrated a high prevalence of *vacA*s1 in their study in Ethiopia and Nigeria respectively. These results are however contrary to the findings noted in African Arabs who are predominantly infected with the s2 type allele (Al Qabandi *et al.,* 2005).

In this study, the prevalence of *vacA*m2 (50%) was higher than *vacA*m1 (8%) (Table 5.3). The results are similar to the findings of Wong *et al.* (2001) who reported a higher prevalence of m2 in their study; however, some authors have documented a higher prevalence of m1 in their study area (Smith *et al.,* 2002; Asrat *et al.,* 2004; Ko *et al*., 2008). This result portrays a high prevalence of *vacA*m1/m2 (36%).

Meanwhile, s1m2 and s2m2 were the most common combinations of the *vacA* gene in this study population (Table 5.3). The genotype s1m1 was detected in 8% of all the strains analysed. These results are in accordance with the findings of Wong *et al*. (2001) that delineated a high prevalence of s1m2 and a low prevalence of s1m1. However, the frequency of *vacA* s1m1 allelic type in this study is lower than those reported from the Netherlands (36%), Hong Kong (26 to 31%) and Nigeria (24%) (Smith *et al*., 2002; van Doorn *et al*.,

1998; Wong *et al.,* 2001). This may be a reflection of the great heterogeneity exhibited by this organism.

In the present investigation, the rare s2m1 allele was also not detected. This finding is in line with several studies in different parts of the world but contrary to the finding of Asrat *et al.* (2004) and Smith *et al.* (2002) who reported the presence of this allele though in low percentages; 2% and 6.7% respectively in their various investigations. However, the fact that the s2m1 allele was not found in this study cannot be completely ruled out for we detected multiple *vacA* genotypes (s2m1m2) of which s2m1 was a makeup. The allele s2m1 has been noted to suffer from a selective disadvantage (Letley *et al*., 1999).

Multiple *H. pylori vacA* genotypes were detected in 46% (46/100) of the strains. The presence of multiple *vacA* genotypes in this study ties with the finding of other studies both in Africa and the world (Morales- Espinosa *et al*., 1999; Wong *et al.,* 2001; Arents *et al*., 2001; Andreson *et al.,* 2002; Asrat *et al.,* 2004). However, this is the first study in South Africa to report the detection of multiple *vacA* genotypes. Infection with these strains has been associated with a higher degree of inflammation and gastroduodenal lesions. This data did not indicate that multiple strain infection increases the risk of developing NUD ($P>0.05$). Furthermore, the middle region of *vacA* was not detected in six of the strains in this study. Genotyping of the *vacA* middle region failed in four strains, probably due to heterogeneity in the *vacA* gene, a finding reported previously (Asrat *et al.,* 2004). *vacA*s2m2 genotype was noted at a higher frequency in NUD; but no significant correlation was observed between s2m2 genotypes and the appearance of NUD. Generally, there was no statistically significant difference between specific genotypes and disease conditions which is in agreement with previous reports (Park *et al.,* 1998; Wong *et al.,* 2001).

H. pylori cagA positive strains have been associated with more severe gastroduodenal diseases (van Doorn *et al.,* 1998). Of the one hundred strains examined in this study, the *cagA* gene was detected in 90%, prevalence similar to that reported in many African countries and other parts of the world (Wong *et al.,* 2001; Smith *et al.,* 2002; Asrat *et al.,* 2004). These results are also consistent with those of a Sowetan study (South Africa) of asymptomatic children aged 6-15years, in which 87% were *cagA*-positive strains (Ally *et al.,* 1999). In this study, *cagA* positivity was not associated with the presence of a specific disease condition ($P > 0.05$).

The present study showed that *iceA2* (58%) allele was predominant among the strains. This corroborates the findings of Yamaoko *et al*. (1999) in the USA and a study among Jordanian patients by Nimri *et al*. (2006) where *iceA2* was predominant, but however different from the strains reported in Hong Kong, Japan, Korea and Nigeria where *iceA1* allele was predominant (Yamaoko *et al*., 1999; Nimri *et al.,* 2006). Twenty-six percent of the strains had both *iceA1* and *iceA2* genotypes. These results corroborates the findings of a study in South Africa by Kidd *et al.* (2001) who demonstrated the presence of *iceA1* and *iceA2* in 68% and 80% respectively of their isolates examined; and also detected combined *iceA* (*iceA1, iceA2*) in approximately 40% of patients. *IceA* positivity was not associated with the presence of a specific disease condition in this study ($P > 0.05$). On comparing the various *vacA* genotypes and the presence of *iceA2* allele, a significant correlation was observed between *vacA* genotype and *iceA*2 ($P < 0.05$).

The association of *cagA, iceA* status and *vacA*s1 genotype which is commonly linked to an increase in *H. pylori* virulence was studied (Arents *et al.,* 2001). There was no association between *cagA, iceA* status and *vacA*s1 genotype ($P > 0.05$). Previous studies have shown a

high association between *cagA* positive genotype and the appearance of PUD (Park *et al*., 1998; Wong *et al.,* 2001). In this study, however, no correlation was observed between *cagA* status and PUD. However, a high frequency of cag-positive strains was observed in PUD patients (Table 5.3), which may indicate that a statistical association could be reached by increasing the number of patients in future studies.

From this, it can be concluded that the absence of a correlation between the virulence genes analysed and the development of gastroduodenal disease might be due to the small number of patients with each disease condition in this study, even though several others have not found any correlation between the presence of *H. pylori* main virulent factor (alone or in combination) and gastroduodenal diseases. It may be that a large population of patients must be studied to reveal statistically significant relations between *H. pylori* virulence genes and patterns of clinical disease.

This study has shown a relatively high prevalence of the main virulence factors (*vacA, cagA* and *iceA* genes) in South African *H. pylori* isolates. It also demonstrates that *vacA*s1, *iceA2* and *cagA*+ are common genotypes of *H. pylori* in this study group. Multiple *vacA* genotypes were found, and the presence of such combined genotypes in infected patients may increase the risk of development of clinical conditions such as peptic ulceration and gastric cancer. Further genetic analysis to determine the homology of *H. pylori* genome in members of the same family in order to study the transmission of the organism from person to person and their clinical implications in this environment is advocated.

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CHAPTER SIX

Characterization of virulence and antibiotic resistant genes in South African *Helicobacter pylori* strains

ABSTRACT

Sequence diversity and population structures can vary widely among pathogenic bacterial species. In some species, all isolates are highly similar, whereas in others most of the isolates are distinguished easily. *H. pylori* is known for its wide genetic diversity amongst the various strains both in genes involved in virulence and antibiotic resistance**.** Intrinsic resistance of antimicrobial agents against *H. pylori* maybe linked to the genes associated with resistance to the various agents**.** Truncation in *rdxA* and *frxA* is associated with metronidazole resistance while mutations in 23SrRNA are associated with clarithromycin resistance. The aim of this study was to evaluate by PCR and sequence analysis the genetic profile of *H. pylori vacA*s1 and *vacA*m2 and also to determine the genetic basis of the genes involved in antibiotic resistance to the drugs (metronidazole and clarithromycin) employed for standard therapy of *H. pylori*. Ninety five percent and 20% of *H. pylori* strains investigated were resistant to metronidazole and clarithromycin respectively (previously described). *rdxA* and *frxA* truncation was found only in metronidazole-resistant strains. Two point mutations in the 23SrRNA genes of clarithromycin-resistant strains were detected. Eighty-six perecent of *vacAs*1 and 85.8% of *vacA*m2 strains respectively were conserved amongst the strains studied. Mutation in the *rdxA* gene may contribute more significantly than *frxA* gene to the high level of resistance to metronidazole. Findings of this study support the need to continue monitoring the genotypes and antibiotic susceptibility of *H. pylori* in the Eastern Cape Province of South Africa to guide empiric treatment and prognosis of such infection.

6.1 INTRODUCTION

H. pylori is a gastric pathogen that infects more than 50% of the world's population; is the major cause of a number of gastro duodenal pathologies in infected patients (Tankovic *et al*., 2000; Andreson *et al.,* 2002; Cover, 2005), and an early risk factor for gastric cancer (Matsuhisa *et al.,* 2003). Sequence diversity is wide among pathogenic bacterial species. *H. pylori* is known to exhibit wide genetic diversity. In some species, all isolates are highly similar, whereas in other species, any two unrelated isolates can be easily distinguished **(**Akopyanz *et al*., 1992; Mukhopadhyay *et al.,* 2000). Strains of *H. pylori* are renowned for their tremendous genetic diversity both in the virulent and antibiotic associated genotypes (Akopyanz *et al.,* 1992; Paul *et al.,* 2001; Marais *et al*., 2003).

Clinical outcome of infection is variable and is considered to relate to bacterial virulence factors which may vary with sequence type (Kidd *et al.,* 2001). Direct sequencing and multilocus sequence typing (MLST) has indicated that different *H. pylori* genotypes predominate in different human populations (Mukhopadhyay *et al.,* 2000). In particular, African strains seem to be distinct from those of other continents (Falush *et al.* 2003). This diversity probably reflects a combination of factors including: mutation, recombination among divergent lineages, gene transfer from unrelated species and preferential transmission among family members (Akada *et al*., 2003). Mukhopadhyay *et al. (*2000) reported tremendous diversities amongst strains of India, ethnic European and East Asia in their study.

Eradication of the organism has been shown to result in the general well being of the patient (Sepulvedo and Coelho, 2002). The recommended triple therapy to eradicate *H. pylori* which includes a bismuth preparation or proton pump inhibitor (PPI) together with amoxicillin and clarithromycin or metronidazole (Mtz) and tetracycline (Kalach *et al.,* 2001), is the most

widely used regimen. Over time, the success rates of cure with the use of these combination therapy range from 85% to 95%. Unfortunately *H. pylori* acquire resistance to many classes of antibiotics after exposure to them (Kwon *et al.,* 2001; Ahmad *et al.,* 2009). Antibiotic resistance remains a problem worldwide and is also considered a primary cause of treatment failure to eradicate infection with this organism. Resistance to Mtz and clarithromycin results in an increased failure rate of therapies (Megraud, 1997). Resistance to metronidazole for example, has been reported to be very high worldwide with about 50% reported in Europe (Ables *et al.,* 2007) and percentages as high as 90% in different parts of Africa (Asrat *et al*., 2004; Ndip *et al*., 2007b).

The activity of Mtz on *H. pylori* is dependent on reduction of its nitro moiety to highly reactive compounds that cause DNA strand breakage (Kwon *et al*., 2001). The acquisition of resistance is highly associated with mutational inactivation of the *rdxA* gene, which encodes an oxygen-insensitive NADPH nitroreductase (Kwon *et al*., 2000; Tankovic *et al*., 2000). Recent evidence has suggested that inactivation of *frxA* encoding NAD(P)H-flavin oxidoreductase also contribute to the resistant phenotype (Jeong *et al.,* 2000; LIanes *et al.,* 2010). Also, clarithromycin-resistant *H. pylori* isolates are becoming increasingly prevalent. Clarithromycin resistance is associated with mutation in the 23SrRNA gene (Lee *et al*., 2005). Several reports have demonstrated that more than 90% of macrolide resistance in *H. pylori* is mediated by either of two transition mutations Adenine to Guanine (A→G) at adjacent positions 2142 and 2143 in one or both of the bacterium's two 23SrRNA genes (Matsuoka *et al.,* 1999). In South Africa, it is estimated that about 50 to 60% individuals are infected with *H. pylori* (Samie *et al*., 2007). Previous observation indicated resistance to the antibiotics widely used for triple therapy (Tanih *et al*., 2010b) and also found a high prevalence of virulence genes (*vacA*s1m2*)* in this study population (Tanih *et al*., 2010c). The

purpose of this study therefore was to investigate the genetic diversity by polymerase chain reaction and sequence analysis of the genes involved in virulence (*vacA*s1m2*)* and antibiotic resistance (*rdxA* and *frxA* for metronidazole, 23SrRNA for clarithromycin) in the strains as a guide for better prognosis and empiric treatment.

6.2 MATERIALS AND METHODS

6.2.1 Study population

H. pylori strains used in this study were isolated from patients suffering from gastric related morbidities with no history of antibiotics treatment, especially those used for conventional treatment of *H. pylori* infection. Previous use of antibiotic was excluded by way of a questionnaire and examination of medical records. Gastric biopsies were collected after informed consent by a resident gastroenterologist. The study was approved by the institutional review board of the University of Fort Hare and the Eastern Cape Department of Health (Protocol number EcDoH-Res 0002).

6.2.2 Bacteriology

The organism was isolated following standard microbiological procedures (Ndip *et al.,* 2008). Briefly, biopsies were homogenised under aseptic conditions in 0.2g/L of cysteine and 20% glycerol in Brain heart infusion broth; a loop full plated on freshly prepared Columbia agar base (Oxoid LTD, Basingstoke, Hampshire, England) supplemented with 7% sheep's blood (Oxoid, UK) and Skirrow's supplement (Oxoid, UK): trimethoprim (2.5 mg), vancomycin (5 mg), cefsulodin (2.5 mg); amphotericin (2.5 mg) was also added to the medium. All plates were incubated at 37ºC for 3–5 days under microaerophilic conditions (5–6% O_2, 10% CO_2, 80–85% N_2) (Anaerocult Basingstoke, Hampshire, England). Isolates were identified based on colony morphology and positive oxidase, urease and catalase tests. A reference strain of *H. pylori* (NCTC 11638) was included as a positive control. Confirmed isolates were suspended in 20% glycerol and stored at -80ºC (Sanyo, Japan) for future experiments.

6.2.3 Antibiotic susceptibility testing

Susceptibility testing was carried out by the disk diffusion (Kirby-Bauer) technique as previously reported in chapter 4. Clarithromycin (15µg) and metronidazole (5µg) antibiotic disk were obtained from Mast Diagnostics, UK. Brain heart infusion (BHI) agar (Oxoid, England) containing 7% horse blood and *Helicobacter pylori* selective supplement (Oxoid, England) were used for bacteria culture. Zone diameters of inhibition were measured. *H. pylori* control strains NCTC 11638 and J99 were included in all the experiments to determine susceptibility or resistance.

6.2.3.1 Determination of Minimum Inhibitory Concentration (MIC)

The MIC was determined by the agar dilution method (EUCAST, 2000). After incubation, the MIC value was read as the lowest concentration of the antibiotic that inhibited bacteria growth (no visible growth). The resistant breakpoint used for metronidazole was >8µg/mL and >1.0 µg/mL for clarithromycin (Osato, 2000).

6.2.4 Molecular Characterisation

6.2.4.1 DNA extraction

DNA was extracted from seventeen strains resistant to metronidazole and three to clarithromycin. DNA was obtained from strains with the virulence gene *vacA*s1 and m2 since the combination s1m2 was the most prevalent in this study. DNA was extracted from the pellets by use of the QIAamp DNA kit (Qiagen, SA) according to the manufacturer's recommendations and stored at -20°C until analysis.

6.2.4.2 PCR Amplification

PCR analysis of the targeted genes was performed using Thermo-stat Taq DNA polymerase (ABgene, UK) and manufacturer-provided reaction buffer. Five microlitres of DNA were added to 50µl of reaction mixture containing 1×PCR buffer, 3mM $MgCl_2$, 0.2mM (each) deoxynucleotide (ABgene, UK) and 0.5µM of oligonucleotide primers (*vacA*s1*, vacA*m2*, rdxA,* 23SrRNA*),* and 0.2µM of oligonucleotide primer *frxA*. Thermo-stat Taq DNA polymerase (ABgene, UK) 1.25U/µl was added to each tube. PCR was performed with a thermal cycler (MJ Research, USA). The amplification cycles consisted of an initial denaturation of target DNA at 95°C for 15min and then denaturation at 94°C for 1 min, primer annealing at 56°C (for *vacA* s1, m2, *rdxA, frxA* and 23SrRNA), for 1 min and extension at 72°C for 1 min. All reactions were performed through 35 cycles**.** The final cycle included an extension step for 5 min. The primers used to amplify the targeted genes are summarised in table 6.1. Negative controls were added to each PCR run including all reagents except template DNA which was substituted with ultra pure water (Sigma-Aldrich, UK). Amplification of DNA was analysed by agarose gel electrophoresis using standard procedures (Akopyanz *et al*., 1992). Briefly, aliquots of amplified samples (5µl) were electrophoresed on 2% high-resolution agarose gel in TAE buffer. The gel was stained with ethidium bromide 0.5ug/ml. The amplified bands were visualised under ultraviolet light and photographed.

Table **6.1:** PCR primers for amplification of *vacA (*s1*,* m2*),* 23SrRNA*, rdxA* and *frxA* genes.

Gene	Primer sequence	PCR product (bp)	Reference
vacA s1	5'-ATGGAAATACAACAAACACAC-3'	259	Smith *et al.,* 2002
	3'-CTGCTTGAATGCGCCAAAC-5'		
vacA m2	5'-GGAGCCCCAGGAAACATTG-3'	290	Smith *et al.,* 2002
	3'- CATAACTAGCGCCTTGCAC-5'		
rdxA	5'- GTTAGGGATTTTATTGTAATG-3'	427	Kwon *et al*., 2001
	3'- ACGCCAAGCATTTGAGCAAA-5'		
frxA	5'- TCTCAAGCGGAAAAATCCGG-3'	445	Kwon *et al.,* 2001
	3'- AATTTTTGATGATTTGAGCG-5'		
23SrRNA	5'- ACGGCGGCCGTAACTATA-3'	307	Wang *et al.,* 2001
	5'- ACAGGCCAGTTAGCTA -3'		

6.2.5 Mutational analysis

Analysis for mutations and genetic diversity was carried out by sequencing of PCR products. Products for sequencing were purified using shrimp alkaline phosphatase. Direct cleaning of amplified DNA was done with 20µl of amplified DNA, 4µl (2µl of shrimp alkaline phosphatase plus 2µl Exol 1 in 10 with water) (Promega, UK) under the following condition; 37°C for 30mins, 72°C for 15mins and 8°C for 5mins. DNA sequencing was carried out by using a Big Dye Terminator DNA sequencing kit v3.1 (Applied Biosystems, UK). Direct sequencing on chromosomal DNA was done with 2µl of chromosomal DNA, 0.25µl of primer (10pmol per µl), 2µl of Big Dye buffer and 2µl of Big Dye under the following conditions: a denaturation at 96°C for 10 s, annealing at 50°C for 20 seconds, and extension at 60°C for 4 min over 30 cycles, followed by Agencourt CleanSeq cleanup and analysed with ABI automated sequencer 3130xl machine (Applied Biosystems, UK). DNA sequence

editing and analysis was performed with the programs Bioedit and EMBL- EBI- Clustalw2. Sequences were aligned using DNAMAN.

6.3 RESULTS

6.3.1 Susceptibility testing and MIC determination

Of the two hundred strains subjected to the antibiotics susceptibility, 95.5% resistance was noted for metronidazole and 20% to clarithromycin. Metronidazole showed MIC values which ranged from 1-256μg/mL. Minimum inhibitory concentration values for clarithromycin ranged from 0.125-256μg/mL. The resistant breakpoint used for metronidazole was >8 μg/mL and >1.0 μg/mL for clarithromycin (Osato, 2000; Ndip *et al*., 2008).

6.3.2 PCR amplification of 23SrRNA, *frxA* and *rdxA genes.*

There was successfully amplification of the expected 307bp fragments in all the strains which presented with clarithromycin resistance. Likewise was the amplification of *rdxA* and *frxA* with expected bands 427bp and 445bp respectively (Figs 6.1 & 6.2). *vacA* s1 and m2 amplification was equally successful as demonstrated in the previous chapter.

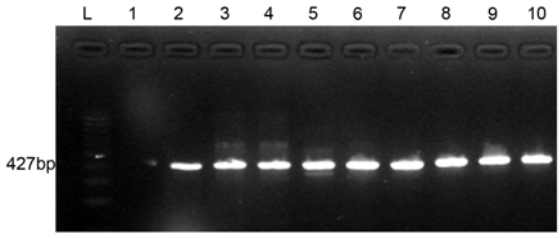

Fig. 6.1. Detection of *rdxA* allele.

Lane L, marker; lane 1, negative control; lanes 2 to 10 are *rdxA* positive samples.

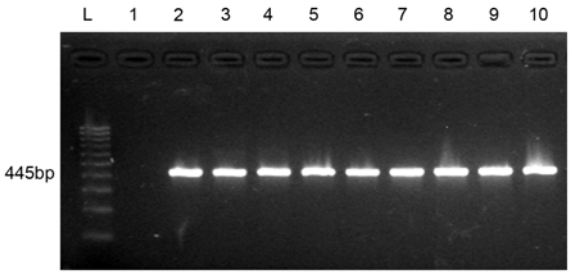

Fig. 6.2. Agarose (2%) gel electrophoresis of PCR products for *frxA* detection.

Lane L, marker; lane 1, negative control; lanes 2 to 10 are *frxA* positive.

6.3.3 Characterisation of *rdxA* and *frxA* genes of Mtz –sensitive and resistant strains

Metronidazole-sensitive strain (#237C) exhibited amino acid substitution Glu(27)-Val (missense mutation) which was not identical to those exhibited by the metronidazole resistant strains and might not be important for resistance. In Mtz-resistant strains, one had a missense mutation (position no. 80-82); thirteen amino acid substitutions were found in this study in nine different strains (#243A, #243C, #265C, #266A, #268C, #293A, #294A, #308A and #308C); seven of the strains had nonsense mutations (#243A, #243C, #266A, #268C, #279A, #293C, and #296C); and four others (#243A, #243CA, #266A and #268) showed both nonsense mutations and amino acid substitutions that resulted in a truncated *rdxA* protein at positions no. 16, 22, 27, 32, 56, 60, 71, 78, 97, 106, 111, 113, 115, 121. Two strains in this study (#238A and #238C) remained unchanged for the *rdxA* gene (Table 6.2).

The open reading frame for the *frxA* gene was disrupted by a deletion of a nucleotide at position 54 and 98 and in the resistant strain #293C by a nucleotide insertion at position 224. These events led to the occurrence of a stop codon at positions corresponding to amino acids 39, 72 or 84. In resistant strains, #265C, #266A, #268C, #308A and #308C, some point mutations were observed (Table 6.2).

Table 6.2: Detection of mutations in the *rdxA* and *frxA* genes in *Helicobacter pylori* strains.

		rdxA		*frxA*	
H. pylori strain	MIC(µg/mL)	Change in nucleotide sequence	Change in amino acid sequence	Change in nucleotide sequence	Change in amino acid sequence
237C	1	-	Glu(27)-val	Frameshift (nt deletion at position 54)	Val(44)-Gly Tyr(60)-Phe
238A	8	-	-	Frameshift (nt deletion at position 54)	Stop codon at position 39
238C	1	-	-	Frameshift (nt deletion at position 54)	Stop codon at position 39
243A	256	-	Leu(71)-Phe Gln(113)- stop codon	Frameshift (nt deletion at position 54)	Stop codon at position 39
243C	256	-	Pro(106)-Ser Ser(111)-leu Arg(16)- stop codon	Frameshift (nt deletion at position 54)	Stop codon at position 84
265C	256	-	Arg(16)- cys Met(56)-Val	Frameshift (nt deletion at position 98)	Stop codon at position 39 His(6)- leu Ser(7)-Phe
266A	64	-	Ser(111)- leu Arg(56)- stop codon		Stop codon at position 39 Thr (110)-Ser Glu(169)-Lys

268C	256	-	Arg(16)-His Thr(16)-Ala Glu(60)- stop codon	Frameshift(nt deletion at position 54)	Thr(26)-Glu
279A	128	-	Leu(121)- stop codon	Frameshift(nt deletion at position 54)	Stop codon at position 39
279C	64	Missense (deletion of three nt 80-82)	-	Frameshift(nt deletion at position 54)	Stop codon at position 39
293A	128	-	Glu(27)- Gln	Frameshift(nt deletion at position 54)	Glu(199)-stop codon
293C	128	-	Glu(60)- stop codon	Frameshift(nt deletion at position 224)	Stop codon at position 72
294A	256	-	Val(32)-Ala	Frameshift(nt deletion at position 54)	Stop codon at position 39
296C	256	-	Glu(78)- stop codon	Frameshift(nt deletion at position 54)	Stop codon at position 39
305C	64	Missense (deletion of four 80-83)	-	Frameshift(nt deletion at position 54)	Stop codon at position 72
308A	64	-	Asn(22)- Ser	-	Ala(112)-stop codon
308C	128		His(97)-Tyr Arg(115)-lle	Frameshift(nt deletion at position 54)	Glu(120)-Lys Met(126)-Phe Thr(26)-Glu

nt = nucleotide

.

6.3.4 Detection of 23SrRNA gene mutation by sequencing.

Mutations of the 23SrRNA gene were analysed by sequencing. Among the three strains investigated, two (66.7%) harboured 23SrRNA mutations at position A2142G while the other had both A2142G and A2143G. These strains exhibited high level resistance to clarithromycin (MIC ≤256 μg/mL).

6.3.5 Genetic diversity of *vacAs1* and *m2*

Comparison of DNA sequence alignments of *vacA*s1 and m2 nucleotides found in *H. pylori* strains in this study area was carried out. Comparison of *vacA*s1 aligned sequences of these strains in comparison with the standard strain NCTC 11638 (U07145) showed a 91% similarity while that between one strain to another varied between 95- 98% .

Of the two hundred and twenty two nucleotides in each *vacA*s1 strain, one hundred and ninety (86%) were highly conserved. Most of the differences observed between the standard strain and the strains enrolled in this study were substitutions, insertions and deletions, for example at position 2, 4, 21, 28 etc (Fig 6.3). Nucleotides of the non conserved regions were similar except for one or more changes. For example, at position 112 there was a single change in the nucleotide for strain 11. A deletion was noticed at position 69 in strain 7 and an insertion at position 211 in strains 3, 4 and 13 (Fig 6.3).

In *vacA*m2 strains, an overall identity of 85.8% was noticed between the strains when compared to the standard NCTC 11638. However, some few differences (14.2%) were observed between the strains studied; mutations were noticed (insertions and deletions). For example, insertions were found at positions 8, 21 and 39 while deletions were observed at positions 3, 9, 17 etc (Fig 6.4).

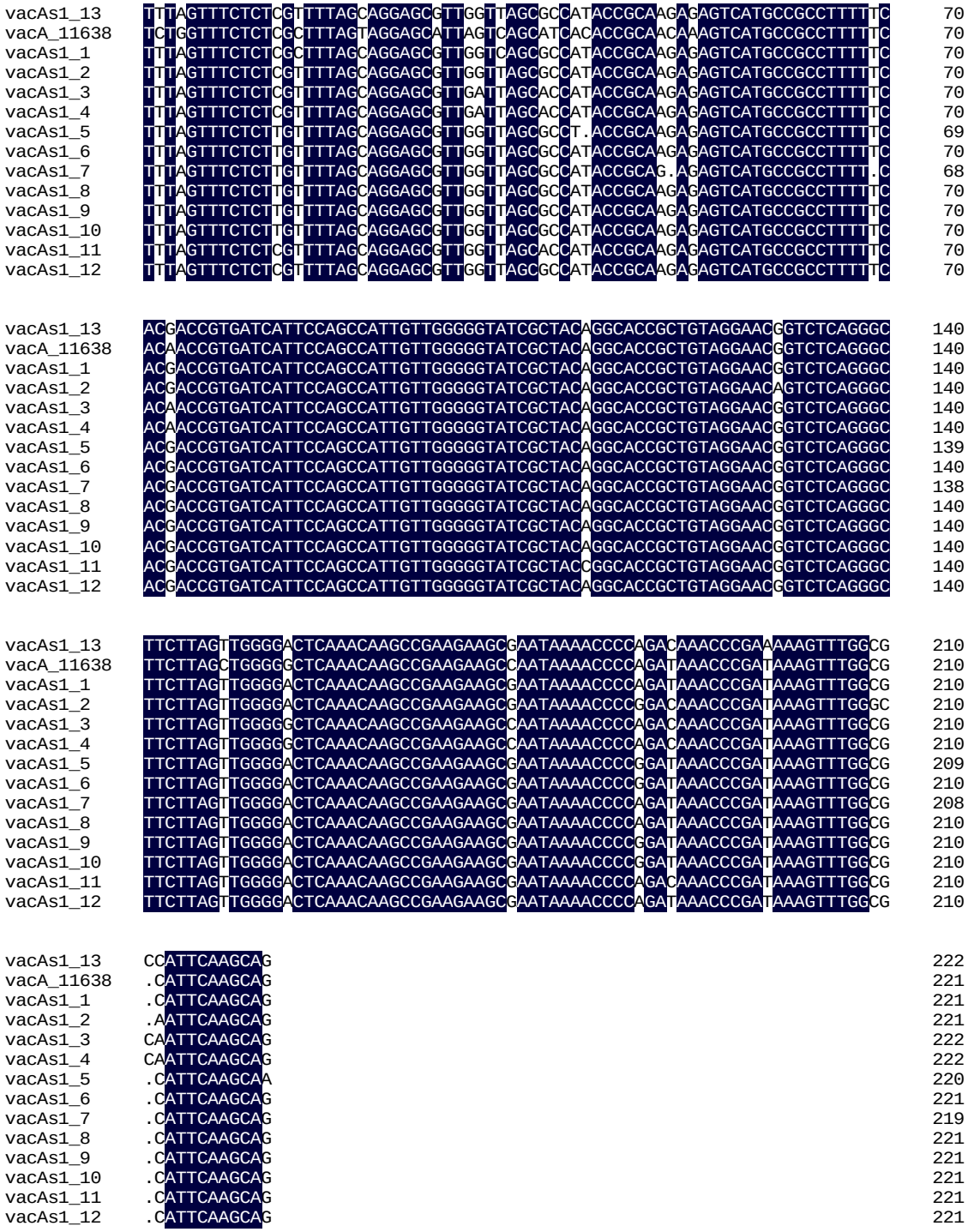

Fig 6.3. DNA sequence alignments illustrating nucleotides found in *H. pylori* strains in this study area. *vacA*s1 region sequences showing differences between the strains studied as compared to the standard.

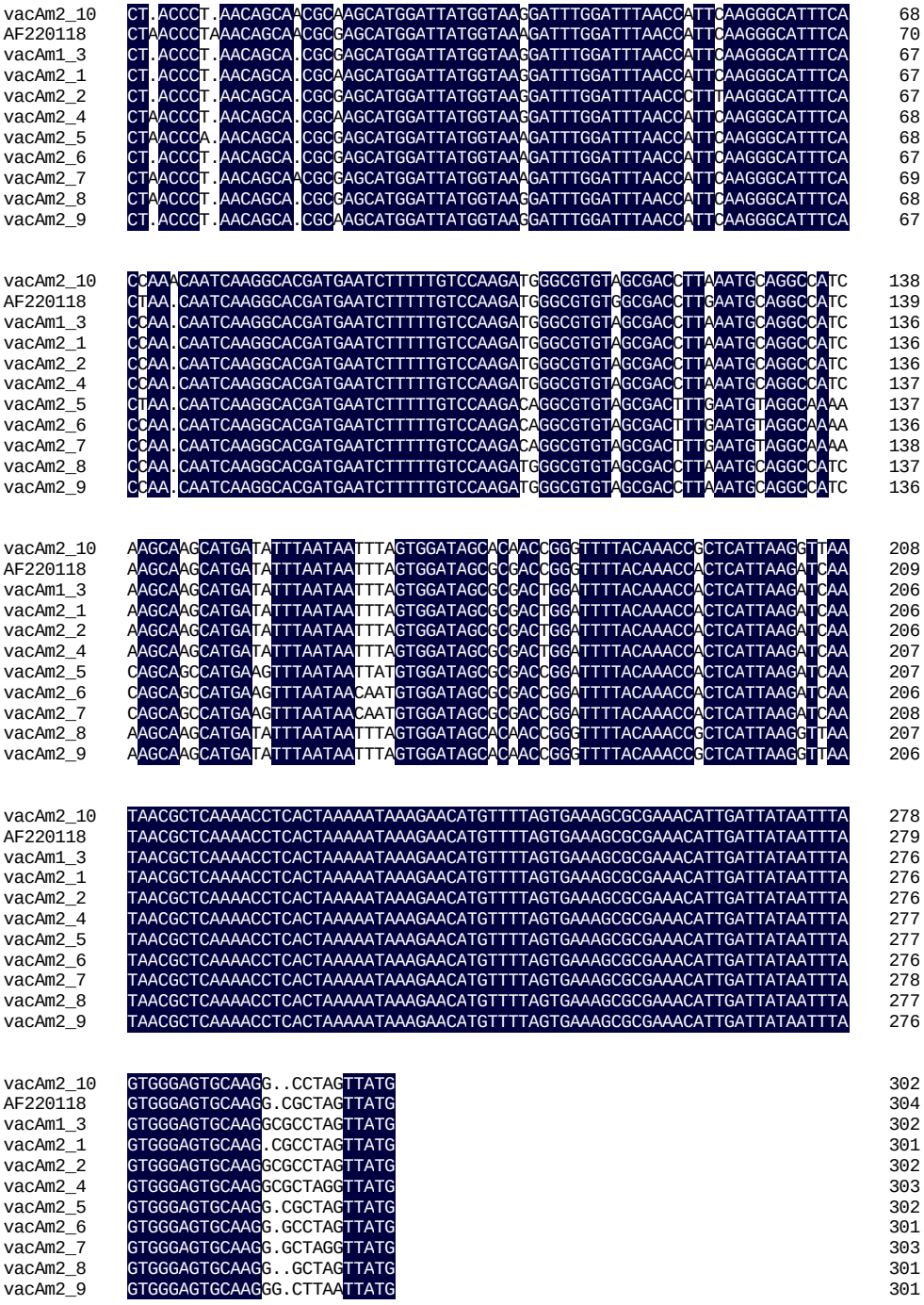

Fig 6.4. DNA sequence alignments illustrating nucleotides found in *H. pylori* strains in this study area. *vacA*m2 region sequences showing differences between the strains studied as compared to the standard.

6.4. DISCUSSION AND CONCLUSION

Previous reports on resistance of metronidazole and clarithromycin to the isolates used in this study exist and also the MIC values of isolates documented (Tanih *et al*., 2010b)**.** Resistance to antimicrobials (metronidazole and clarithromycin) as detected in culture is of particular concern with *H. pylori,* as it represents a major cause of eradication failure. Resistance to Mtz is the most common type of resistance in this pathogen (Nahar *et al.,* 2004, Tanih *et al*., 2010b). The high prevalence of resistance to metronidazole in South African strains of *H. pylori* might be due to its frequent use for intestinal parasites and gynaecological disorders, as this drug is one of the most widely used antibiotics by South African patients attending the primary health care system (Tanih *et al*., 2010b).

Mtz- resistance has been associated with inactivation of *rdxA* and or *frxA* (Tankovic *et al*., 2000; Kwon *et al.,* 2001; Jeong *et al.,* 2000). Neither *rdxA* nor *frxA* is a lethal gene for *H. pylori* survival (Kwon *et al*., 2000)**,** and mutations in Mtz has been constantly reported in *H. pylori,* which is most likely responsible for the observation that clinical use of Mtz often results in the development of resistance (Kwon *et al.* 2001; Jeong *et al.,* 2000; Tanih *et al*., 2010b). A mutation Glu(27)-Val was detected in a Mtz-susceptible strain which was different from those observed in the Mtz resistant ones; and might not be essential for resistance to occur. The following mutations were found in the *rdxA* gene sequenced, Arg(115)-lle, Arg(16)-His, Ser(111)-Leu, Arg(16)-Cys; these have also been described previously (Wang *et al.,* 2001; Kwon *et al.,* 2001; Yang *et al.,* 2004).

Several mutations in the *rdxA* gene were detected in the Mtz-resistant strains in this study which have not been previously described in literature: Thr(16)-Ala, Glu(27)- Gln, Val(32)-Ala, Asn(22)-Ser, His(97)-Tyr, Met(56)-Val, Ser(111)- Leu, Leu(71)-Phe, Pro(106)-Ser,

Gln(113)- stop codon, Arg(16)- stop codon, Arg(56)- stop codon, Glu(60)- stop codon, Leu(121)- stop codon, Glu(78)- stop codon; and in the *frxA* gene: [Val(44)-Gly, Tyr(60)-Phe, His(6)- Leu, Ser(7)-Phe, Thr (110)-Ser, Glu(169)-Lys, Thr(26)-Glu, Glu(120)-Lys, Met(126)-Phe, Thr(26)-Glu, Ala(112)-stop codon, Glu(199)-stop codon]; it is likely that these might be associated with resistance as mutation has been reported to cause changes which may introduce phenotypic characteristics amongst which is antibiotic resistance (Jeong *et al.,* 2000).

Some reports established that inactivation of *frxA* without mutations in *rdxA* could not cause Mtz resistance (Kwon *et al*., 2001; Yang *et al*., 2004). These results suggest that alterations of *frxA* alone produced resistance, e.g. strain #238A. However, as the *frxA* mutations were also observed in Mtz-susceptible strains, there is speculation that these are unlikely to contribute to the Mtz resistance of these strains (Table 6.2). The results of this study are in accordance with the notion that metronidazole susceptibility is dependent on the level of nitroreductase activity produced by *rdxA* and *frxA*, and the dual inactivation of these genes are required to confer high level resistance (Kwon *et al*., 2001).

Clarithromycin is one of the antibiotics used in the triple therapy regimen for the treatment of *H. pylori* infection. Resistance to this antibiotic considerably reduces the success rate of standard triple therapies (Duck *et al.,* 2004; Ahmad *et al*., 2009). A very high rate (81.5%) of mutation has been reported worldwide for A2142G or A2143C in clarithromycin resistant isolates of *H. pylori* (Mégraud, 2004). In the current investigation, two mutation types A2142G and A2143G were detected. Two strains exhibited A2142G mutation while one had both A2142G and A2143G. Results of several studies have reported that A2142G and 2143G

mutations of 23SrRNA genes in *H. pylori* are associated with resistance to clarithromycin (Versalovic *et al* 1996; Matsuoka *et al*., 1999; Mégraud, 2004; Ahmad *et al*., 2009).

Substantial evidence suggests that *H. pylori* eradication will prevent the majority of gastric related morbidities that are caused by gastric inflammation and many that are associated with gastric atrophy. Population screening and treatment for *H. pylori* infection is an appealing strategy (Moayyedi and Hunt, 2004) as the aim is to prevent the disease and its complications. Findings of this study are critical to guide clinicians on the effectiveness of treatment regimens and provide great insight for evaluation and implementation of standard therapy for treatment of this infection.

Sequence analysis has shown that different genotypes of *H. pylori* prevail in different geographical locations (Mukhopadhyay *et al.,* 2000). The allele *vacA*s1 was sequenced which is the most toxigenic strain of the vacuolating cytotoxic gene and m2 which is one of the mild alleles of the *vacA* gene. The combination *vacA*s1m2 was of interest being the most prevalent combination in this study; this has also been reported to be the second most toxigenic combination of the *vacA*s&m after s1m1 (Tanih *et al.,* 2010c). Interestingly, a lot of similarities were found between the *vacA* in this study in terms of s1 and m2 strains when compared to the standard strain (U07145) (Figs. 6.3 and 6.4). Eighty- seven percent of the nucleotides in the *vacA*s1 strains in this study were conserved while 13% were different. This finding corroborates that of Cover *et al.* (1993) who reported diverse *vacA* sequence and suggested that there may be considerable sequence diversity among strains in the middle region of the *vacA* gene.

High level of conservation of the nucleotides was found in *vacA*m2 (85.8%) strains which were analysed as opposed to 14.2% dissimilarity. The results of this study are in line with the findings of Mukhopadhyay *et al.* (2000) who reported a considerable difference between the *vacA*m2 allele in their study. None of the strains in both *vacA*s1 and m2 were identical (100 %) to the standard strains used for comparison. This is in accordance with the finding of Wirth *et al.* (2003) who reported wide genetic diversity in *H. pylori* strains from region to region and even between ethnic groups in the same geographical region. However, close similarity was found between the *vacA*m2 strains with the *vacA*m2 of *H. pylori* AF220118 and AF220119 accession numbers derived from strains in India (Mukhopadhyay *et al.,* 2000). Sequences from this study await submission in the genbank for accession numbers.

The high level of genetic diversity in the *vacA*s1 and m2 type's alleles in South African *H. pylori* strains is in agreement with the high prevalence of this organism in this population and also by the fact that evolutionary consideration and high rates of transmission favour the emergence of more virulent strains of the organism. The differences observed are thought to have resulted due to selection pressures in different ancestral animal host rather than in humans (Mukhopadhyay *et al.,* 2000). Studies in cell culture and animal models to gain further insight on the different *H. pylori* strains (proteins), and their role in bacterial pathogenesis is recommended.

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CHAPTER SEVEN

General discussion, Conclusions and Recommendations

7.1 GENERAL DISCUSSION

H. pylori is a common human gastric pathogen causing chronic gastritis and duodenal ulcers (Kuipers and Michetti, 2005; Ndip *et al.,* 2008). Gastric infections with this organism are common and ubiquitous in Africa (Segal *et al*., 2001). The prevalence of *H. pylori* infection as well as the incidence of gastric cancer has been reported in other continents in the world such as Asia, South America, Europe and the United States (Hisada *et al*., 2001). The prevalence of *H. pylori* infection is generally higher in developing countries (Holcombe, 1992). Several studies have documented a high prevalence of *H. pylori* in South Africa. There is a dearth of knowledge on the molecular epidemiology of *H. pylori* in the Eastern Cape Province of South Africa. The current study investigated the phenotypic and molecular characterisation of *H. pylori* from patients with gastroduodenal pathologies in the Eastern Cape Province of South Africa.

The results from this study delineated a high prevalence of *H. pylori* (66.14%) in this study population, which is amongst the highest when compared with other studies in South Africa (Samie *et al*., 2007) and other parts of the world. However, this is similar to other studies documented in Cote d'Ivoire, Egypt, Ghana, Kenya, Malawi, Nigeria, Cameroon, Zaire, Zimbabwe and parts of Korea [(60–94%) Kidd *et al*., 1999; Ndip *et al*., 2008)]. Kidd *et al*. (2001) had also reported that in Africa, endoscopic studies in dyspeptic individuals revealed the presence of the organism in most of the subjects.

The prevalence of *H. pylori* infection was highest among the coloured 66.92% (87/130) followed by the blacks 65.85% (54/ 82). The high prevalence of *H. pylori* infection among the coloured and black South Africans compared to the white can partially be explained by other known risk factors for *H. pylori,* particularly socioeconomic determinants such as lower income, lower educational level and greater household crowding as previously suggested (Louw *et al*., 1993a). *H. pylori* prevalence was highest (100%) amongst patients with duodenitis (1/1), GC (4/4) and gastric erosion (5/5). However, patients with gastritis (75%; 6/8), GU (70.8%; 17/24), DU (65%; 26/40), NUD (64.7%%; 55/85), and GERD (64%; 29/45) also had high prevalence rates of the organism. This corroborates the findings of other investigators (Kidd *et al*., 1999; Matsuhisa *et al*., 2003; Onyekwere *et al*., 2008) who reported either one or two of the above clinical conditions as the most common endoscopic findings in *H. pylori* infection.

Females, particularly blacks, had a higher prevalence (71.69%) than males (55.17%). A few other studies have found a higher prevalence among women but numerous studies have found no sex difference (Replogle *et al*., 1995; Malaty *et al*., 2002; Chong *et al.,* 2008). It is possible that the risk of acquisition is not different between the sexes. The prevalence was probably higher in females than males because more females were recruited in the study than males; it is speculated that females are more likely to have infections (such as genitourinary infections).

Another important finding of this study was increased infection rate with age which peaked at around 45-54yrs. This is similar to the results reported by Chong *et al*. (2008) in which the prevalence of infection increased with age and was highest between the age ranges 20–25 years. The organism is ubiquitous with acquisition in childhood being the rule. The results of

this study also indicated that alcohol consumption and smoking were not significantly associated with *H. pylori* infection (P> 0.05). Although the OR (OR= 1.15; 95% CI: 0.64 - 2.07) indicates that alcohol consumption may be a risk factor for *H. pylori* infection. However, a previous study had reported that *H. pylori* infection was slightly lower in smokers than in non smokers (Iso *et al*., 2005); and this has been attributed to the promotion of acid secretion by smoking and resultant generation of an environment not conducive to *H. pylori* survival (Ogihara *et al*., 2000).

This study indicated monoresistance to metronidazole and erythromycin and multidrug resistance of the other drugs studied (Table 4.2, Chapter 4). These results revealed marked antimicrobial susceptible rates of 100% for ciprofloxacin, and 97.5% for amoxicillin. These findings corroborate those of other investigations (Eltahawy *et al.,* 2002; Mirza *et al.,* 2007) who also reported marked susceptibility (100%) to amoxicillin or ciprofloxacin in their studies. Isolates were resistant (95.5%) to metronidazole (Chapter 4). Smith *et al.* (2001) in Nigeria also reported 100% resistance to metronidazole. The high prevalence of metronidazole resistance in developing countries is probably because of the frequent use of nitroimidazole derivatives for the treatment of protozoa infections and gynaecological problems (Loffeld & Fijen 2003). A total of 19 antimicrobial resistance patterns were observed with the most prevalent pattern being metronidazole (MET^R) accounting for 49 (26.06%) of *H. pylori* isolates studied. Multidrug resistance by the isolates used in this study may be due to indiscriminate use of these drugs for the treatment of other infections, and may serve as a major selective pressure for antibiotic resistant bacteria (Ndip *et al*., 2005).

With the high prevalence of *H. pylori* in the Eastern Cape Province this study investigated the genetic diversity of the isolates by genotyping the genes involved in virulence of *H. pylori*

[*vacA* (s and m subtypes), *cagA and iceA*]. A high prevalence of these genotypes in the strains studied was identified suggesting that they maybe associated with gastroduodenal pathologies of patients in the Eastern Cape Province of South Africa (Chapter 5). These findings are in line with those of Kidd *et al.* (2001) who previously described South African *H. pylori* isolates by the universal presence of *cagA* but with differences in the vacuolating cytotoxin gene (*vacA*) alleles. Also, *vacA*s1 (the most virulent) allele was found to be predominant in this study population 58(58%) a finding which has also been observed in other studies in South Africa and the world (Letley *et al*., 1999; Smith *et al*., 2002).

Drug resistance has been a major concern in the treatment of *H. pylori* infections and antimicrobials like metronidazole have been reported with high level of resistance in many studies in Africa and different parts of the world (Tanih *et al*., 2010b). Clarithromycin resistance is also of concern. Genes involved in metronidazole (*rdxA* and *frxA*) and clarithromycin (23SrRNA) resistance, were amplified and sequenced (Chapter 6). Sequence typing has indicated unique genotypes in different human populations. African strains seem different when compared to other strains worldwide. This study indicated genetic diversity (*vacA*s1 and *vacA*m2) among the strains employed for sequencing. Mutations were also found in *rdxA* and *frxA* which might be responsible for metronidazole resistance. A2142G and A2143G mutations highly implicated in clarithromycin resistance were detected in this study.

7.2. CONCLUSIONS

Based on this study, the following conclusions can be drawn;

1. A high prevalence (66.14%) of *H. pylori* was delineated in this study population.
2. Coloured and blacks ethnic populations are more likely to become infected with the organism than whites.
3. Ciprofloxacin and amoxicillin were significantly active against the isolates. However, there is a high level of resistance of *H. pylori* to classical antibiotics used in South Africa most especially metronidazole.
4. Evaluation of the genes involved in metronidazole (*rdxA, frxA*) and clarithromycin (23SrRNA) resistance indicated that a number of mutations maybe associated with resistance to these antibiotics.
5. A high prevalence of the main virulence factors (*vacA*, *cagA* and *iceA* genes) was found in this study. Multiple *vacA* genotypes were also observed in this study. There was no statistical significant difference between the association of these genotypes and disease status.
6. *vacA*s1 and m2 sequences derived from this study showed great diversity from one another though closely related to the standard strain used for comparison.

7.3. RECOMMENDATIONS

Based on these findings the following can be recommended;

1. Improved environmental and socioeconomic conditions (either by way of education which will lead to employment and improved living standards) which have been associated with increased prevalence of *H. pylori.* This is of particular importance in this study population which is predominantly rural with deprived living conditions.

2. Antimicrobial susceptibility pattern should be carried out prior to treatment of infected patients especially those who have not been on *H. pylori* therapy to overcome the problem of resistance because such patients easily select for resistant strains. Ciprofloxacin should be included in the treatment regimen of *H. pylori* in this study area.
3. Further studies in cell culture and animal models should be conducted to gain insight on the different *H. pylori* strains (proteins), and their role in bacterial pathogenesis.
4. There is a need to conduct further studies in the area of genetic analysis to determine the homology of *H. pylori* genome in members of the same family in order to study the transmission of the organism from person to person and their clinical implications in this environment.
5. A comparative analysis of the sequences derived from this study in the Eastern Cape Province of South Africa with those of other regions in South Africa and other parts of the world would form an important basis of disease prognosis.

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APPENDICES

APPENDIX 1:

ETHICAL CLEARANCE

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

ECDoH-Res0001_

Eastern Cape Department of Health

Enquiries: Zonwabele P. Merile
Tel No: 040 609 3408
Date: 08th May 2008
Fax No: 040 609 3784
e-mail address: zonwabele.merile@mpilo.ecprov.gov.za

Dear Prof. Roland N. Ndip

Re: Genotypes of Helicobacter pylori in South Africa: understanding transmission and disease

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

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 "Genotypes of *Helicobacter pylori* circulating in South Africa: Understanding disease and transmission", 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

APPENDIX 2

CONSENT FORM

TITLE OF STUDY: "Molecular and phenotypic characterization of *Helicobacter pylori* isolates from patients with gastroduodenal pathologies in the Eastern Cape Province of South Africa"**.**

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: " Molecular and phenotypic characterization of *Helicobacter pylori* isolates from patients with gastroduodenal pathologies in the Eastern Cape Province of South Africa " 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's Name: _____

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: "Molecular and phenotypic characterization of *Helicobacter pylori* isolates from patients with gastroduodenal pathologies in the Eastern Cape Province of South Africa".

PURPOSE: Four gastric biopsies will be taken from you when you undergo endoscopy and tested for *H. pylori*.

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: Endoscopy may be uncomfortable. The biopsies will be taken by trained medical personnel who will try to minimize this discomfort.

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: " Molecular and phenotypic characterization of *Helicobacter pylori* isolates from patients with gastroduodenal pathologies in the Eastern Cape Province of South Africa" under the direction of Prof Roland Ndip. You may at any time change your mind and withdraw yourself from the study.

Subject's Printed Name: _____

Subject's Signature/Mark/Fingerprint: _____ Date: _____

Study Number: _____

Witness's Signature:_____ Date: _____

Witness's Name: _____

APPENDIX 3

QUESTIONNAIRE

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

SECTION A: PERSONAL

1. Patient name……..Date…….
1. Age……Sex……
2. Place of birth……
3. Ethnic background……...
4. Area of residence…….Tel……..
5. Educational level……..Occupation……
6. Number of persons sharing accommodation……..
7. Contact Address…….
8. Socio-economic status (income) Low ☐ Moderate ☐ High ☐

SECTION B: PATIENT'S CHARACTERISTICS

10. Do you smoke? Yes ☐ No ☐ Used to ☐
11. Do you drink alcoholic beverages Yes ☐ No ☐ Used to ☐
12. How many meals do you eat per day 1 ☐ 2 ☐ 3 ☐

SECTION C: CLINICAL HISTORY

13. Indication for endoscopy
    - ☐ Nausea
    - ☐ Vomiting
    - ☐ Belching
    - ☐ Retrost chest pain
    - ☐ Epigastric pain

    Others…….

14. Clinical diagnosis of current disease
    - ☐ Non-ulcer dyspepsia
    - ☐ Gastric ulcer
    - ☐ Duodenal ulcer
    - ☐ Gastro-oesopharyngeal reflux disease

15. Duration of current episode……

16. Are you on any regular medication? ☐ Yes ☐ No

If Yes, please specify and for what reason……

17. Have you had any course of antimicrobials in the last 3 months with regard to the current disease? Yes ☐ No ☐

If Yes, please specify……

18. Where were you treated?

☐ Hospital ☐ Self medication ☐ Traditional Doctors Others

19. Which drugs did you take?…….

SECTION D: COLLECTION OF GASTRIC BIOPSY

20. Number of specimens obtained…….

21. Site of collection:

	Antrum	Corpus
	☐ 1	☐ 1
	☐ 2	☐ 2

APPENDIX 4

PUBLICATIONS, MANUSCRIPTS IN PREPARATTION AND CONFERENCE PRESENTATION.

LIST OF PUBLICATIONS

1. **Tanih NF**, Clarke AM, Mkwetshana N, Green E, Ndip LM, Ndip RN (2008). *Helicobacter pylori* infection in Africa: Pathology and microbiological diagnosis. African Journal of Biotechnology. **7**(25):4563-4662.
2. **Tanih NF**, Dube C, Green E1, Mkwetshana N, Clarke AM, Ndip LM, Ndip RN (2009). *Helicobacter pylori* prevalence in Africa: drug resistance and alternative approaches to treatment. Annals of Tropical Medicine and Parasitology. **103**(3):189-204.
3. **Tanih NF**, Ndip LM, Clarke AM, Ndip RN (2010a). An overview of pathogenesis and epidemiology of *Helicobacter pylori* infection. Africa Journal of Microbiology Research. **4**(6):426-436.
4. **Tanih NF**, Okeleye BI, Naidoo N, Green E, Mkwetshana N, Clarke AM , Ndip LM, Ndip RN (2010b). Marked susceptibility of South African *Helicobacter pylori* strains to ciprofloxacin and amoxicillin: clinical implications. South African Medical Journal. **100**(1):49-52.
5. **Tanih NF**, McMillan M, Naidoo N, Ndip LM, Weaver LT, Ndip RN (2010c). Prevalence of *Helicobacter pylori vacA, cagA* and *iceA* genotypes in South African patients with upper gastrointestinal diseases. Acta Tropica. **116**(1):68-73
6. **Tanih NF**, Okeleye BI, Naidoo N, Green E, Mkwetshana N, Clarke AM, Ndip LM, Ndip RN. (2010d). *Helicobacter pylori* prevalence in dyspeptic patients in the Eastern Cape Province of South Africa: ethnicity and disease status. South African Medical Journal (In press).

MANUSCRIPTS IN PREPARATION

1. Characterisation of metronidazole (*rdxA* and *frxA*) and clarithromycin resistance (23SrRNA) genes in South African *H. pylori* strains.

2. Genetic diversity of *vacA*s1 and m2 in South African *H. pylori* strains

CONFERENCE PRESENTATIONS

1. **Tanih NF,** Okeleye BI, Green E, Mkwetshana N, Clarke AM, Ndip LM, Ndip RN (2010). *Helicobacter pylori* prevalence in dyspeptic patients in the Eastern Cape Province of South Africa: An analysis of ethnicity and disease status. Paper presented at Bio2Biz SA 2009 and SASM 09 conference, ICC, Durban, South Africa, 20-23 September 2009 (Poster presentation).
2. **Tanih NF,** Okeleye BI, Green E, Mkwetshana N, Clarke AM, Ndip LM, Ndip RN (2010). Prevalence of *Helicobacter pylori vacA* and *cagA* genotypes in South African dyspeptic patients. Paper presented at Bio2Biz SA 2009 and SASM 09 conference, ICC, Durban, South Africa, 20-23 September 2009 (Oral talk).
3. Okeleye BI, **Tanih NF,** Green E, Mkwetshana N, Clarke AM , Ndip LM , Ndip RN. (2010). *Helicobacter pylori* strains recovered from dyspeptic patients in the Eastern Cape Province of South Africa: Antibiogram of isolates. Paper presented at Bio2Biz SA 2009 and SASM 09 conference, ICC, Durban, South Africa, 20-23 September 2009 (Poster presentation).