PREVALENCE OF CYTOTOXIN-ASSOCIATED GENE A (CagA) POSITIVE *HELICOBACTER PYLORI* AMONG SEROPOSITIVE ASYMPTOMATIC CHILDREN ATTENDING KENYATTA NATIONAL HOSPITAL, NAIROBI, KENYA.

A DISSERTATION SUBMITTED TO THE UNIVERSITY OF NAIROBI IN PART FULFILLMENT OF THE DEGREE OF MASTER OF MEDICINE IN HUMAN PATHOLOGY

Declaration

This dissertation represents an original study, and has not been presented to any other institution for review and approval.

Sign: …….. Date: …….

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DEDICATION

To the memory of my father, for the extraordinary sacrifice he made to help me realize my potential and pursue my dream and to whom I owe my achievement.

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LIST OF ABBREVIATIONS

Ab	Antibody
Ag	Antigen
CC	Calibrator
CagA	Cytotoxin-associated gene A
DU	Duodenal Ulcer
EGFR	Epidermal Growth Factor Receptor
ELISA	Enzyme Linked Immunosorbent Assay
EQA	External Quality Assurance
ERC	Ethics and Review Committee
FlaA	Flagellin A
FlaB	Flagellin B
GERD	Gastro-Esophageal Reflux Disease
GIT	Gastro-intestinal tract
GU	Gastric Ulcer
Hp	*Helicobacter pylori*
IL	Interleukin
IQC	Internal Quality Control
KNH	Kenyatta National Hospital
LPS	Lipopolysaccharide
MALT	Mucosa Associated Lymphoid Tissue
ml	milliliters
NC	Negative Control
NSAID	Non-Steroidal Anti-inflammatory Drug
PAI	Pathogenicity Island
PC	Positive Control
PCL	Positive Control Low

PCH	Positive Control High
PI	Principle Investigator
POPC	Pediatric Out-Patient Clinic
PPIs	Proton Pump Inhibitors
PUD	Peptic Ulcer Disease
RA	Research Assistant
rpm	rotations per minute
RSV	Respiratory Syncitial Virus
S.E.	Socio-economic
TK	Tyrosine Kinase
TLR	Toll-Like Receptor
TMB	Tetramethylbenzidin
TNF-α	Tumor Necrosis Factor – α
UoN	University of Nairobi
VacA	Vacuolating Antigen
WGO	World Gastroenterology Organization

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ABSTRACT

Background: *Helicobacter pylori is* a curved, microaerophilic, gram negative bacterium that was isolated in 1983 from gastric biopsy specimens of patients with chronic gastritis. The bacterium colonizes the gastric mucosa of 20-80% of humans worldwide. In many developing countries, >50% of children and >70% of adults are *H. pylori* positive as compared with <15% of children and <40% of adults in most developed countries. Locally, studies done in asymptomatic children have shown a seroprevalence of between 45% and 51%. Infection is usually acquired in early childhood, is usually life-long unless eradicated and about 12-24% of those infected ultimately develop peptic ulcers and gastric malignancies. The mode of transmission from person to person is often feco-oral, and infection rate is highest in the developing world due to socio-economic factors. Risk factors significantly associated with *H. pylori* infection include: lack of clean water supply, poor sanitation, overcrowding, low maternal education level and low socio-economic status. Initial infection causes an acute superficial gastritis which may be followed by a chronic active gastritis, more severe forms of disease, such as peptic ulcer disease, atrophic gastritis, and gastric adenocarcinoma and mucosa associated lymphoid tissue (MALT) lymphoma in a minority of cases. Extra-gastric associations include: food allergy and chronic tonsillitis in children, hypertension and chronic prostatitis. Approximately 60-70% of *H.pylori* strains possess the Cytotoxin-associated gene A (CagA gene) and express the CagA protein, an oncoprotein and a highly immunogenic virulence factor that has been linked to gastric disease. Globally, few studies done on the prevalence of CagA positive strains in children have shown about 70% seropositivity. Eradication of *H. pylori* infection is of importance in prevention of the long-term severe forms of gastric disease. Studies have shown a high prevalence of *H. pylori* infection and acquisition early in life, thus, there is need to determine the local prevalence of the CagA positive *H. pylori* strains in the pediatric population in order to formulate strategies aimed at infection eradication.

Objective: To determine the prevalence of CagA positive *H. pylori* among asymptomatic children at Kenyatta National Hospital, Nairobi.

Study design: Cross-sectional descriptive study.

Study area: This study was conducted at Kenyatta National Hospital (KNH), a tertiary, referral and teaching hospital in Nairobi, Kenya, between October 2012 and May 2013. The study

participants were recruited from the general pediatric outpatient clinic and the pediatric wards. Laboratory analysis was done at the Immunology laboratory, University of Nairobi.

Study population: Children aged 2-13 years attending KNH, mainly drawn from Nairobi and its environs and presenting with non-GIT conditions.

Materials and methods: Socio-demographic information was collected by direct interview of the participants' parents/guardians (respondents). A study questionnaire was administered to all the participants to collect the socio-demographic information. Two (2) milliliters of venous blood samples were drawn from the subjects and serum separated. The serum samples were tested by ELISA for the anti-*H. pylori* IgG antibody. Those that tested positive were tested for anti-CagA IgG antibody by ELISA. The data collected was entered into SPSS v.18 software for analysis.

Results: A total of 175 children aged 2-13 years were enrolled on the study. 57.3% (101/175) were males with a male to female ratio of 1.4:1. The mean age was 7.5 years (±3.68) with a median (IQR) of eight years for both gender. The overall prevalence of *H. pylori* among the participants was 50.3% (88/175). CagA positive *H. pylori* prevalence among those who tested positive for *H. pylori* was 64.8% (57/88).

65% (113/175) of the participants lived in permanent houses with 66.9% (117/175) being urban dwellers. 70.9% (124/175) used tap water as their source of water. 70.3% (123/175) treated their drinking water, mainly by boiling. 32.6% (57/175) reported keeping domestic animals as pets with 29 keeping cats. As regards human waste disposal, 38.3% (67/175) used individual toilets and only1.1% (2/175) disposed of their human waste in the bush.

Socio-demographic data was provided by the parents/guardians (respondents) of the study participants, 84.7% (148/175) of whom were females. 74.9% (131/175) had up to three children. 46.3% (81/175) had secondary education while 19.4% (34/175) had tertiary education. 5 (9/175) had not attained any formal education. 81.7% (143/175) were in marriages, 45.1% (79/175) were in formal employment with 48.1% (38/79) earning a maximum monthly income of Ksh.10,000. 22.3% (39/175) were the sole bread winners in the family.

There was positive correlation between *H. pylori* seropositivity with increasing age ($p<0.001$). Education level of the parent/guardian significantly correlated with infection ($p = 0.030$), seropositivity being highest among parents/guardians of study participants' with informal and basic education. In addition, there was significant association between the participants' human waste disposal practices and *H. pylori* infection, seropositivity was highest in those who lacked human waste disposal facilities ($p=0.031$). Participants in the rural areas had a significantly higher seroprevalence of *H. pylori* than the urban and semi-urban dwellers ($p=0.043$). Regarding gender, males had a higher prevalence of *H. pylori*, (53.5%) compared to females (45.9%), however, females had a slightly higher prevalence of CagA seropositivity (67.6%) than males (63.0%). However both findings were not statistically significant. There was no significant correlation between socio-demographic characteristics of the study participants and CagA status.

Conclusions:

1. Prevalence of *H. pylori* was 50.3% and among the positive patients, prevalence of virulent strains was 64.8%.
2. *H. pylori* seropositivity increases with age and low social economic factors play a key role in contributing to the risk of *H. pylori* infection.
3. There was no significant correlation between socio-demographic characteristics of the study participants and *H. pylori* CagA status.

Recommendations:

H. pylori testing currently practiced in children should focus on the virulent strains only since half of the young population is infected by both virulent and non-virulent strains while only two thirds of infections are caused by the clinically significant virulent strains. Improvement of the socio-economic status of the population will reduce risk of infection acquisition.

1. INTRODUCTION

Helicobacter pylori is a Gram negative, spiral bacterium. In the 1870s, despite some reports on finding of spiral bacteria in gastric tissues, stress and diet were thought to be the only causes of peptic ulcers. In 1979, Warren found unidentified Campylobacter-like organisms in inflamed gastric tissues and in 1982, his co-worker, Marshall, isolated the bacteria. In 1985, Marshall performed self-inoculation by Campylobacter-like organisms and proved their ability to cause gastritis (1,2). Originally called *C. pyloridis* and then corrected to *C. pylori,* the bacteria were renamed again due to taxonomic data as *H. pylori* in a new genus Helicobacter. Since its discovery, various invasive and non-invasive diagnostic tests, susceptibility testing methods and treatment regimens for the infection have been developed and improved (3,4).

H. pylori particularly inhabit the gastric antrum and pylorus and cause a chronic low-level inflammation of the mucosal lining, and are strongly linked to the development of gastric and duodenal ulcers and gastric malignancies. The role of *H. pylori* in pathogenesis of gastric adenocarcinoma and MALT lymphoma has been proven (5). Moreover, *H. pylori* has been associated with several extra gastric diseases (6,7,8). The enormous genetic diversity of the bacteria and their numerous virulence factors has been revealed and genomes of many strains have been sequenced (9,10). Presently, chronic gastritis and peptic ulcers are treated as bacterial infections with antibiotics combined with acid inhibitors. The gift that Warren and Marshall's discovery has given to human medicine has been the consequent detection of the link between chronic bacterial infections and malignancy and the option to prevent a tumor by eradicating the associated microorganisms.

Improvement in eradication of *H. pylori* has been obtained by triple, quadruple, sequential or other regimens (3). However, at present, the treatment of *H. pylori* is not easy and still no vaccines are available for prevention (11). Infection is more prevalent in developing countries due to socio-economic factors and, despite the fact that infection is often asymptomatic in 80% of those infected; *H. pylori* infects more than half of the global population (12). About 20% eventually develop severe disease. Thus in the field of pathogenesis, epidemiology, diagnosis, prevention and treatment of the infection, there are still approaches to be optimized and many questions to be answered.

This study aims to determine the local seroprevalence of *H. pylori* among asymptomatic children and to correlate infectivity with known risk factors associated with infection acquisition. Since most of the pathology is attributable to the virulent strains of the bacteria, the study aims to determine the prevalence within the same population of the CagA positive strain in those who test positive for *H. pylori.*

2. LITERATURE REVIEW

2.1. Microbiology of *Helicobacter pylori*

Helicobacter pylori is a Gram negative, spiral bacterium that undergoes coccoid transformation under hostile conditions. It is about 3 micrometer long with a diameter of about 0.5 micrometer. It is a microaerophilic bacterium, that is, it requires oxygen but at lower concentration than is found in the atmosphere. It contains hydrogenase which oxidizes molecular hydrogen produced by intestinal bacteria to provide energy (13). It produces oxidase, catalase and urease enzymes and is capable of forming biofilms (14) and can convert from spiral to a viable coccoid form (15). All these factors enable the bacterium to adapt to the gastric mucosa and favor its survival. The coccoid form, though not culturable, (15) has been shown to adhere to gastric epithelial cells in vitro (16).

H. pylori possesses five major outer membrane protein families (5). The largest protein family includes known adhesins that aid the bacteria to adhere to epithelial cells. The other four families include porins, iron transporters, flagellum-associated proteins and proteins of unknown function. Like other typical Gram negative bacteria, the outer membrane of *H. pylori* consists of phospholipids and lipopolysaccharides (LPS). The O antigen of LPS may be fucosylated and mimic Lewis blood group Ags found on the gastric epithelium. The outer membrane also contains cholesterol glucosides, which are found in few other bacteria (5). *H. pylori* has 4-6 liphotrichous flagella; all gastric and enterohepatic Helicobacter species are highly motile due to the flagella (17). The characteristic sheathed flagellar filaments of *Helicobacter* are composed of two copolymerized flagellins, FlaA and FlaB (18).

2.2. Genome and Virulence Factors

H. pylori consist of a large diversity of strains with large genetic differences (19). The genomes of three including strains 26695 and NCTC11638 have been completely sequenced (9, 10). The genome of 26695 strains consists of a circular chromosome of 1,667,867 base pairs and has several genes of different sizes compared with strain NCTC11638. Several regions within the genome contain genes involved in various bacterial processes including DNA processing. Another region at the end of the sequence has been shown to contain a single contiguous 40kb-long *cag* pathogenicity island (a common gene sequence believed to be responsible for pathogenesis) that contains over 40 genes largely eliciting production of interleukin (IL)-8 by

gastric epithelial cells (9). The pathogenicity island (PAI) is usually absent from *H. pylori* strains isolated from asymptomatic human carriers (20).

The CagA gene codes for one of the major *H. pylori* virulence proteins, CagA protein. Bacterial strains that have the gene have been associated with an ability to cause ulcers (21). The CagA gene codes for a relatively long 1186 amino acid protein. CagA, an oncoprotein, alters cell-signaling pathways and induces morphological changes, chromosomal instability, cell proliferation and apoptosis, IL release and proto-oncogene activation. *H. pylori* spiral shape, urease, motility, LPS and outer membrane adhesins enable the establishment of infection. *H. pylori* genetic diversity and induced immunomodulation contribute to the infection chronicity. Vacuolating antigen, VacA, another virulence factor, causes vacuolation, pore formation, disruption of endo-lysosomal activity, apoptosis in gastric cells and immunomodulation.

Although infection outcomes have shown strong association with Cytotoxin-associated gene A (CagA) and Vacuolating antigen (vacA) status of the strain, the combined activity of all virulence factors appears to be crucial for the infection pathogenesis. By the complex and well-coordinated interplay of its virulence factors, *H. pylori* adapts to the changing gastric environment. Targeting the virulent strains in a country or region is important to better explain the clinical significance of some virulence factors and their interaction, to choose local diagnostic markers, to imply aggressive eradication strategies in the concerned patients and to provide new prophylactic and therapeutic agents and improved regimens to control the infection (22).

2.3. Pathophysiology

To colonize the stomach, *H. pylori* must survive the acidic pH of the lumen and burrow into the mucus on the gastric epithelium. The bacterium has flagella to enable movement through the mucoid lining (23). It produces adhesins which bind to membrane-associated glycolipids and help it adhere to epithelial cells (24). *H. pylori* produce large amounts of the enzyme urease, which breaks down urea (normally secreted into the stomach) into carbon dioxide and ammonia. The ammonia is converted to ammonium by taking a proton (H^+) from water, which leaves a hydroxyl ion, OH^-, which in turn reacts with carbon dioxide, producing bicarbonate, which neutralizes gastric acid. Thus the survival of the bacterium is dependent on urease. The ammonia

produced is toxic to epithelial cells, and, along with other products of *H. pylori* - including proteases, vacA and certain phospholipids – damages the cells (25).

Colonization of the stomach by *H. pylori* results in chronic gastritis. Ulceration results when the consequences of inflammation allow the acid and pepsin in the lumen to overwhelm the mechanisms that protect the gastric and duodenal mucosa from these caustic substances. The inflammatory response to the bacteria induces G cells in the antrum to secrete gastrin which stimulates the parietal cells in the corpus to secrete even more acid into the lumen. High levels of gastrin over a long time cause hyperplasia of the parietal cells, further escalating the amount of acid secreted (26). Gastric ulcers, in contrast, are often associated with normal or reduced gastric acid production, due to defective protective mechanisms. Chronic inflammation induced by the bacteria causes reduction of acid production and eventually, atrophy of the stomach lining, which may lead to gastric ulcer and increase the risk of malignancy (27).

About 50-70% of *H. pylori* strains carry the cagPAI (28). Patients infected with these strains have a stronger inflammatory response and are at a greater risk of developing peptic ulcers and malignancies than those infected with strains lacking the island. Following attachment of the bacteria gastric epithelial cells, these strains inject an inflammation-inducing peptidoglycan from their cell wall into the epithelial cells leading to stimulation and expression of cytokines that promote inflammation (29). The cagPAI-encoded protein CagA is also injected into the epithelial cells disrupting the cytoskeleton, adherence to adjacent cells, intercellular signaling, cell polarity and other cellular activities. It also activates the epidermal growth factor receptor, EGFR, a membrane protein with a tyrosine kinase (TK) domain. Activation of EGFR is associated with altered signal transduction and gene expression in host epithelial cells and may contribute to pathogenesis(20,21).

Two related mechanisms by which *H. pylori* could promote carcinogenesis are under investigation. One mechanism involves enhanced production of free radicals and an increased rate of host cell mutation. The other mechanism, the peri-genetic mechanism, involves enhancement of the transformed host cell phenotype by means of alterations in cell proteins, such as adhesion proteins. The bacterium induces inflammation and locally high levels of TNF-α and/or IL-6. These inflammation-associated signaling molecules can alter gastric epithelial cell adhesion and lead to the dispersion and migration of mutated epithelial cells without the need for

additional mutations in the tumor suppressor genes, such as genes that code for cell adhesion proteins (30).

2.4. Immunology of *H. pylori* infection

H. pylori infection induces both innate and acquired immunity. Bacterial virulence factors stimulate Toll-like receptors (TLR) to induce innate and adaptive cell mediated immune response. Balance of Th1/Th2 response is of great importance in host protection and in pathogenesis of *H. pylori* mediated diseases. A predominant activation of Th1 cells plays a key role in tissue damage. Th2 response appears to be protective against gastric inflammation. Cytotoxic activities of T cells are important for the outcome of *H. pylori* infection. Protection due to anti - *H. pylori* humoral, local and systemic immune responses is minimal. Furthermore, the Abs may promote colonization of gastric mucosa (22).

2.5. Epidemiology of *H. pylori* infection

H. pylori is one of the most common causes of chronic bacterial infections in humans; at least 50% of the world's population is infected by the bacterium(5,12). Infection is usually acquired in early childhood, is usually life-long unless eradicated and about 12-24% of those infected ultimately develop peptic ulcers and gastric malignancies (5). Actual infection rates vary from country to country, being higher in the developing countries, most likely due to socio-economic factors (31,32). Person to person transmission by either oral-oral or feco-oral route is the most likely mode of transmission (33). In developing countries, infection is usually acquired via environmental contamination, leading to higher infection prevalence in these regions (22). As a whole, the infection prevalence is still high in countries with poor socio-economic status (12,33). Risk factors significantly associated with *H. pylori* infection include: lack of clean water supply, poor sanitation, overcrowding, low maternal education level and low socio-economic status (34).

In many developing countries, >50% of children and >70% of adults are *H. pylori* positive as compared with <15% of children and <20-40% of adults in most developed countries (22). In an epidemiological study on seroprevalence of *H. pylori* in healthy children aged 1-18 years in South Germany, none of the children less than four years old were positive for anti-*H. pylori* specific IgG Abs. In the older subjects, seropositivity increased significantly and linearly with age ranging from 8.3% to 47.2% (35). A case control study to determine the risk factors associated with *H. pylori* infection in Butajira area of Ethiopia on 242 children aged 2-4 years

showed an overall seroprevalence of 48%. Several factors such as crowding, water, animals and sanitation strongly correlated to seropositivity (36). In Kampala, Uganda, a study on anti - *H. pylori* antibodies in 427 apparently healthy children aged 0-12 years showed an overall seroprevalence of 44.3%. By type of housing, those who lived in permanent houses had a prevalence of 38.5% compared with 48.6% for those living in semi-permanent houses (37).

In Northern Nigeria, a study to determine *H. pylori* seroprevalence in 268 subjects found 85% had IgG Abs to *H. pylori* and the majority, 82%, of these were children aged between 5-10 years (38). A study in Thailand on 159 children aged 0-15 years found 34.6% *H. pylori* seroprevalence and of the seropositive subjects, 96.4% were anti-CagA positive (39). 386 healthy children aged 1-15 years studied in Iran had an overall seroprevalence of 46.6%. The prevalence of CagA protein on those who were infected was 72.8% (40).

Locally, a few studies on *H. pylori* seroprevalence in children as well as in adults have been done. In 2003, Shmuely *et al* found a high prevalence level of *H. pylori* infection in both dyspeptic patients (71%) and asymptomatic controls (51%) (41). A more recent study based on stool antigen test in children less than three years of age attending well baby clinics in Nairobi, Kenya found a prevalence of 45.6% and a significant correlation between family income and *H. pylori* infection (42). However, there is no data on the local prevalence of virulent *H. pylori* strains which would form the basis for further studies and give further insight on prevention and treatment modalities as well as vaccine development.

Region	**Prevalence (%)**
United States and Canada	30
Mexico, Central and South America	70 – 90
Africa	70 – 90
Asia	70 – 80
Eastern Europe	70
Western Europe	30 – 50
Australia	20

Table 1: Global seroprevalence of *H. pylori* infection (43)

As illustrated in Table 1, there is a higher prevalence of *H. pylori* in developing countries in Africa, Asia, Central and South America compared with low seroprevalence in the developed world - United States, Canada, Europe and Australia (43).

2.6. *H. pylori* associated diseases

H. pylori plays a main role in the development of gastritis all over the world. In addition, it is well known that *H.pylori* is associated with many non-malignant and malignant GI and extra-gastric diseases. It is one of the commonest causes of PUD, gastric MALT lymphoma and gastric cancer (22). It is associated with a 1-2% lifetime risk of gastric cancer and <1% risk of gastric MALT lymphoma (20). Recent clinical data shows a relationship between *H. pylori* infection and GERD, NSAID-induced gastritis, and functional dyspepsia. Several local studies have shown a high prevalence of the bacteria in dyspeptic patients with gastritis ranging from 87.5% to 91% (44). One such study showed all cases of PUD had evidence of *H. pylori* infection while dyspeptic patients with normal endoscopic mucosal findings had *H. pylori* in 80.5% of cases (45). Eradication of *H. pylori* in patients with functional dyspepsia offers modest but significant benefit. An inverse relationship between infection and reflux esophagitis and Barrett esophagus has been confirmed. Eradication of *H. pylori* infection has been recommended for NSAID users and for those treated with anti-platelet therapy. The beneficial effects of eradication of the bacterium on MALT lymphoma and on gastric cancer have been proven (22).

On the other hand, an increasing amount of evidence for extra-gastric manifestation of *H. pylori* infection has been shown. Eradication of the infection has been shown to improve blood pressure, particularly diastolic blood pressure in patients with hypertension (46). Studies have shown a positive association between infection and food allergy in children (6), as well as a possible role of the bacteria in chronic prostatitis – possibly due to an immune response to the infection and presence of increased pro-inflammatory cytokines in seminal fluid (7). In addition, adenotonsillar tissue may constitute an extra-gastric reservoir for *H. pylori* in symptomatic children with chronic and recurrent adenotonsillitis (8).

2.7. Diagnosis of *H. pylori* infection

Diagnostic approaches to infection include direct identification of the bacterium by means of microscopy and/or culture from biopsy specimens of gastric mucosa and, indirectly, by serology and the urea breathe test. Sero-diagnostic evaluation of *H. pylori* infection, though less sensitive, is most commonly used in epidemiological studies (22).

2.8. Treatment of *H. pylori* associated diseases

Treatment of *H. pylori* infection remains a significant clinical problem despite the extensive research on the topic over the last 25 years. For its eradication, combined regimens of non-antibiotic (bismuth compounds and/or PPIs) and usually two or more antibiotics are used (3). A number of factors such as duration of treatment, choice of antibiotics, new drug combinations, improved patient compliance and novel agents may help to improve the eradication rates. However, bacterial resistance to the antibiotics, poor patient compliance and host genetic polymorphism can strongly reduce the success of eradication (22). Recent data have shown that the rates of eradication have decreased worldwide.

2.9. Prevention of *H. pylori* associated diseases

Eradication of virulent *H. pylori* strains in individuals prevents the development of gastric diseases. Rising antibiotic resistance increases the need for a prevention strategy for the bacteria (4). Research on different adjuvants, antigens, and routes of immunization are recently moving from animal to human trials (11). An effective vaccine is needed to improve the success of anti-*H. pylori* therapy. In recent years, many attempts, using various *H. pylori* antigens, many adjuvants and different routes of immunization have been made to create vaccines against the bacteria. Although promising, no effective and safe vaccine is currently available. CagA protein is highly immunogenic and therefore is a major antigen in vaccine development. Immunization with vaccines containing the *H. pylori* virulence factors: VacA, CagA, and neutrophil-activating protein (NAP), alone or in combination, have been shown to prevent experimental infection in animals. Specifically, an intra-muscular vaccine consisting of recombinant VacA, CagA, and NAP has shown 100% Ab response to 1 or 2 of the antigens and 86% Ab response to all the 3 antigens while producing mild local and systemic adverse effects (47).

3. RATIONALE

H. pylori infection is usually acquired early in childhood and is one of the commonest causes of chronic bacterial infections, with the highest prevalence in developing countries. Studies have shown virulent strains of the bacteria are responsible for the etiology of gastric pathology. CagA protein, a highly immunogenic oncoprotein is one of the major virulence factors possessed by some strains of the bacteria. Globally, over half of all *H. pylori* infections are due to CagA-positive strains. Most infections of both virulent and non-virulent bacteria are asymptomatic and serological studies of large populations have been utilized to establish the prevalence of the bacterial infection.

Since most of *H. pylori* infections are asymptomatic, studies in asymptomatic individuals present the actual prevalence of infection in the general population. Local data on the prevalence of *H. pylori* and possible risk factors associated with infection is available; however, no local studies on prevalence of virulent CagA positive *H. pylori* have been done.

The aim of this study was to determine the prevalence of virulent CagA positive *H. pylori* among asymptomatic children in the local population and to correlate infectivity with known risk factors associated with infection acquisition. Asymptomatic patients were screened for the presence of *H. pylori.* All those found positive with *H. pylori* were screened for the presence of CagA. Participants with positive CagA serology test were advised on confirmatory antigen testing and if indicated, appropriate therapy given by the clinician to those with active infection.

4. STUDY OBJECTIVES

The broad objective of the study was to determine the prevalence of CagA positive *H. pylori* among seropositive asymptomatic children attending Kenyatta National Hospital.

The specific objectives were:

1. To determine the prevalence of *H. pylori* among asymptomatic children attending Kenyatta National Hospital.
2. To determine the prevalence of CagA positive *H. pylori* in the asymptomatic children infected with *H. pylori.*

The secondary objectives were:

1. To correlate *H. pylori* seropositivity with the risk factors associated with *H. pylori* infection including age, crowding, low education level, unemployment and low monthly income, living in rural areas, housing type, lack of clean water supply, poor sanitation, domestic animals and human waste disposal facilities.
2. To correlate CagA positivity with the risk factors associated with *H. pylori* infection.

5. MATERIALS AND METHODS

5.1. Study Design

This was a cross-sectional descriptive study conducted between October 2012 and May 2013.

5.2. Study Area

The study was conducted at Kenyatta National Hospital (KNH), a tertiary, referral and teaching hospital in Nairobi, Kenya. The study participants were recruited from the general pediatric outpatient clinic and the pediatric wards at Kenyatta National Hospital. All specimens collected from the study participants were processed and analyzed at the Immunology laboratory, University of Nairobi.

5.3. Study Population

Participants were children aged 2-13 years drawn from KNH as out-patients or in-patients and presenting with non-gastro-intestinal disease. Those children aged above two years were enrolled into the study because of their ability to develop and express antibodies following infection.

5.4. Selection Criteria

Inclusion Criteria

Children aged 2-13 years who were on follow-up or admitted at KNH for non-GIT diseases and whose parents/guardians gave informed consent for their children to participate in the study were included in the study.

Exclusion Criteria

Children who were on treatment for chronic gastric disorders, that is, patients presenting with chronic symptoms (over two weeks) including nausea, vomiting, abdominal pain/discomfort, indigestion, diarrhea or diagnosed gastric diseases including chronic gastritis and peptic ulcer disease or previously treated for *H. pylori* - associated disorders and those whose parents/guardians declined to consent to participate in the study were excluded.

Sample Size Calculation

Fisher's formula for sample size calculation was used as shown below:

$$n = \frac{Z^2 \times P(1 - P)}{d^2} \quad (48)$$

n = sample size (n = 170) where:-

Z confidence level at 95% (Z = 1.96)

P prevalence of *H. pylori* infection in children at 45.6% (P = 0.46) (42).

d margin of error at 7.5%.* (d = 0.075)

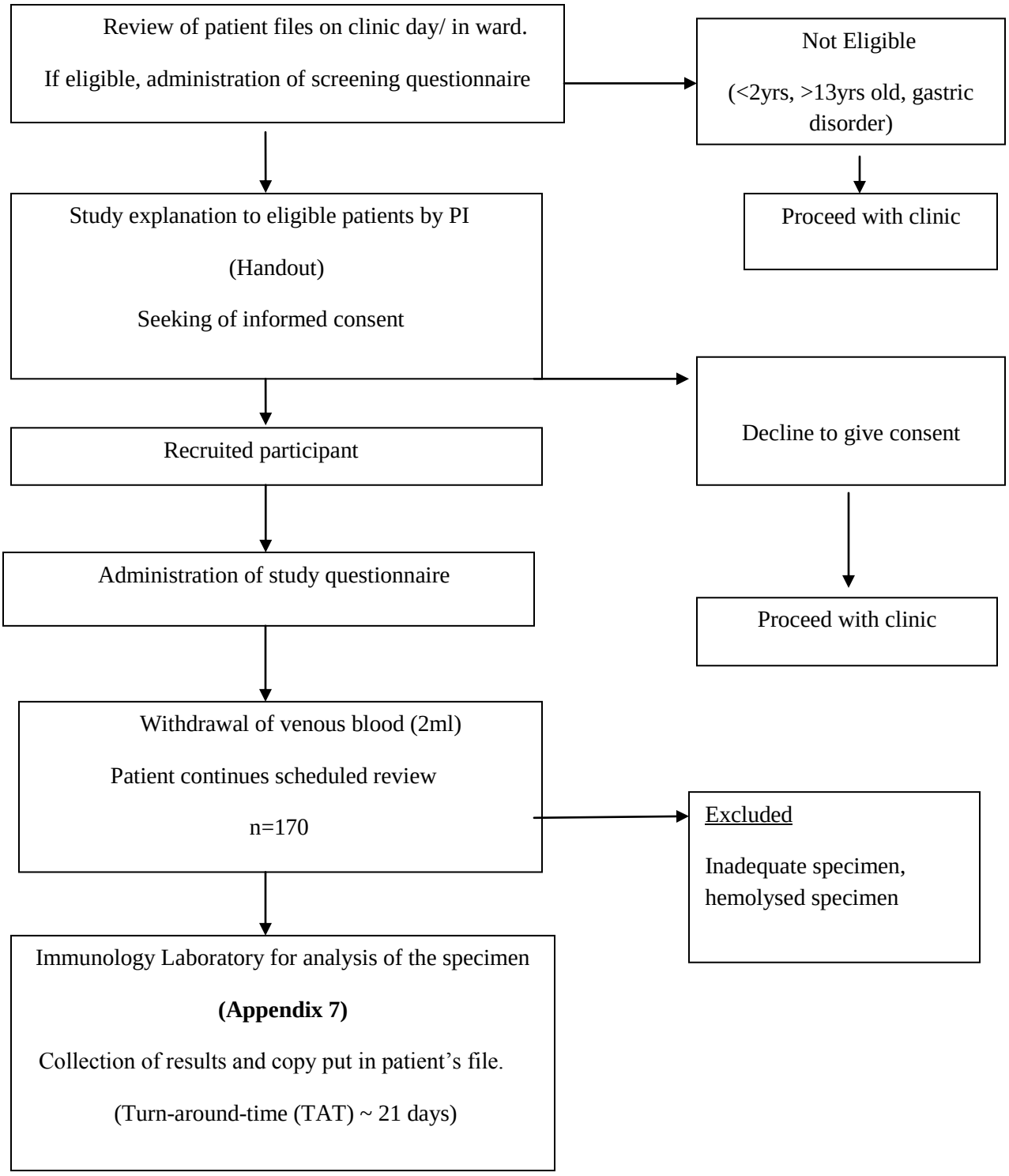
5.5. Recruitment and Screening

Recruitment of study participants was done at the general pediatric out-patient clinic (POPC) and pediatric wards by the PI assisted by a trained research assistant (registered clinical officer). Prior to commencement of the study, medical personnel at these sites were notified about the study via oral and written communication to facilitate the recruitment of patients.

During the clinic day all the files retrieved from the records office for patients scheduled for review on that particular day were obtained from the attending nurse and reviewed before the start of the clinic. Files for the in-patients were randomly obtained and reviewed at the nurses' stations in all the four pediatric wards to assess eligibility (inclusion and exclusion criteria) for recruitment into the study. All patients who met the inclusion criteria were recruited into the study until the desired sample size was achieved. The recruitment procedure is outlined in the flow chart (figure 1). The study participants were further screened by direct interview of their parents/guardians using a screening questionnaire (**Appendix 1**). To those who fulfilled the selection criteria, the PI explained the purpose of the study and the benefits/risks involved. Informed consent was sought from the parents/guardians and the older children assented to participate in the study. (**Appendices 2, 3, 4 & 5**).

*Use of `d' > 5% due to limitation of resources (48).

Figure 1: Data Collection Flow Chart



5.6. Data Collection Procedures

5.6.1. Questionnaire administration

After obtaining informed consent, the patient's as well as the parent's/guardian's demographic data were obtained by direct interview and review of the patient's file. Socio-demographic data associated with risk of acquiring infection was sought. Venous whole blood was then collected for the laboratory tests by the Principal Investigator and/or research assistant. The data collected from each patient was captured in a pre-designed structured study questionnaire (**Appendix 6**).

Specimen Collection and Handling

Verbal consent for specimen collection was sought. Two (2) milliliters of blood was drawn using standardized procedure by the PI and/or research assistant with no adverse events encountered. The 2 ml of blood was then put in well labeled plain vacutainer bottle, promptly transported to the Immunology laboratory for analysis, and let to clot undisturbed for 1 hour at room temperature. Serum was separated from the clotted blood by centrifugation at 3000 rpm, aliquoted into well labeled cryovials and frozen at -80°C for storage and batch testing. The precipitant was safely discarded as per the laboratory protocol.

5.6.2. Laboratory Testing

All frozen specimens were tested in batches. Before testing, frozen specimens were thawed on the bench or in a water bath at room temperature, and then inverted several times to ensure homogeneity before testing for *H. pylori* using ELISA method.

Anti-H. pylori IgG

This was performed in the Immunology laboratory using human *Helicobacter pylori* IgG ELISA (Human Gesellschaft für Biochemica und Diagnostica mbH, Germany). The ELISA has a sensitivity and specificity of 94.3% and 98.9%, respectively and has shown no cross reactivity with RSV IgG, Adenovirus IgG and Yersinia IgG **(Appendix 7)**. All reagents were brought to room temperature before use. The specimens were thawed and homogenized and where particulate matter was found, centrifugation of the specimen was performed. The thawed specimens were diluted in 1:100 with the dilution buffer and mixed thoroughly. 100µl of the specimen was carefully applied on the microtiter plate and all controls were run in duplicate. The microtiter plate was covered with adhesive strip and incubated for 60 minutes at room

temperature. Washing was done three times to remove unbound components. Conjugate was added, covered with adhesive strip and incubated for 30 minutes at room temperature. Washing three times was done before adding the substrate and incubating for 20 minutes at room temperature. Stop solution was added and carefully mixed to stop the reaction.

The automated HUMAREADER plate reader was checked before measuring the absorbance of controls and specimens at 450nm wavelength within half an hour. Results for patient specimens were obtained by comparison with a cut-off value (**Appendix 7**).

Anti - H. pylori CagA IgG

This was performed sequentially on the anti - *H. pylori* positive samples using anti - *H. pylori* CagA ELISA (IgG) (EUROIMMUN Medizinische Labordiagnostika AG, Germany). The test uses the same principle and procedure as the anti - *H. pylori* IgG ELISA, has a sensitivity and specificity of 98% and100%, respectively and has shown no cross reactivity **(Appendix 7).** Absorbance of calibrators, controls and test samples were read at 450nm wavelength using the Humareader plate reader. The results were evaluated semi-quantitatively by calculating the ratio of the absorbance of the control or patient sample over the absorbance of the standard and interpreted accordingly (**Appendix 7**).

5.6.3. Quality Assurance

Specimen collection

Proper identification, labeling and matching of collected specimens with the participants was done to ensure there was no mix-up. There was strict adherence to protocol and standard procedure (see Specimen Handling) during sample collection. Adequate blood volume was obtained, checked to ensure it was not hemolysed.

Specimen transport

Specimens were promptly transported to the Immunology laboratory in well labeled plain sterile vacutainer bottles on a rack to prevent breakage and placed on the bench top to clot undisturbed for one hour at room temperature.

Specimen separation

All specimens were centrifuged at 3000rpm and only separated serum was obtained by pipetting. The serum was put in sterile well labeled cryovials, tightly sealed and promptly frozen at -80° C. The temperature of the freezer was routinely monitored to ensure appropriate temperature was maintained.

Testing

Prior to testing, all specimens were retrieved from the freezer, thawed once, thoroughly mixed and testing was only done on homogenized specimens. Use of suitable unexpired reagents brought to room temperature before testing was employed. Precaution was taken to avoid mixing cryovial caps and contaminate the specimens and reagents. A record of the specimens and controls was made on a spread sheet prior to testing. Prompt pipetting in the same order, while ensuring there were no air bubbles was done to minimize reaction time differences between wells. One pipette tip was used for each well to minimize contamination. After each wash, the microtiter plate was tapped upside down on tissue paper to remove any remaining liquid in the wells. The plate reader was checked and blanked using substrate blank before measuring absorbance. All tests were performed in accordance with manufacturers' recommendations. Anti - *H. pylori* IgG test was performed in two batches on the same day and simultaneously to minimize within-day and between-day variation. Anti-CagA IgG test was performed in one batch. Internal quality control (IQC) materials: - positive control (PC) – low (PCL)/ high (PCH) and negative control (NC)) and calibrators (CC1, CC2 and CC3) were included during analysis; these were provided by the manufacturers and were analyzed with each run concurrent with the test samples. Absorbance was promptly measured on termination of the reaction with the stop solution ensuring there were no air bubbles prior to reading. Test results for both anti - *H. pylori* IgG and anti-CagA IgG were considered valid only if IQCs and calibrators were within the manufacturer's set cut-off values. To ensure reproducibility of the results, calibrators, controls and test samples for both anti - *H. pylori* IgG and anti-CagA IgG were run in duplicate. Standard deviation (SD) and coefficient of variation (CV) were calculated and were within acceptable limits.

Recording and archiving

Post-analytical data interpretation was done as per recommendations based on the cut-off values and ranges given by the manufacturers. Matching results generated by the reader were carefully entered on the spread sheet ensuring there was no mix-up. The research assistant and the PI counter-checked the results to ensure there were no transcription errors. Data entry and analysis was counter-checked by the statistician and the PI to ensure there were no errors.

In addition, the Humareader equipment is routinely serviced and calibrated and the Immunology laboratory participates in EQA activities.

5.7. Data Management

Data Analysis

Using the study questionnaire, variables were categorized into independent and dependent variables. Independent variables included demographic characteristics – age, gender and socio-economic characteristics – number of children, educational level, marital status, employment, housing, child care, water source and treatment, pets and waste disposal. Dependent variables included laboratory characteristics – anti - *H. pylori* IgG and anti-CagA IgG antibodies.

Socio-demographic data and test results were entered into MS Excel computer database, checked to ensure correct entries. Where indicated, the data was grouped and then imported into SPSS (v.18) statistical software for analysis. Descriptive statistics on socio-demographic characteristics was presented using percentages and frequencies for categorical or nominal data. Continuous data was presented using means, medians and standard deviation (SD). Tables and graphs were used to display the results.

Pearson's chi-square tests for independence were used to assess association between two nominal or categorical variables. The level of significance was set at 5% with p-values of ≤ 0.05 being considered statistically significant. Correlation analysis to assess for any linear association was done using Pearson correlation coefficient for the continuous variables, and considered significant at 5% level.

5.8. Ethical Considerations

Approval for the study was obtained from KNH/UON-Ethics and Research Committee prior to commencement of the study (**P496/12/2011, Appendix 8**). In addition, informed consent and

assent (where applicable) were obtained from the study participants. Samples were collected according to standard procedure. Patient details including results were kept confidential. The results, including recommendations for further tests in those who tested positive were provided to the clinic for use during the review of the participants during their scheduled return visits. The participants were not charged for the tests.

6. STUDY LIMITATIONS

The anti - *H. pylori* CagA ELISA kit is not available locally and therefore had to be sourced from outside the country. The local suppliers of laboratory equipment and reagents were unwilling to import the kit on our behalf mainly due to logistical reasons and therefore we had to source for it ourselves. This delayed and prolonged the duration of the study. There was limited reagent to carry out anti - *H. pylori* CagA ELISA test validation, being a new test in the Immunology laboratory. Confounders affected the interpretation of socio-demographic data especially noting that the participants were schooling and the information provided in regard to their homes may not have applied to their schools as well.

7. RESULTS

A total of 178 eligible participants met the inclusion criteria and were enrolled into the study. One participant was excluded due to mislabeling of the specimen cryovial, two had insufficient samples. This was recorded in the patients' files. The remaining 175 samples were analyzed in the laboratory.

7.1. Socio-demographic characteristics of the study population

57.3% of the study participants were males. The age groups were evenly distributed among the study participants, 2-5yrs = 61 (34.9%), 6 – 10yrs = 60 (34.3%) and 11 – 13yrs = 54 (30.9%). 77.7% of the study participants were under the care of their parents/guardians with 20% being under the care of house helps while 2.3% were cared for by their siblings, aunties, uncles or grandparents. 65% lived in permanent houses and 66.9% were urban and semi-urban dwellers.

70.9% used tap water as their source of water while 8% sourced their water from rivers. 70.3% treated their water mainly by boiling. However, sharing of water sources might have been possible especially in school going children. 32.6% reported keeping domestic animals as pets with 50.9% (29/57) mainly keeping cats. 38.3% reported using individual toilets and only 1.1% disposed their human waste in the bush.

The average number of siblings in the family was three with 74.9% having 1 - 2 siblings and 19.4% having 3 – 5 siblings. 65.7% (115/175) of the study participants' parents/guardians had attained post-primary education, 46.3% had secondary education while 19.4% had tertiary education. 5.1% had no formal education. 81.7% were from families in marriages, 45.1% were in formal employment with 48.1% (38/79) earning a maximum monthly income of Ksh.10,000. 22.3% of those in employment were the sole bread winners in the family.

7.2. Prevalence of H. pylori in asymptomatic participants

We analyzed serum from 175 participants for the presence of anti - *H. pylori* IgG antibodies and found a prevalence of 50.3%. Amongst those who tested positive for *H. pylori* antibodies, we found that prevalence of *H. pylori* increased with increasing age with a p-value of <0.001 indicating that age is determinant in acquisition of *H. pylori* (**Figure 2**).

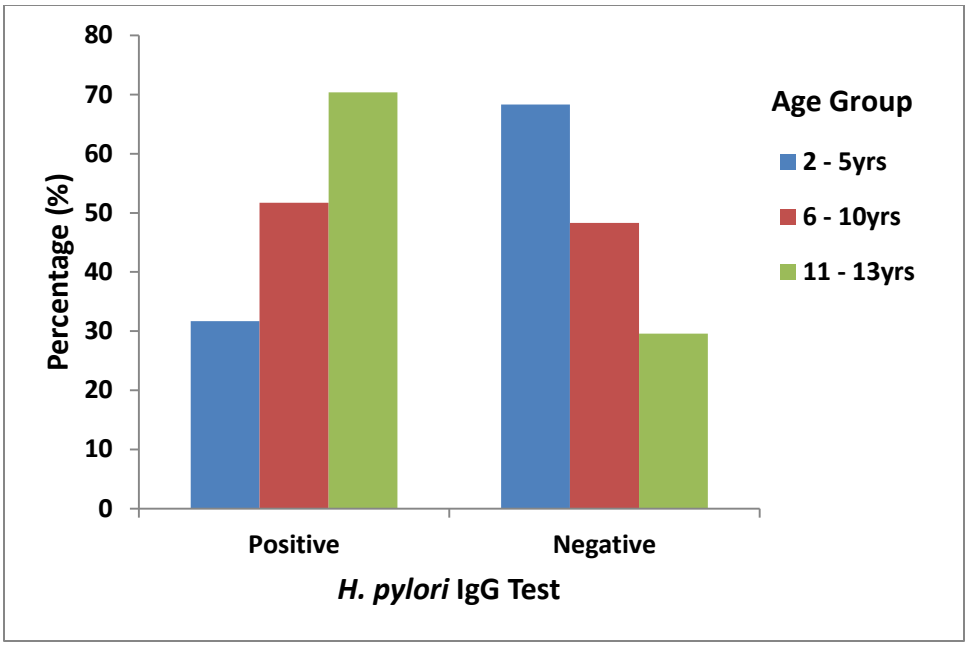


Figure 2: *H. pylori* IgG test results by age group, (n=175)

7.3. Association of key socio-demographic factors and prevalence of H. pylori

Socio-demographic characteristics	*H. pylori* Negative n (%)	*H. pylori* Positive n (%)	Total (n)	OR (95% CI)	p-value
1. Age (yr):					
2 – 5	42(68.3)	19(31.7)	61		
6 – 10	29(48.3)	31(51.7)	60	3.8	<0.001
11 – 13	16(29.6)	38(70.4)	54	(1.8-8.3)	
2. Gender:					
Male	47(46.5)	54(53.5)	101	1.4	0.326
Female	40(54.1)	34(45.9)	74	(0.7-2.5)	
3. No. of children:					
1 – 3	69(52.7)	62(47.3)	131		
4 – 6	16(47.1)	18(52.9)	34	2.9	0.130
6+	2(20.0)	8(80.0)	10	(0.8-12.2)	
4. Education level of parent/guardian:					
None	3(33.3)	6(66.7)	9		
Primary	18(35.3)	33(64.7)	51	2.8	0.030
Secondary	44(54.3)	37(45.7)	81	(0.9-9.7)	
Tertiary	22(64.7)	12(35.3)	34		
5. Marital status of parent/ guardian:					
Married	75(52.4)	68(47.6)	143	1.8	0.126
Single/Widowed/Divorced	12(37.5)	20(62.5)	32	(0.8-4.0)	

Socio-demographic characteristics	*H. pylori* Negative n (%)	Positive n (%)	Total (n)	OR (95% CI)	p-value
6. Employment:					
Yes	43(54.4)	36(45.6)	79	1.4	0.258
No	44(45.8)	52(54.2)	96	(0.8-2.6)	
7. Monthly income (Ksh.):					
0 – 9,999	19(50.0)	19(50.0)	38		
10,000 – 19,999	14(56.0)	11(44.0)	25	1.5	0.568
20,000+	10(62.5)	6(37.5)	16	(0.5-5.1)	
8. Housing type:					
Permanent	60(53.1)	53(46.9)	113	1.5	
Semi-permanent	27(43.5)	35(56.5)	62	(0.8-2.7)	0.227
9. Domicile:					
Rural	23(39.7)	35(60.3)	58	1.8	
Urban & Semi-urban	64(54.7)	53(45.3)	117	(0.9-3.5)	0.043
10. Water treatment:					
Yes	66(53.7)	57(46.3)	123	1.7	
No	21(40.4)	31(59.6)	52	(0.9-3.3)	0.108
11. Pets:					
Yes	28(49.1)	29(50.9)	57	1.0	
No	59(50.0)	59(50.0)	118	(0.6-2.0)	0.913
12. Human waste disposal:					
Community pit latrine	18(50.0)	18(50.0)	36		
Community toilet	13(34.2)	25(65.8)	38		
Individual toilet	42(62.7)	25(37.3)	67	3.9	0.031
Individual pit latrine	14(43.8)	18(56.2)	32	(0.9-49.2)	
Other - Bush	0(0.0)	2(100.0)	2		

Table 2: Correlation between socio-demographic characteristics and *H. pylori* infection

We cross tabulated prevalence of *H. pylori* among the study population with socio-demographic factors contributing to infection acquisition. Correlations were done using Pearson Chi-Square tests and statistical significance determined at 5% level. **Table 2** shows significant positive correlation existed between age of the patient and *H. pylori* infection ($p<0.001$), as well as education level of parent/guardian and *H. pylori* infection ($p = 0.030$). Males had a higher seroprevalence (53.5%) compared with females, (45.9%), however this was not statistically significant ($p=0.326$). Seropositivity increased with increase in the number of children per household, even though there was no statistical significance ($p=0.130$). Single parenthood, unemployment and low income level were associated with a higher prevalence; however, this was not statistically significant (p-values 0.126, 0.258 and 0.568, respectively).

There was significant association between place of domicile of the study participants and human waste disposal facilities with *H. pylori* infection ($p = 0.043$ and 0.031, respectively). Children

who lived in permanent houses as well as where drinking water was treated were found to have a lower prevalence of *H. pylori* seropositivity; however this was not statistically significant (p-values 0.227 and 0.108, respectively). There was no difference in prevalence between those who kept pets at home and those who did not (p=0.913).

7.4. Prevalence of CagA in H. pylori positive participants

Serum of 88 participants who tested positive for *H. pylori* antibodies was analyzed for CagA IgG antibodies and out of these, 64.8% tested positive. **Figure 3** shows prevalence of CagA IgG Ab in different age groups. The highest prevalence (77.4%) was found in the 6 – 10 year age group while the least (47.4%) was in the 2 – 5 year age group. We however found that age was not a significant determinant of CagA positivity (p=0.094).

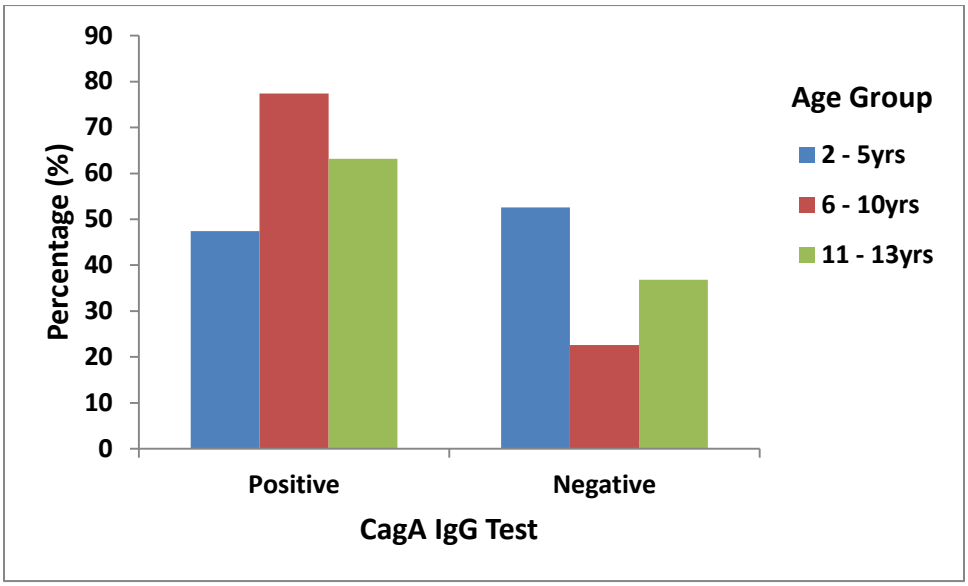


Figure 3: CagA IgG test results by age group, (n=88)

Comparison in terms of gender showed males had a higher prevalence of *H. pylori* positivity 53.5% compared with 45.9% in females. Females had a slightly higher prevalence of CagA antibodies than males (67.6% and 63.0%, respectively) however; there was no statistical significance in the differences (**Figure 4**).

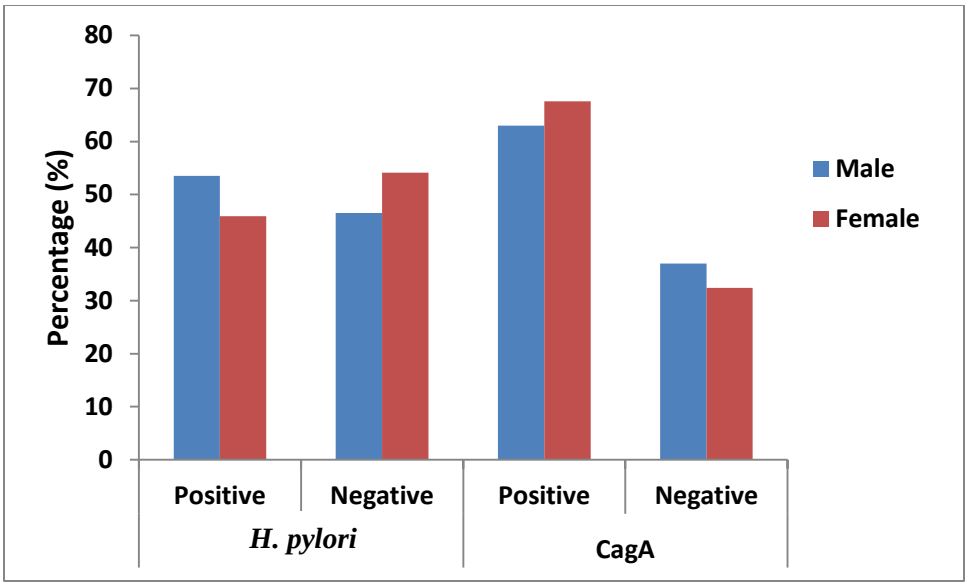


Figure 4: *H. pylori* and CagA IgG test results by gender

7.5. Association of key socio-demographic determinants with CagA positivity

Socio-demographic characteristics	CagA Negative n (%)	CagA Positive n (%)	Total (n)	OR (95% CI)	p-value
1. Age (yr):					
2 – 5	10 (52.6)	9 (47.4)	19		
6 – 10	7 (22.6)	24 (77.4)	31	2.9	0.094
11 – 13	14 (36.8)	24 (63.2)	38	(0.9-9.5)	
2. Gender:					
Male	20(37.0)	34(63.0)	54	1.2	0.654
Female	11(32.4)	23(67.6)	34	(0.5-3.0)	
3. Housing type:					
Permanent	18 (34.0)	35 (66.0)	53	0.9	
Semi-permanent	13 (37.1)	22 (62.9)	35	(0.4-2.1)	0.760
4. Domicile:					
Rural	15 (42.9)	20 (57.1)	35	0.6	
Urban & Semi-urban	16 (30.2)	37 (69.8)	53	(0.2-1.4)	0.223
5. Water treatment:					
Yes	22 (38.6)	35 (61.4)	57	1.5	
No	9 (29.0)	22 (71.0)	31	(0.6-4.0)	0.370
6. Pets:					
Yes	11 (37.9)	18 (62.1)	29	0.8	
No	20 (33.9)	39 (66.1)	59	(0.3-2.1)	0.710

Table 3: Correlation between socio-demographic characteristics and CagA positivity

We cross tabulated CagA positivity with socio-demographic factors associated with *H. pylori* infection. As shown in **Table 3**, there was no association between age, gender, housing type and

keeping of pets with CagA status (p-values 0.094, 0.654 and 0.760, respectively). Urban and semi-urban dwellers and those who treated their drinking water had a higher prevalence of CagA positive *H. pylori.* However, there was no statistical significance in this association (p-values 0.222 and 0.370, respectively).

8. DISCUSSION

This study evaluated 175 patients, most of who were males (57.7%). The age of the children ranged from two to thirteen years with a mean age of 7.5 years (±3.68). Several studies done in many developing countries have demonstrated a seroprevalence of *H. pylori* of 50-70% (22). The overall *H. pylori* prevalence in the study population was 50.3%. This compares well with a study on prevalence of *H. pylori* in children aged less than three years in Nairobi province where the overall prevalence was 45.6% (42). A study done in Kampala, Uganda compared well with this study. In that particular study on apparently healthy children aged one to twelve years, Hestvik *et al* (2010) found an overall seroprevalence of 44.3% (37). During analysis, 17 (10%) patients were borderline. This may have been due to the test characteristics (sensitivity of 94%) or the patients may not have developed sufficient antibodies to reach the cut-off value. Thus a repeat test was recommended for those patients.

As observed in other studies, there was a higher prevalence of *H. pylori* infection among males; however the difference was not statistically significant. In the Ugandan study, there was a significant difference in the prevalence of *H. pylori* by gender with a higher proportion of males having a prevalence of 49.8% as compared to 38.5% of females, (37). A similar finding was observed by Jafarzadeh *et al* (2007) in South East Iran, (40). However, there has been no inference made from these findings.

Infection with *H. pylori* is usually acquired in early childhood and the prevalence increases with age. This study shows significant correlation between age of child and *H. pylori* infection, children were infected early in life with slightly less than a third, 31%, of children aged two to five years being seropositive. In a 1997 study in South Germany on children aged one to eighteen years, seropositivity increased significantly and linearly with age ranging from 8.3% to 47.2% (35). This is due to increased exposure time to risk factors of infection as the child grows.

Three quarters of the study population had up to three children in the family which is relatively a small family size. Seropositivity increased with increased family size; however, there was no significant correlation between the number of children per family and *H. pylori* prevalence. The increasing infection rates with increase in the family size as established in this study may be attributable to crowding within the family. Large families may also be economically strained, thus predisposing the children to risk of infections. Overcrowding in homes and schools has been established as a risk factor for infection (34). Lindkvist *et al* (1998) in a study on *H. pylori* infection in children aged two to four years in rural Ethiopia found overall seroprevalence of 48%. In that study it was established that overcrowding, lack of clean water, domestic animal keeping and poor sanitation significantly correlated with seropositivity (36).

The respondents are fairly educated, two thirds of whom have acquired secondary and tertiary education. There was significant association between the level of education and *H. pylori* prevalence, there was a higher positivity, 67%, in children of respondents who had not attained formal education compared with 35% in children of those who had attained tertiary education. Langat *et al* (2006) found significant correlation between family income and *H. pylori* infection (42). From this study, the population's economic status is relatively average, other than education level, nearly half of the participants are in employment or in marriages where the spouses are in a position to provide basic needs, good hygienic standards, clean water and a clean environment to their children.

In correlating *H. pylori* infection with the type of housing that children lived in, the Ugandan study found a high likelihood of infection in those children who lived in semi-permanent houses (prevalence of 48.6%) compared with a prevalence of 38.5% for those living in permanent houses (37). In comparison with this study, 46.9% of those living in permanent houses were seropositive compared with 56.5% seropositivity in those living in semi-permanent houses. The type of housing has a bearing on the socio-economic status of the family and relates with availability of clean, safe water and human waste disposal facilities in Kenya.

Infection rates were significantly higher in the rural population than in the urban and semi-urban population. This may be attributed to lack of clean, safe water, poor hygienic standards and lack of proper human waste disposal facilities in the rural areas. Lindkvist *et al* in 1998 found a higher prevalence of *H. pylori* infection in children living in the rural areas (36). This study

however contrasts a similar study by Vivatvakin *et al* (2004) in Thailand who found a higher prevalence in the urban areas compared to the rural areas. The finding was attributed to the local water supply (39). In Kenya, tap water treatment by local authorities has been in practice and this may explain the lower prevalence of *H. pylori* in the urban and semi-urban areas supplied with treated tap water.

Studies on the mode of transmission of *H. pylori* have established that person to person transmission by either oral-oral or feco-oral route is the most likely (33), thus infection rates are likely to be high in areas which lack human waste disposal facilities or where individuals are sharing these facilities. There was a higher prevalence, 59.6%, in those respondents that drank untreated water regardless of its source. However this finding was not statistically significant. Although the principal reservoir for *H. pylori* infection appears to be humans, *H. pylori* has been isolated from domestic animals, especially cats and thus these could act as reservoirs of the bacteria (33). However, in this study there was no difference in prevalence between those who kept pets at home and those who did not. In the seropositive group, there was no difference in terms of the animals kept as pets and *H. pylori* infectivity. Significant correlation between *H. pylori* infection and human waste disposal facilities explains the association between high prevalence and poor sanitation. However, since these children are schooling, confounders like sharing meals, latrines and toilets and drinking untreated water both in the neighborhood and schools may have impacted on the outcome of this study.

About 50 – 70% of *H. pylori* strains carry the cagPAI, the gene that codes for the CagA protein (28). Infection outcomes have shown strong association with cagA, one of the major virulence factors. In this particular study, 65% of those infected with *H. pylori* were infected with the CagA positive strains. Three participants had borderline results and due to unavailability of CagA ELISA test kits locally, a recommendation for *H. pylori* antigen testing to establish presence of active infection and treatment if indicated was made. These findings compare with the global prevalence of CagA positive strains (50 – 70%), however, other studies in the Middle East and Asia found higher prevalence, suggestive of regional differences in CagA positive *H. pylori* prevalence (39,40).

The prevalence of CagA positive *H. pylori* was lowest in the 2–5 year age group (47.4%) and highest in the 6–10 year age group (77.4%). The 11–13 year age group had a lower prevalence of

63.2%. Though the differences were not statistically significant, the younger children had a much lower seropositivity. Jafarzadeh *et al* in 2007 observed that seroprevalence of CagA antibodies increased significantly with age from 59.6% in the 1-5 year age group to 79.5% in the 11-15 year age group (40). This association between infection and age has been observed on the *H. pylori* infection in general. In this study, CagA positive *H. pylori* prevalence was only marginally higher in females (67.6% versus 63%). This contrasts with the Iranian study where it was observed males had a significantly higher CagA prevalence (78.4% versus 66.3%) (40).

From this study, there was a higher prevalence of CagA positive *H. pylori* in urban and semi-urban areas than in the rural areas (69.8% versus 57.1%); however, this finding was not statistically significant to conclude that urban and semi-urban areas have a higher prevalence of virulent *H. pylori* strains. Other socio-demographic characteristics for instance, housing type, water treatment and keeping of pets in the home showed no significant association with CagA positive *H. pylori* infection. However, individuals living in semi-permanent houses, those who did not treat their drinking water and those who kept pets within their homes tended to have higher prevalence. This association was observed to apply to all *H. pylori* strains irrespective of their virulence status.

9. CONCLUSIONS

1. Prevalence of *H. pylori* was 50.3% and among the positive patients, prevalence of the virulent strains was 64.8%.
2. *H. pylori* seropositivity increases with age and low social economic factors play a key role in contributing to the risk of *H. pylori* infection.
3. There was no significant correlation between socio-demographic characteristics of the study participants and *H. pylori* CagA status.

10. RECOMMENDATIONS

H. pylori testing currently practiced in children should focus on the virulent strains only since half of the young population is infected by both virulent and non-virulent strains while only two thirds of infections are caused by the clinically significant virulent strains. Improvement of the socio-economic status of the population will reduce risk of infection acquisition.

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APPENDICES

APPENDIX 1: SCREENING QUESTIONNAIRE

Prevalence of CagA positive *H. pylori* among asymptomatic children at KNH

Age: 2–13 years (circle one) Yes No

Gender: 1. Male ….. 2. Female …..

Diagnosis:	**YES**	**NO**
a) Chronic GIT symptoms/disease*		
b) On medication for GIT conditions		
c) Previous treatment for *H. pylori*		
Eligibility		
1. Are you willing to participate in this study?		

If answers on age and willingness to participate in the study are YES, and answers to questions on diagnosis are NO, recruit and issue Study No.

*Chronic gastric disorders presenting with nausea, vomiting, abdominal pain and discomfort, indigestion, diarrhea and diagnosed gastric diseases including chronic gastritis and peptic ulcer disease.

FOR OFFICAL USE:

RECRUITED (encircle) YES NO

STUDY NUMBER: | | | |

Once recruited, proceed to Consent Explanation (Appendix 4).

APPENDIX 2: CONSENT EXPLANATION

Prevalence of CagA positive *H. pylori* among asymptomatic children at KNH

Introduction and objectives of the study:

I am Dr. Sava S, a master's student in Human Pathology at the University of Nairobi. I am conducting a study to determine the number of children attending Kenyatta National Hospital, and infected with a small organism known as *H. pylori* but which they are not suffering from or having symptoms, as a percentage of the sample size. *H. pylori* is a bacterium that commonly infects and lives in the stomach. Most of the infections associated with *H. pylori* do not show symptoms, occur in early childhood through eating food contaminated by stool containing the bacteria and unless treated, infection lasts for a long time. CagA is a protein possessed by some of the *H. pylori* bacteria and those that possess the protein are more likely to cause ulcers or wounds in the stomach and intestines in adulthood, and in a few patients, stomach cancers. Identification and treatment of the bacterial infection by use of medicines that kill the bacteria plays an important role in prevention of these late medical conditions.

The study aims to:

i. Determine the number of children aged 2-13 years attending Kenyatta National Hospital that are infected by the bacteria *H. pylori* but without symptoms, expressed as a percentage of the sample size.

ii. Provide a basis for further research on the factors that increase chances of getting infected with *H. pylori*, its long-term complications and beneficial effects of its eradication.

Benefits and risks of the study to your child:

- By your child participating, he/she will benefit by having the laboratory test done on him/her at no additional cost.

You can come for the results of your child's *H. pylori* infection status after 3 weeks at the department of Human Pathology Room 1 Monday to Friday between 9am and 4.30pm.

A copy of the results will be put in the child's file. This will be reviewed by the attending doctor during your child's scheduled visit to the clinic and he/she will advice you on what will be done with the results for your child.

Risk: 2 ml of blood will be drawn from the front part of the forearm at the elbow. The prick will be painful and a swelling at the site from where blood was drawn may form, but this is not common. Should this happen, you may contact me and I will attend to your child and provide the appropriate medical care.

Voluntary Participation:

Participation is voluntary and you can withdraw your child's participation at any time before taking of the blood sample or if the sample has already been taken, in which case it will be discarded and not used for further study. Withdrawal will be through a written notification and the quality of health care management of your child's medical condition in this institution and any other medical facility will not be interfered with.

Confidentiality:

Any information given to us will remain confidential and the privacy of your child will be respected. The sample will only bear the study number and it is I as a doctor who will connect the name to the results to be able to give your child's results.

You may ask me questions regarding the study now or at any time during the study. If you have any question relating to the study, kindly Contact:

1. Dr. S. Sava (PI), Registrar, Department of Human Pathology, University of Nairobi, P.O. Box 19676-00202, Nairobi. Tel- 0722-414528 E-mail: savasolo@yahoo.com
2. My supervisors: Prof. C. Kigondu 0733-730796, Dr. C. Gontier 0716-199138 and Dr. W. Waweru 0722-759523.
3. The Secretary to the KNH/UON-Ethics and Research Committee: Prof. A. Guantai KNH Tel No. 020-726300-9.

Maelezo kuhusu Utafiti:

Jina langu ni Dr. Sava S., mwanafunzi wa Chuo Kikuu cha Nairobi. Ninahusika katika utafiti wa kuchunguza kiwango cha maambukizi ya viini au bacteria inayoitwa *H. pylori* katika watoto wasiokuwa na dalili za ugonjwa unaosababishwa na viini hivyo. Maambukizi ya tumbo yasipotibiwa kwa dawa za kuuwa hivyo viini husababisha vidonda katika tumbo wakati mtoto anapokuwa mtu mzima na kwa wachache huweza kusababisha saratani au cancer ya tumbo.

Utafiti wangu utaweza kuonyesha kiwango cha maambukizi katika watoto wanaokuja kutibiwa hospitali ya Kenyatta. Pia nitachunguza uwezekano wa sababu zinazofanya mtu kuwa katika hatari ya kupata maambukizi ya viini hivyo.

Utafiti huu hautakugharimu pesa zozote na utaweza kupata matokeo baada ya wiki tatu ukija department ya Human Pathology chumba cha kwanza siku yoyote kuanzia jumatatu hadi ijumaa saa tatu hadi saa kumi na nusu. Matokeo pia yatatumwa kwa faili ya mtoto kwa kliniki na daktari atakaye muona atamuelezea jinsi mtoto atakavyo hudumiwa kulingana na matokeo.

Damu kiwango cha mililita mbili itatolewa kutoka sehemu ya mbele ya mkono au mguu kiunoni na itakuwa na uchungu. Ingawa itatolewa kwa maakini, uvimbe mdogo usiodumu kwa mda mrefu unaweza ukatokea mahali sindano itadunga.

Kushiriki katika utafiti huu utakuwa wa kujitolea na unaweza kumuondoa mtoto wako wakati wowote bila kupoteza haki yake ya kuhudumiwa vilivyo katika hospitali.

Maelezo yoyote utakayotoa yatakuwa siri na yatatumika katika utafiti huu pekee.

Ukiwa na maswali yoyote unaweza kuuliza wakati wowote kwa kutumia nambari zifuatazo:

1. Dr. Sava S. (mtafiti) 0722-414528 Barua pepe: savasolo@yahoo.com
2. Wasimamizi wangu: Dr. C. Kigondu 0733-730796, Dr. C. Gontier 0716-199138 au Dr. W. Waweru 0722-759523.
3. Prof. A. Guantai, Katibu wa KNH/UON-ERC, KNH namba ya simu 020-726300-9.

APPENDIX 3: CONSENT FORM

Prevalence of CagA positive *H. pylori* among asymptomatic children at KNH

I……
…….after reading and being explained to on the study purpose by Dr. Sava S. S., do hereby give informed consent for my child to participate in the study on SEROEPIDEMIOLOGY OF H. PYLORI AND CAGA PROTEIN IN ASYMPTOMATIC CHILDREN AT KNH.

Signed/Thumbprint by parent/Guardian:…… Date: ……..

Signed by the PI ……...

Witness: …… Date: ……

APPENDIX 4: GUARDIAN VERIFICATION FORM (to completed by the legal guardian)

I …….. affirm that I am the legal guardian of the study subject. I have lived with him/her for …… year(s). My relationship with him/her is by virtue of ……. Blood/Adoption/Other (specify)…… I take sole responsibility of the child.

……..	……
Signature of Guardian	Date
…….	……
Signature of Witness	Date
……	……
Signature of PI	Date

APPENDIX 5: ASSENT FORM (to be completed by children aged 7 years and above) (49)

Prevalence of CagA positive *H. pylori* among asymptomatic children at KNH

I have been explained to by the Principal Investigator on the nature and procedure of the study in a language I clearly understand and my parent and/or legal guardian agrees that I can participate in this study. My parent and/or legal guardian has been given a signed and dated copy of this assent form.

I have asked any questions I have about the study and my questions have been answered.

I identify the person with me as my (tick one): (a) Mother (b) Father (c) Auntie (d) Uncle (e) Grandparent (f) Other (specify)……

I recognize _____ as my legal guardian.

_____ _____

Child's Name Date

_____ _____

Signature of Parent/Legal Guardian Date

_____ _____

Signature of PI Date

_____

Witness name

_____ _____

Witness signature Date

APPENDIX 6: STUDY QUESTIONNAIRE

Prevalence of CagA positive *H. pylori* among asymptomatic children at KNH

Date ☐☐☐

dd / mm / yy

A. Demographic data (Child)

Name …… ☐☐ Age (Years):

Gender: 1. Male ☐ 2. Female ☐

Study No. ☐☐☐

Hospital No. ☐☐☐☐☐☐☐☐☐

Return date for results: ☐☐☐ Clinic …….

B. Demographic data (Parent/Guardian)

1. How many children do you have?

☐☐

2. What is your level of education? (tick one)

0 None

1 Primary

2 Secondary

3 Tertiary

3. What is your marital status?

0 Married 1 Single

4. Are you employed? 0 Yes 1 No

If yes, what is your approximate total monthly income? Ksh…….

If yes, are you the sole bread winner in the family? 0 Yes 1 No

5. Who takes care of your child on most occasions? 0 Parent 1 House-help

2 Day-care 3 Other (specify)……..

6. What type of housing do you live in?

0 Permanent

1 Semi-permanent

7. Place of domicile….. 0 Rural 1 Urban/Semi-urban

8. What is the source of the water you use for drinking/cooking?

0 River 1 Tap 2 Combination (tap/river) 3 Other (specify)……..

9. Do you boil/treat your drinking water? 0 Yes 1 No

10. Do you keep any pets in the house? 0 Yes 1 No

If yes, which ones? .....

11. How do you dispose of your human waste?

0 Communal pit latrine 1 Communal toilet 4 Other (specify)

2 Individual toilet 3 Individual pit latrine

Definitions

- Risk factors associated with *H. pylori* infection include: age, crowding, education level, unemployment and low monthly income, living in rural areas, housing type, lack of clean water supply, poor sanitation, domestic animals and human waste disposal facilities.
- Permanent houses were defined as either brick/stone-walled and/or with iron sheet or tile roofing. Semi-permanent houses were defined as having mud, wood or iron sheet walls and/or grass thatched.
- Urban and semi-urban areas were defined as the relatively populated areas within the city/towns and the surrounding residential estates serviced by the city/town/municipal councils in terms of water, sewerage and waste collection services. Rural areas were defined as those sparsely populated areas away from the urban areas that were not provided with the above services.

Laboratory Results

Study No…….	**RESULTS**		
	NEGATIVE	**POSITIVE**	**BORDERLINE**
H. pylori			
CagA Protein			

Comment:……
……..
.....

Signed…….. Date……..

Dr. Sava S. S.

APPENDIX 7: TEST PRINCIPLES AND PROCEDURES

H. PYLORI IgG

ELISA for the Detection of anti-IgG Antibodies to Helicobacter pylori in Human Serum

Package Size

REF	51222	96 Tests	Complete Test Kit
IVD			

Intended Use

Helicobacter pylori (H. pylori) is a spiral gram-negative bacterium which colonises the human gastric mucosa.
The bacterium can cause various gastrointestinal diseases.
More than 90% of patients with chronic gastritis (Type B) / ulcer patients and gastric carcinoma patients are infected with *Helicobacter pylori*. The early detection and treatment can eradicate the bacterium.
The detection of IgG and IgA antibodies is preferred as an initial test in the case of a chronic infection.

Principle - Sandwich EIA -

The HUMAN Helicobacter pylori IgG ELISA is based on the sandwich ELISA technique. The microtiter strip wells as a solid phase are coated with cell culture derived *H. pylori* antigens (H.pylori-Ag). In the first incubation step corresponding specific antibodies (anti-H.pylori-IgG-Ab) present in patient specimens or controls bind to the antigens at the solid phase. At the end of the incubation unbound components are washed out. For the second incubation step anti-IgG conjugate (anti-human IgG antibodies, peroxidase conjugated) is added which binds specifically to IgG class antibodies resulting in the formation of typical immunocomplexes. After a second washing step to remove excess conjugate, TMB/Substrate is added (Step 3). A blue colour develops changing to yellow after stopping the reaction. The intensity of the colour is directly proportional to the anti-*H. pylori* - IgG-Ab concentration in the specimen.
The absorbance of controls and specimen is determined by using ELISA microplate readers or automated ELISA systems (like HUMAN's HUMAREADER or ELISYS line). Results for patient samples are obtained either by comparison with a cut-off value or by expression in units per ml.

Reagents and Contents

MIC	12	**Microtiter Strips** (in 1 strip holder) 8-well snap-off strips coated with H.pylori antigen (cell culture derived)	
NC	2 ml	**H.pylori IgG Negative Control** (green cap), ready for use, human	< 9 U/ml
CC	2 ml	**H.pylori IgG cut-off Control** (yellow cap), ready to use, human	10 U/ml
PCL	2 ml	**H.pylori IgG Low Positive Control** (blue cap), ready to use, human	25 U/ml
PSI	2 ml	**H.pylori IgG High Positive Control** (red cap), ready for use, human	150 U/ml
DIL	2x50 ml	**Dilution Buffer** Ready for use, coloured blue PBS/BSA Buffer with Tween 20	pH 7.0 – 7.5
CON	13ml	**Anti-IgG Conjugate** ready for use, coloured red Anti-human IgG (rabbit), peroxidase-conjugated	
WS	2x50ml	**Washing Solution** Concentrate PBS Buffer with Tween 20	pH 6.5 – 7.0
SUB	13 ml	**Substrate Reagent** (brown bottle) 3,3', 5,5'-tetramethylbenzidin (TMB)	
STOP	13 ml	**Stop Solution** Sulphuric acid, ready for use	0.5 mol/l
	2	**Adhesive Strips**	

Preservatives: Total concentration < 0.1%

Safety Notes

Do not swallow the reagents. Avoid contact with eyes, skin and mucous membranes. All patient specimens and controls should be handled as potentially infectious. The controls have been checked on donor level for HCV and HIV-1/2 antibodies and HBsAg and found negative. Wear protective clothing and disposable gloves according to Good Laboratory Practices.
All materials contaminated with patient specimens or controls should be inactivated by validated procedures (autoclaving or chemical treatment) in accordance with applicable regulations.
STOP, SUB could irritate eyes, skin and mucous membranes. Upon contact, rinse thoroughly with copious amounts of water and consult a doctor.

Stability

The reagents are stable up to the stated expiry dates on the individual labels when stored at 2...8°C.

MIC
- sealed in an aluminium bag with a desiccant.
- must be at room temperature before opening.
- unused: return with the desiccant to the zip-lock bag and store in this way at 2...8°C.

Do not touch the upper rim or the bottom of the wells with fingers.

Reagent Preparation

Bring all reagents to room temperature (15...25°C) before use.
Reagents not in use should always be stored at 2...8°C.

Working Wash Solution WASH
- dilute WS 1+9 with fresh deionised water, e.g. 30 ml WS + 270 ml = 300 ml.
- Stability: **4 weeks at 2...8°C.**

Specimen

Serum
Do not use highly lipemic or hemolysed specimens.
Specimens may be stored for 7 days at 2...8°C or longer at -20°C. **Freeze and thaw once only.** Thawed specimen must be homogenised. Eliminate particulate matter by centrifugation or filtration.

Procedure

Follow the procedure exactly as described.

Procedural Notes

P1: Do not mix caps of vials (risk of contamination). Do not use reagents after their expiration date.
P2: Do not use reagents that could be contaminated or look or smell different than usual.
P3: Record specimens and controls carefully on the spread sheet supplied with the kit.
P4: MIC - select the required number of Microtiter Strips.
P5: **Run duplicates** for controls and specimens. Pipette controls and specimens **on the bottom** in the microwells.
P6: **Always add reagents in the same order** and timing to **minimise reaction time differences between wells.** This is important for reproducible results. Pipetting of specimens should not exceed 5 minutes. Otherwise pipette the calibration curve in the indicated positions at half way time of the series. If more than 1 plate is used, repeat the dose response curve for each plate.
P7: Avoid/remove air bubbles prior to incubations and reading of absorbance.
P8: SUB – incubate in the dark. SUB initiates a kinetic reaction, which is terminated by STOP.

Wash Procedure

The wash procedure is critical. Insufficient washing will result in poor precision or falsely high absorbance.

W1: Remove Adhesive Strips, aspirate off the contents into 5% sodium hypochlorite solution and add [WASH] to each well, aspirate off after 30 sec. soak time and repeat washing twice.

W2: In case of automatic washers fill and prime with [WASH]. Subsequently wash strips 3 times. Ensure the washer fills all wells completely and aspirates off efficiently after 30 sec. (remaining liquid: < 15 µl).

W3: After washing, remove remaining liquid by tapping the plate upside down on tissue peper.

Pipetting Scheme

Reagents and specimens should be at room temperature before use.				
Sample Preparation: Dilute the patient's sera 1 + 100 with [DIL], e.g. 10 µl serum + 1 ml [DIL], mix thoroughly. Controls are ready for use.				
Step 1	Well [µl]			
	A1 Blank	B1/C1 [NC]	D1...A2 [CC]	B2... Sample
[NC] in duplicate	--	100	--	--
[CC] in duplicate	--	--	100	--
[PCL] in duplicate	--	--	100	--
[PCH] in duplicate	--	--	--	100
Diluted samples	--	--	--	--
[MIC] cover with Adhesive Strips				
Incubate 60 min. at 17...25°C				
Wash 3 times as described (see W1 - W3)				
[WASH]	300	300	300	300
Step 2				
[CON]	--	100	100	100
[MIC] cover with Adhesive Strips				
Incubate 30 min. at 17...25°C				
Wash 3 times as described (see W1 - W3)				
[WASH]	300	300	300	300
Step 3				
[SUB]	100	100	100	100
Incubate 20 min. at 17...25°C (see P8)				
[STOP]	100	100	100	100
Mix carefully				
Zero the ELISA microtiter plate reader (HUMAREADER) using the substrate blank in well A1.				
Measure the absorbance at 450 nm as soon as possible or within 30 min. after terminating of the reaction, using a reference wavelength of 630-690 nm (if available).				

Interpretation of Results

The mean absorbance values are calculated after subtraction of the substrate blank value (well A1).

The test run may be considered valid provided that the following criteria are met:

1. [NC] ≤ 0.150
2. [CC] ≥ 0.200
3. [PCL] ≥ 0.450
4. [PCH] ≥ 0.750

Samples are considered **POSITIVE** if the absorbance value is more than 20% above the cut-off.
Samples with an absorbance value of 20% above or below the cut-off should not be considered as clearly positive or negative – **GREY ZONE**.
Samples are considered **NEGATIVE** if the absorbance value is lower than 20% below the cut-off.

A_{450} (patient) ≥ cut-off + 20%: anti-H.pylori-IgG-Ab-positive
A_{450} (patient) < cut-off - 20%: anti-H.pylori-IgG-Ab-negative

Anti-H. pylori IgG Antibody Units

The results found in patient's sera can be expressed in units per ml (U/ml):
The values for controls in Units are printed on the labels of the vials. For the quantitative evaluation the absorbance of the controls are graphically drawn against their concentrations. From the resulting reference curve the concentration values for each patient sample can be extracted in relation to their absorption.

Performance Characteristics

Typical performance data can be found in the Verification Report, accessible via
www.human.de/data/gb/vr/el-hpylg.pdf or
www.human-de.com/data/gb/vr/el-hpylg.pdf

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Anti-*H.pylori* CagA ELISA (IgG)

EUROIMMUN Medizinische Laboratoriumsdiagnostika AG

Anti-Helicobacter pylori CagA ELISA (IgG)
Test instruction

ORDER NO.	ANTIBODIES AGAINST	IG-CLASS	SUBSTRATE	FORMAT
EI 2661-9601 G	Helicobacter pylori CagA	IgG	Ag coated microplate with	96 x 8 (1 x 12)

Principles of the test: The ELISA test kit provides a semiquantitative or qualitative in vitro assay for human antibodies of the IgG class against Helicobacter pylori CagA. In serum or plasma. The test kit contains microtiter strips each with 8 break-off reagent wells coated with recombinant Helicobacter pylori CagA antigen. In the first reaction step, diluted patient samples are incubated in the wells. In the case of positive samples, specific IgG antibodies (also IgA and IgM) will bind to the antigens. To detect the bound antibodies, a second incubation is carried out using an enzyme-labelled anti-human IgG (enzyme conjugate), which is capable of promoting a colour reaction.

Contents of the test kit:

	Component	Colour	Volume	Symbol
1.	Microplate wells coated with antigens: 12 microplate strips each containing 8 individual break-off wells in a frame, ready for use	—	12 x 1	
2.	Calibrator 1 200 RU/ml (IgG, human), ready for use	dark red	1 x 2.0 ml	CAL 1
3.	Calibrator 2 20 RU/ml (IgG, human), ready for use	red	1 x 2.0 ml	CAL 2
4.	Calibrator 3 2 RU/ml (IgG, human), ready for use	light red	1 x 2.0 ml	CAL 3
5.	Positive control (IgG, human), ready for use	blue	1 x 2.0 ml	POS CONTROL
6.	Negative control (IgG, human), ready for use	green	1 x 2.0 ml	NEG CONTROL
7.	Enzyme conjugate peroxidase-labelled anti-human IgG (rabbit), ready for use	green	1 x 12 ml	CONJUGATE
8.	Sample buffer ready for use	light blue	1 x 100 ml	SAMPLE BUFFER
9.	Wash buffer 10x concentrate	colourless	1 x 100 ml	WASH BUFFER 10x
10.	Chromogen/substrate solution TMB/H$_2$O$_2$, ready for use	colourless	1 x 12 ml	SUBSTRATE
	Stop solution 0.5 M sulfuric acid, ready for use	colourless	1 x 12 ml	STOP SOLUTION
11.	Test instruction	—	1 booklet	
12.	Protocol with target values	—	1 protocol	
LOT	Lot		storage temperature	
	In vitro determination		unopened usable until	

Storage and stability: The test kit has to be stored at a temperature between +2°C to +8°C. Do not freeze. Unopened, all test kit components are stable until the indicated expiry date.

Waste disposal: Patient samples, calibrators, controls and incubated microplate strips should be handled as infectious waste. All reagents should be disposed of according to official regulations.

EUROIMMUN · Medizinische Laboratoriumsdiagnostika AG · Seekamm 31 · D-23560 Lübeck · Telefon 0451/5855-0 · Telefax 0451/5855-555

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Preparation and stability of the reagents

Note: All reagents must be brought to room temperature (+18°C to +25°C) around 30 minutes before use. After first use, the reagents are stable until the indicated expiry date if stored at +2°C to +8°C and protected from contamination, unless stated otherwise below.

- **Coated wells:** Ready for use. Tear open the resealable protective wrapping of the microplate at the recesses above the grip seam. Do not open until the microplate has reached room temperature to prevent the individual strips from misting. Immediately replace the remaining wells of a partly used microplate in the protective wrapping and tightly seal with the integrated grip seam (Do not remove the desiccant bag).
  Once the protective wrapping has been opened for the first time, the wells coated with antigens can be stored in a dry place and at a temperature between +2°C and +8°C for a minimum of 4 months.
- **Calibrators and controls:** Ready for use. The reagents must be mixed thoroughly before use.
- **Enzyme conjugate:** Ready for use. The enzyme conjugate must be mixed thoroughly before use.
- **Sample buffer:** Ready for use.
- **Wash buffer:** The wash buffer is a 10x concentrate. If crystallization occurs in the concentrated buffer, warm it to 37°C and mix well before diluting. The quantity required should be removed from the bottle using a clean pipette and diluted with deionized or distilled water (1 part reagent plus 9 parts distilled water).
  For example, for 1 microplate strip: 5 ml concentrate plus 45 ml water.
  The ready-to-use diluted wash buffer is stable for 1 month when stored at +2°C to +8°C and handled properly.
- **Chromogen/substrate solution:** Ready for use. Close the bottle immediately after use, as the contents are sensitive to light. The chromogen/substrate solution must be clear on use. Do not use the solution if it is blue coloured.
- **Stop solution:** Ready for use.

Warning: The control sera used have tested negative for HBsAg, anti-HCV, anti-HIV-1 and anti-HIV-2 using enzyme immunoassays and indirect immunofluorescence methods. Nonetheless, all materials should be treated as being a potential infection hazard and should be handled with care. Some of the reagents contain the toxic agent sodium azide. Avoid contact with the skin.

Preparation and stability of the patient samples

Sample material: Human serum or EDTA, heparin or citrate plasma.

Stability: Patient samples to be investigated can generally be stored at +2°C to +8°C for up to 14 days. Diluted samples should be incubated within one working day.

Sample dilution: Patient samples are diluted 1:101 in sample buffer. For example: dilute 10 µl serum in 1.0 ml sample buffer and mix well by vortexing (sample pipettes are not suitable for mixing).

NOTE: Calibrators and controls are prediluted and ready for use, do not dilute them.

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Incubation

For semiquantitative analysis incubate **calibrator 2** along with the positive and negative controls and patient samples. For quantitative analysis incubate calibrators 1, 2 and 3 along with the positive and negative controls and patient samples.

Sample incubation: (1. step)
Transfer 100 µl calibrators, positive and negative controls or diluted patient samples into the individual microplate wells according to the pipetting protocol. Incubate for **30 minutes** at room temperature (+18°C to +25°C).

Washing:
Manual: Empty the wells and subsequently wash 3 times using 300 µl of working strength wash buffer for each wash.
Automatic: Wash reagent wells 3 times with 400 µl working strength wash buffer (program setting: e.g. TECAN Columbus Washer "Overflow Mode").

Leave the wash buffer in each well for 30 to 60 seconds per washing cycle, then empty the wells. After washing (manual and automated mode), thoroughly dispose of all liquid from the microplate by tapping it on absorbent paper with the openings facing downwards to remove all residual wash buffer.

Note: Residual liquid (> 10 µl) remaining in the reagent wells after washing can interfere with the substrate and lead to falsely low extinction values. Insufficient washing (e.g., less than 3 wash cycles, too small wash buffer volumes, or too short reaction times) can lead to falsely high extinction values.
Free positions on the microplate strip should be filled with dumb wells of the same plate format as that of the parameter to be investigated.

Conjugate incubation: (2. step)
Pipette 100 µl of enzyme conjugate (peroxidase-labelled anti-human IgG) into each of the microplate wells. Incubate for **30 minutes** at room temperature (+18°C to 25°C).

Washing:
Empty the wells. Wash as described above.

Substrate incubation: (3. step)
Pipette 100 µl of chromogen/substrate solution into each of the microplate wells. Incubate for 15 minutes at room temperature (+18°C to 25°C) (protect from direct sunlight).

Stopping the reaction:
Pipette 100 µl of stop solution into each of the microplate wells in the same order and at the same speed as the chromogen/substrate solution was introduced.

Measurement:
Photometric measurement of the colour intensity should be made at a wavelength of 450 nm and a reference wavelength of between 620 nm and 650 nm within 30 minutes of adding the stop solution. Prior to measuring, slightly shake the micro-plate to ensure a homogeneous distribution of the solution.

Pipetting protocol

	1	2	3	4	5	6	7	8	9	10	11	12
A	C 1	P 6	P 14	P 22			C 1	P 4	P 13	P 20		
B	pos.	P 7	P 15	P 23			C 2	P 5	P 13	P 21		
C	neg.	P 8	P 16	P 24			C 3	P 6	P 14	P 22		
D	P 1	P 9	P 17				pos.	P 7	P 15	P 23		
E	P 2	P 10	P 18				neg.	P 8	P 16	P 24		
F	P 3	P 11	P 19				P 1	P 9	P 17			
G	P 4	P 12	P 20				P 2	P 10	P 18			
H	P 5	P 13	P 21				P 3	P 11	P 19			

The pipetting protocol for microtiter strips 1-4 is an example for the semiquantitative analysis of patient samples (P 1 to P 24).
The pipetting protocol for microtiter strips 7-10 is an example for the quantitative analysis of 24 patient samples (P 1 to P 24).

The calibrators (C 1 to C 3), the positive (pos.) and negative (neg.) controls, and the patient samples have each been incubated in one well. The reliability of the ELISA test can be improved by duplicate determinations for each sample.
The wells can be broken off individually from the strips. This makes it possible to adjust the number of test substrates used to the number of samples to be examined and minimizes reagent wastage.
Both positive and negative controls serve as internal controls for the reliability of the test procedure. They should be assayed with each test run.

Calculation of results

Semiquantitative: Results can be evaluated semiquantitatively by calculating a ratio of the extinction value of the control or patient sample over the extinction value of the calibrator 2. Calculate the ratio according to the following formula:

$$\frac{\text{Extinction of the control or patient sample}}{\text{Extinction of calibrator 2}} = \text{Ratio}$$

EUROIMMUN recommends interpreting results as follows:

Ratio <0.8:	**negative**
Ratio ≥0.8 to <1.1:	**borderline**
Ratio ≥1.1:	**positive**

Quantitative: The standard curve from which the concentration of antibodies in the patient samples can be taken is obtained by point-to-point plotting of the extinction values measured for the 3 calibration sera against the corresponding units (linear/linear). Use "point-to-point" plotting for calculation of the standard curve by computer. The following plot is an example of a typical calibration curve. Please do not use this curve for the determination of antibody concentrations in patient samples.

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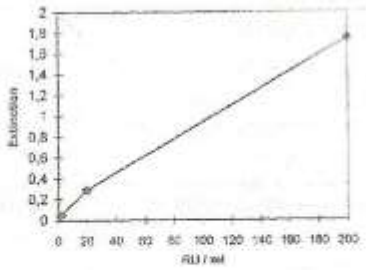

If the extinction of a serum sample lies above the value of calibrator 1 (200 RU/ml), the result should be given as ">200 RU/ml". It is recommended that the sample be re-tested at a dilution of 1:400. The result in RU/ml read from the calibration curve for this sample must then be multiplied by a factor of 4.

The upper limit of the normal range of non-infected persons (**cut-off value**) recommended by EUROIMMUN is **20 relative units (RU)/ml**. EUROIMMUN recommends interpreting results as follows:

<16 RU/ml:	**negative**
≥16 to <22 RU/ml:	**borderline**
≥22 RU/ml:	**positive**

For duplicate determinations the mean of the two values should be taken. If the two values deviate substantially from one another the sample should be retested.

For diagnosis, the clinical symptoms of the patient should always be taken into account alongside the serological results.

Test characteristics

Calibration: As no international reference serum exists for antibodies against Helicobacter pylori CagA, the calibration is performed in relative units (RU).

For every group of tests performed, the extinction values of the calibrators and the relative units and/or ratios determined for the positive and negative controls must lie within the limits stated for the relevant test kit lot. A protocol containing these target values is included. If the values specified for the controls are not achieved, the test results may be inaccurate and the test should be repeated.

The activity of the enzyme used is temperature-dependent and the extinction values may vary if a thermostat is not used. The higher the room temperature during substrate incubation, the greater will be the extinction values. Corresponding variations apply also to the incubation times. However, the calibration sera are subject to the same influences, with the result that such variations will be largely compensated in the calculation of the result.

Antigen: The microplate wells were coated with recombinant Helicobacter pylori CagA antigen. The corresponding cDNA was expressed in E. coli as a full length protein.

Linearity: The linearity of the test was investigated using series dilutions of patient sera with high antibody concentrations. The Anti-Helicobacter pylori CagA ELISA (IgG) is linear in the measurement range 2 - 200 RU/ml.

Detection limit: The detection limit is defined as a value of three times the standard deviation of an analyte-free sample and is the smallest detectable antibody titer. The detection limit of the Anti-Helicobacter pylori CagA ELISA (IgG) is approximately 1 RU/ml.

Cross reactivity: This ELISA showed no cross reactivity.

Interference: Haemolytic, lipaemic and icteric samples showed no influence at the result up to a concentration of 10 mg/ml for hemoglobin, 20 mg/ml for triglycerides and 0.4 mg/ml for bilirubin in this ELISA.

Reproducibility: The reproducibility of the test was investigated by determining the intra- and inter-assay coefficients of variation (CV) using 3 sera. The intra-assay CVs are based on 20 determinations and the inter-assay CVs on 4 determinations performed on 6 different days.

Intra-Assay Variation, n = 20		
Serum	Mean value (RU/ml)	CV (%)
1	62	8.6
2	89	6.9
3	173	4.5

Inter-Assay Variation, n = 4 x 6		
Serum	Mean value (RU/ml)	CV (%)
1	62	7.0
2	91	6.3
3	182	3.9

Specificity and sensitivity: 16 uncharacterized sera were investigated with the EUROIMMUN Anti-Helicobacter pylori CagA ELISA (IgG) and the EUROIMMUN Anti-Helicobacter pylori Westernblot (IgG) was used as a reference. The test showed a specificity and a sensitivity of 100% each with regard to the Westernblot. The results of 5 samples were borderline and were not accounted for in the evaluation.

n = 16		Westernblot: anti-CagA		
		positive	borderline	negative
ELISA	positive	6	4	0
	negative	0	1	5

Reference range: The levels of the anti-Helicobacter pylori CagA antibodies (IgG) were analyzed with this EUROIMMUN ELISA in a panel of 300 healthy blood donors. With a cut-off of 20 RU/ml, 24.2% of the blood donors were anti-Helicobacter pylori CagA positive (IgG).

Clinical significance

Helicobacter pylori (synonyms Campylobacter pylori or C. pyloridis) was first cultured fr mucosa of patients with chronic gastritis in 1982. The presence of bacteria in the stom been known for about 100 years and a connection with gastritis and stomach ulcers has since the forties.

Today, Helicobacter pylori is considered the etiological agent of chronic gastritis type B mainly colonization of the antrum mucosa, while the bacterium is rarely detected in the or the duodenum. Diseases associated with a H. pylori infection include, in addition to g and duodenum ulcers and MALT lymphoma of the stomach. Infections are also ass increased risk of stomach adenocarcinoma. The majority of infections proceed clinically pylori infections do not heal spontaneously and the pathogen can persist lifelong. Rare result from recolonization by pathogens persisting in the mucous membrane crypts. eradication of the bacteria in diagnosed H. pylori infections reduces recurrence by 80% t 20% for duodenal ulcers. 50% of people worldwide are infected with H. pylori, and ey an increase in infections with age has been determined.

Helicobacter pylori is a gram-negative, spiral-shaped bacterium with unusually strong ure It colonizes the epithelial cells on the luminal side of the stomach mucosa intracellularly appears in two forms: a bent spiral form which is proven to be infectious and a long-live H. pylori is the only human pathogen of the genus Helicobacter and it is found wo isolates of H. pylori can be divided into two types: H. pylori strains of type I can exp vacuolating cytotoxin (VacA) and an associated protein (CagA). Strains which are synthesising these proteins are classified as type II.

Infections with type I pathogens appear to be associated with higher pathogenicity: 60% gastritis have type I infections, while in patients with duodenal ulcers this can be up respect, an infection with CagA-positive H. pylori strains can increase the risk of develc three to six fold. Therefore, infections with type 1 pathogens (CagA positive) and ty (CagA negative) need to be differentiated. Since the CagA protein is highly immunogen serological detection of antibodies against CagA is highly suitable for differentiating b subtypes.

Following contact with H. pylori, antibodies of classes IgA, IgG and IgM against appear in the serum. The specific IgM disappear after a week, while antibodies of cl detectable for a longer time period. Increased IgG titers are often found only after th decreased, and they can persist for years. Antibodies of class IgA are formed loca always detectable in patient serum. Positive IgA results correlate well with gastritis eased IgG antibody titers are considered a marker for chronic infection.

Antibodies against H. pylori occur in 70% of patients with chronic active gastritis, and in 6 they are associated with ulcerous complaints. 80% of stomach and intestinal ul consequent therapy with suitable antibiotics. The determination of specific IgG antibo pylori is suitable for demonstrating complete eradication of the pathogen. A significant rec liter around 6 weeks after treatment shows that the therapy has been successful.

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Qualitätskontrollzertifikat
Quality Control Certificate

Produkt: *Product:* Anti-Helicobacter pylori CagA ELISA (IgG)
Best.-Nr.: *Order No:* EI 2081-9601 G
Ch.-B.: *Lot:* E121012AX
Verw. bis: *Exp. Date:* 11-Oct-2013

		Referenzwert *Reference value*		Valider Bereich *Valid range*	
Kalibrator 1 *Calibrator 1*	200 RU/ml	1,610	O.D.	> 0,700	O.D.
Kalibrator 2 *Calibrator 2*	20 RU/ml	0,275	O.D.	> 0,140	O.D.
Kalibrator 3 *Calibrator 3*	2 RU/ml	0,043	O.D.		
Pos. Kontrolle 1 *Pos. Control 1*	quantitativ *quantitative*	120	RU/ml	84 - 156	RU/ml
Pos. Kontrolle 1 *Pos. Control 1*	semiquantitativ *semiquantitative*	3,7	Ratio	2,0 - 5,4	Ratio
Neg. Kontrolle *Neg. Control*	quantitativ *quantitative*	2	RU/ml	0 - 15	RU/ml
Neg. Kontrolle *Neg. Control*	semiquantitativ *semiquantitative*	0,1	Ratio	0 - 0,7	Ratio

O.D. Kalibrator 1 > O.D. Kalibrator 2 > O.D. Kalibrator 3
O.D. Calibrator 1 > O.D. Calibrator 2 > O.D. Calibrator 3

Die Charge wurde von der Qualitätskontrolle getestet und erfüllt alle Spezifikationen.
The lot has been tested by the quality control laboratory and meets the specifications.

12.8. APPENDIX 8: ETHICAL APPROVAL

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KENYATTA NATIONAL HOSPITAL
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Date: 22 May 2012

Dr. Sava Solomon D.
Dept. of Human Pathology
School of Medicine
University of Nairobi

Dear Dr. Sava

Research proposal: "Seroepidemiology of Helicobacter pylori and CagA protein among asymptomatic children at Kenyatta National Hospital" (P496/12/2011)

This is to inform you that the KNH/UoN-Ethics & Research Committee (ERC) has reviewed and approved your above revised research proposal. The approval periods are 22nd May 2012 to 21st May 2013. This approval is subject to compliance with the following requirements:

a) Only approved documents (informed consents, study instruments, advertising materials etc) will be used
b) All changes (amendments, deviations, violations etc) are submitted for review and approval by KNH/UoN ERC before implementation.
c) Death and life threatening problems and severe adverse events (SAEs) or unexpected adverse events whether related or unrelated to the study must be reported to the KNH/UoN ERC within 72 hours of notification.
d) Any changes, anticipated or otherwise that may increase the risks or affect safety or welfare of study participants and others or affect the integrity of the research must be reported to KNH/UoN ERC within 72 hours.
e) Submission of a request for renewal of approval at least 60 days prior to expiry of the approval period. (*Attach a comprehensive progress report to support the renewal*).
f) Clearance for export of biological specimens must be obtained from KNH/UoN-Ethics & Research Committee for each batch of shipment.
g) Submission of an *executive summary* report within 90 days upon completion of the study. This information will form part of the data base that will be consulted in future when processing related research studies so as to minimize chances of study duplication and/or plagiarism.

For more details consult the KNH/UoN ERC website www.uonbi.ac.ke/activities/KNHUoN

Yours sincerely

PROF. A.N. GUANTAI
SECRETARY, KNH/UON-ERC

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The Principal, College of Health Sciences, UoN
The Dean, School of Medicine, UoN
The Chairman, Dept. Human Pathology, UoN
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