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Detection of *Helicobacterpylor*i Antigen and Cytotoxic Associated Gene in Stool Samples from Suspected Patients with Upper Gastroduodeneal Diseases in Shendi City

A thesis Submitted in Complete Fulfillment for the Requirement of MSc Degree in Microbiology

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الآيـــة

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سورة الأنعام

الآية (١٦٢)

Dedication

To……all of my family

To…… all my teachers

Specially

Dr. Hadia Abass Eltaib

To all my friends

For their patience and support

Acknowledgment

First of all I thank to Allah who helped me to complete this study. I would like to express endless thanks to my supervisor, Dr, Hadia Abass Altiab Ahmed for her great efforts and guidance. I would like also to thank Dr. Ebrahim Bakhet and Dr. Hamza Hassan and Dr. Mosabomer and Us. Tsabeh Mohamed for their great helps and endless support. I thank the staff of Altameyez medical analysis centre.

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List of abbreviation

Abbreviation	Term
AL	Alkaline lysis buffer
ASL	A stool lysis buffer
BD2	Beta-Defensin 2
Bp	Base pair
CagA	Cytotoxin associated gene
cox-2	Cyclooxygenase 2
DC	Dendritic cells
DNA	Deoxyribo Nucleic Acid
DSBs	DNA double-Strand Breaks
DU	Duodenal Ulcer
EGFR	Epidermal Growth Factor Receptor
ELISA	Enzyme-Linked Immuno Sorbent Assay
ERK1	Extracellular signal-Regulated Kinase 1
FISH	Fluorescent In Situ Hybridization
G	Gram
GU	Gastric Ulcer
Hcp	*Helicobacter* Cysteine-rich Proteins
Hsp	Heat Shock Proteins
ICT	Immuno Chromatographic Test
IFN-γ	Interferon-γ
Ig	Immunoglobulin
IgG	Immunoglobulin gamma
IHC	ImmunoHisto Chemistry
IL	InterLeukin
JNK	Jun N-Terminal Protein Kinase

LPS	Lipo Poly Saccharide
MALT	Mucosa-Associated Lymphoid Tissue
MCP-I	Monocyte Chemo-attractant Protein I
MMP-7	Matrix Metallo Proteinase-7
OipA	Outer inflammatory Protein A
PAI	Pathogenicity Island
PCR	Polymerase Chain Reaction
PG	Peptido Glycan
PMNs	PolyMorph Nuclear s
PUD	Peptic Ulcer Disease
RUT	Rapid Urease Test
SAT	Stool Antigen Test
Treg	Regulatory T cells
T4SS	Type IV Secretion System
TE	TrisEdta
Th	T helper cell
TK	Tyrosine kinase
TNF	Tumor Necrosis Factor
TNF-α	Tumor Necrosis Factor alpha α*
TRAIL	Tumor necrosis factor-Related Apoptosis-Inducing Ligand
UBT	Urea Breath Test
VacA	Vacuolating cytotoxins A
A	Alpha
β	Beta
γ	Gamma
ε	Epsilon
Ml	Micro little

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ABSTRACT

Helicobacter pylori was worldwide distributed with high prevalence rate and it is common bacterial infections in human gastro-duodenal tract. It possess several specific virulence factors greatly increases the risk of disease, the best recognized of these is the cytotoxic associated gene A (*cagA*) which induced more inflammation, ulceration and oncogenesis.

This study a cross-section study carried out to determine the frequency of *H. pylori* in-patient suspected with upper gastro-duodenal disease using Stool Antigen Test and nested PCR, and determination of cytotoxic associated gene(*CagA* gene) by polymerase chain reaction (PCR) as most virulent factor. Moreover, to determine the sensitivity and specificity of stool antigen test (SAT) as diagnostic methods. Stool samples were collected from Shendi city from January2018 to 2020 from 100 patients(60% Males and 40% females) with mean age (37.2±11.8), statically analyzed by (SPSS version 22) (standard deviation, P value, Mean) There are significant association according to age group (P=0.027) and no significant association with gender (P = 0.919).The study reveals the frequency of *H. pylori* infection was 89%for SAT and 65% for PCR indicating low specificity for SAT (63.6%) with reasonable sensitivity (93.8%).Regarding detection the present of *H.pylori* and correlation of ICT and semi nested PCR with Symptoms, there was no statistical correlation with ICT (P.value 0.472) and with Significant Statistical Correlation with PCR (P.value 0.005).

The study shows expression of *CagA* gene is 58% (35/65) indicating high virulent rate of *H. pylori* infection in study group.

The study concludes that high frequency and virulent rates of *H. pylori* infection.

In this study there are no statistical correlation between present of CagA gene and age, gender, symptoms with (P.value 0.532, 0.834, 0.472).
So properly managed to prevent chronic complication.
Moreover, PCR recommended for diagnosis.

مستخلص البحث

الملوية البوابية متوزعة في جميع أنحاء العالم بمعدل انتشار مرتفع والالتهابات البكتيرية الشائعة في الجهاز الهضمي البشري. الملوية البوابية تمتلك العديد من عوامل الضراوة المحددة بشكل كبير التي تزيد من خطر الإصابة بالمرض، وأفضل ما يمكن التعرف عليه هو الجين المرتبط بالتسمم الخلوي الذي يسبب المزيد من الالتهاب والتقرح وتوليد الأورام.

أجريت هذه الدراسة الوصفية القطعية لتحديد تواتر الملوية البوابية للمرضي المشتبه في إصابتهم بأمراض الجهاز الهضمي العلوي باستخدام اختبار مستضد البراز واختبار تفاعل البلمرة المتسلسل وتحديد الجين المرتبط بالتسمم الخلوي بواسطة تفاعل البلمرة المتسلسل باعتباره العامل الأكثر ضراوة ,علاوة علي ذلك لتحديد حساسية وخصوصية مستضد البراز كطريقه تشخيصيه تم جمع عينات البراز من ١٠٠مريض (٦٠% ذكور و٤٠% إناث) بمتوسط عمر,تم تحليلها بشكل ثابت بواسطة برنامج الحزمة الإحصائية للعلوم الاجتماعية)٣٧,٢±١١,٨(الذي يعرف ببرنامج لتحليل بيانات الدراسة (الإصدار ٢٢) (قيمه احتماليه، الانحراف المعياري، المتوسط) هناك ارتباط حسب الفئة العمرية بمعدل قيمة احتمالية (٠،٠٢٧) ولا يوجد ارتبط بالجنس بمعدل قيمة احتمالية (٠،٩١٩)وكشفت الدراسة أن تكرار الإصابة بالبكتيريا كانت ٨٩% لاختبار مستضد البراز و٦٥ % لاختبار تفاعل البلمرة المتسلسل مما يشير إلى انخفاض النوعية لاختبار مستضد البراز (٦٣,٦%)بحساسية معقولة (٩٣,٨%). فيما يتعلق بوجود الملتوية البوابية وعلاقة اختبار البراز وتفاعل البلمرة المتسلسل مع الأعراض لا توجد علاقة ذات دلاله إحصائية مع اختبار مستضد البراز بمعدل قيمه احتماليه (٠.٤٧٢) مع وجود علاقة ذات دلاله إحصائية مع تفاعل البلمرة المتسلسل بمعدل قيمه احتماليه (٠,٠٠٥).

أظهرت الدراسة التعبير عن الجين المرتبط بالتسمم الخلوي هو٥٨ % (٣٥/٦٥) مما يشير إلي ارتفاع معدل الإصابة بالملتوية البوابية في الدراسة.

وخلصت الدراسة إلى أن الإصابة بالملتوية البوابية عالية التردد.

في هذه الدراسة لا يوجد ارتباط إحصائي بين وجود الجين والجنس والعمر والأعراض (P.value 0.532.، 0.834، (0.472 بمعدل قيمه احتماليه

علاوة علي ذلك يوصي باستخدام تفاعل البلمرة المتسلسل للتشخيص.

Chapter One

Introduction

Rationale

Objectives

1.1 Introduction

Its worldwide distribution and high level of prevalence and it is importance of associated pathologies make the elimination of *H. pylori* a very useful approach to treating and controlling these gastro duodenal diseases, In 1982 when Barry Marshall and Robyn Warren first isolated the gastric pathogen *Campylobacter pyloridis*, few if any gastroenterologists would have predicted that almost 20 years later, this bacterium would have been shown to be one of the most common bacterial infections in humans and the etiologic agent of the majority of upper gastroduodenal disease, Today, *Helicobacter pylori* as it is now known, is firmly established as the etiologic agent of acute or chronic gastritis and a predisposing factor in peptic ulcer disease, gastric carcinoma, and B-cell mucosa-associated lymphoid tissue (MALT) lymphoma.[(1)]

Individuals infected with *H. pylori* have a 10 to 20% lifetime risk of developing peptic - ulcers and a 1 to 2% risk of acquiring stomach cancer. Inflammation of the pyloric-antrum is more likely to lead to duodenal ulcers, while inflammation of the corpus (body of the stomach) is more likely to lead to gastric ulcers and gastric carcinoma.[(2)]

However, *H. pylori* possibly plays a role only in the first stage that leads to common chronic inflammation, but not in further stages leading to carcinogenesis, ameta-analysis conducted in 2009 concluded the eradication of *H. pylori* reduces gastric cancer risk in previously infected individuals, suggesting the continued presence of *H. pylori* constitutes a relative risk factor of 65% for gastric cancers; in terms of absolute risk, the increase was from 1.1% to 1.7%. *Helicobacter pylori* are a human-specific bacterium, colonizing the stomach of approximately 50% of the modern human population, infection is associated with several gastro-duodenal pathologies, including chronic gastritis, peptic ulcers, and even gastric cancer in a subset

of individuals, depending on the variation of bacterial virulence, host genetics and/or environmental factors, *H.pylori* is the most genetically diverse pathogenic bacteria, is a gram negative, non-spore forming spiral bacterium, flagellate bacteria, which colonizes the human stomach and is prevalent worldwide.[3]the organism is also thought to be involved in other human illnesses such as hematologic and autoimmune disorders, insulin resistance and the metabolic syndrome Although nearly 50% of the population is infected with *H. pylori* worldwide, the prevalence, incidence, age distribution and sequels of infection are significantly different in developed and developing countries, the prevalence of *H. pylori* infection is decreasing in developed countries; however, the prevalence is still high in developing countries.[4] In Eastern Sudan the prevalence of infection was estimated to be 80%.[5]

Due to the small amount of bacteria that colonizes the stomach, the direct test sensitivity decreases. Thus, several indirect tests, including antibody-based tests such as serology and urine test, urea breath test (UBT), and stool antigen test (SAT) have been developed to diagnose *H. pylori* infection.[6]

H. *pylori* strains differ, and possess several specific virulence factors greatly increases the risk of disease, the best recognized of these is the cytotoxic associated gene *A* (*cagA*) which induced more inflammation, ulceration andoncogenesis.[7]

1.2 Rational

Helicobacter pylori is remains one of the most common worldwide human disease and is associated with a number of a major cause of gastritis, peptic ulcer and also associated with gastric carcinoma. Several assays have been developed to detect *H. pylori*, so there is no data regarding *H. pylori* infection in Shendi city, in this study we attempt to detect the presence of infection among patients suspected with uppergasteroduodneal disease and to evaluate the specificity and sensitivity of stool antigen test (AST), one of the non invasive methods for detection.

H. *pylori* strains differ, and possess several specific virulence factors greatly in creases the risk of disease, the best recognized of these is the cytotoxic associated gene A (*cagA*) which induced more inflammation, ulceration andoncogenesis. In Shendi, no studies have yet been conducted to determine the prevalence of virulence factors of *H. pylori*, and their association with disease. One objective of this project to detect the presence of this virulence factor which plays a major role in pathogen city.

1.3 Objectives

1.3.1General Objectives

To detect *Helicobacterpylor*i antigen and *CagA* gene in stool samples from out patients suspected with upper gastroduodeneal diseases in Shendi.

1.3.2 Specific Objectives

- To determine *Helicobacter pylori*using stool antigen test and seminested PCR.
- To detect *Cag A* gene using polymerase chain reaction technique.
- To evaluate the sensitivity and specificity of Stool Antigen test using PCR as golden standard method.
- To correlate the presence of *H.pylori* infection with age &gender.

Chapter Two

Literature review

2. Literature Review

2.1 History of *H.pylori*

By the late 19th and early 20th centuries, several investigators had reported the presence of spiral microorganisms in the stomachs of animals.[8]Soon afterward similar spiral bacteria were observed in humans, some of whom had peptic ulcer disease or gastric cancer, The etiological role of these bacteria in the development of peptic ulcer disease and gastric cancer was considered at the time, and patients were sometimes even treated with high doses of the antimicrobial compound bismuth.[9]this possibility was later discarded as irrelevant, probably because of the high prevalence of these spiral bacteria in the stomachs of persons without any clinical signs, The bacteria observed in human stomachs were thus considered to be bacterial overgrowth or food contaminants until the early 1980s At this time, Warren and Marshall performed their groundbreaking experiments, leading to the identification of a bacterium in 58 of 100 consecutive patients, with successful culture and later demonstration of eradication of the infection with bismuth and either amoxicillin or tinidazole.[9] The organism was initially named "*Campylobacter*-like organism," "gastric *Campylobacter*-like organism," "*Campylobacter pyloridis*," and "*Campylobacter pylori*" but is now named *Helicobacter pylori* in recognition of the fact that this organism is distinct from members of the genus *Campylobacter*[10].

2.1.1Morphology

H. pylori is a helix-shaped (classified as a curved rod, not spirochete) Gram-negative bacterium about 3μm long with adiameter of about 0.5μm*H. pylori* can be demonstrated in tissue by Gram stain, Giemsa stain, haematoxylin-eosin stain, Warthin-Starry silver stain, acridine orange stain, and phase-contrast microscopy, It is capable of forming biofilms, and can convert from spiral to a possibly viable but nonculturablecoccid form.[11]

H. pylori has four to six flagella at the same location; all gastric and enterohepatic *Helicobacter* species are highly motile owing to flagella,The characteristic sheathed flagellar filaments of *Helicobacter* are composed of two copolymerized flagellins, FlaA and FlaB.[12]

Scientific classification:	
Bacteria	Domain:
Proteobacteria	Phylum:
Epsilonproteobacteria	Class:
Campylobacterales	Order:
Helicobacteraceae	Family:
Helicobacter	Genus:
H. pylori	Species:
Binomial name:	
Helicobacter pylori.[13]	

2.2 Microbiology

2.2.1Genus Description and Phylogeny

The genus *Helicobacter*belongs to the ε subdivision of the *Proteobacteria*, order *Campylobacterales*, family *Helicobacteraceae*. This family also includes the genera *Wolinella*, *Flexispira*, *Sulfurimonas*, *Thiomicrospira*, and *Thiovulum*, the genus *Helicobacter* consists of over 20 recognizedspecies, Members of the genus *Helicobacter* are all microaerophilic organisms and in most cases are catalase and oxidase positive, and many but not all species are also urease positive.[14]

Helicobacter species can be subdivided into two major lineages, the gastric *Helicobacter* species and the entero-hepatic (non-gastric) *Helicobacter* species. Both groups demonstrate a high level of organ specificity, such that

gastric helicobacter in general are unable to colonize the intestine or liver, and vice versa. An extensive review of non-pylori *Helicobacter* species is available.[15]

2.2.2 Genome, plasmids, and strain diversity

The size of the two sequenced *H. pylori* genomes is approximately 1.7 Mbp, with a G+C content of 35 to 40%. The *H. pylori* strain 26695 genome includes 1,587 genes,Both genomes contain two copies of the 16S, 23S, and 5S rRNA genes. Many strains carry one or more cryptic plasmids, which do not seem to carry antibiotic resistance genes or virulence genes. Some of these plasmids form the basis of *H. pylori-E. coli* shuttle vectors used in molecular cloning experiments.[16]

The existence of *H. pylori*-infecting bacteriophages has been reported, but detailed characterization is lacking.In contrast to other bacterial pathogens that are highly clonal (such as *Shigelladysenteriae* and *Mycobacterium tuberculosis*), *H. pylori* is genetically heterogeneous, suggesting a lack of clonality, This results in every *H. pylori*-positive subject carrying a distinct strain, although differences within relatives may be small. The genetic heterogeneity is possibly an adaptation of *H. pylori* to the gastric conditions of its host, as well as to the distinct patterns of the host-mediated immune response to *H. pylori* infection.[17] genetic heterogeneity is thought to occur via several methods of DNA rearrangement and the introduction and deletion of foreign sequences. The latter usually have an aberrant G+C content and often carry genes involved in virulence. A striking example of this in *H. pylori* is the *cag* PAI, but other plasticity regions have also been suggested to play a role in the pathogenesis of *H. pylori* infection, Several virulence genes, such as the *sabA*, *sabB*, *hopZ*, and *oipA* outer membrane protein-encoding genes, display such phenotypic variation, as do lipopolysaccharide (LPS) biosynthetic enzymes.[17]

2.3 Physiology

H. pylori is microaerophilicthat is, it requires oxygen, but at lower concentration than in the atmosphere. It contains a hydrogenase that can produce energy by oxidizing molecular hydrogen (H_2) made by intestinalbacteria.[18]

It produces oxidase, catalase, and urease. *H. pylori* possess five major outer membrane protein families, The largest family includes known and putative adhesins, The other four families are 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 lipopolysaccharide (LPS), The O antigen of LPS may be fucosylated and mimic Lewis bablood group antigens found on the gastric epithelium, the outer membrane also contains cholesterolglycosides, which are present in few other bacteria.[19]

2.3.1 Metabolism

H. pylori exhibit a narrow host and target organ range, but infection is usually lifelong. This suggests strong adaptation to its natural habitat, the mucus layer overlying the gastric epithelial cells, *H. pylori* lacks several of the biosynthetic pathways commonly found in less specialized bacteria, such as many enteric bacteria.[20] it has been inferred from genomic comparisons and metabolic studies that *H. pylori* has a stripped-down metabolic route with very few redundancies and lacks biosynthetic pathways for some amino acids, *H. pylori* can be grown only in chemically defined medium with the additional amino acids arginine, histidine, isoleucine, leucine, methionine, phenylalanine and valine, and some strains also require alanine, *H . pylori* is urease, catalase, and oxidase positive, characteristics which are often used in identification of *H. pylori, H. pylori* can catabolize glucose, and both genomic and biochemical information indicates that other sugars cannot be catabolized by *H. pylor*i.[21]

2.4 Biochemical Characteristics

Helicobacters are chemoorganotrophs and show a respiratory type of metabolism. They are asaccharolytic when sugar catabolism is examined by standard methods (neither oxidation nor fermentation is observed). Recent studies have, however, indicated that glucose oxidation occurs in at least,gelatin, starch, casein, and tyrosine are not hydrolyzed*Helicobacters*are methyl red and Voges-Proskauer negative, Oxidase activity is present in all species, Strains of most species produce catalase, Many species produce urease, alkaline phosphatase, or both. There is no production of pigments.[22]

2.5 Epidemiology

2.5.1 Prevalence and geographical distribution

The prevalence of *H. pylori* shows large geographical variations. In various developing countries, more than 80% of the population is *H. pylori* positive, even at young ages.[23]The prevalence of *H. pylori* in industrialized countries generally remains under 40% and is considerably lower in children and adolescents than in adults and elderly people Within geographical areas, the prevalence of *H. pylori* inversely correlates with socioeconomic status, in particular in relation to living conditions during childhood, In Western countries, the prevalence of this bacterium is often considerably higher among first-and second-generation immigrants from the developing world, While the prevalence of *H. pylori* infection in developing countries remains relatively constant, it is rapidly declining in the industrialized world.[23]The latter is thought to be caused by the reduced chances of childhood infection due to improved hygiene and sanitation and the active elimination of carrier ship via antimicrobial treatment.[23] In developing countries, *H. pylori* infection rates rise rapidly in the first 5 years of life and remain constantly high thereafter, indicating that *H.pylori* is acquired early in childhood ,however, in industrialized countries the prevalence of *H. pylori* infection is low early in childhood and slowly rises with increasing age, This increase

results only to a small extent from *H. pylori* acquisition at later age, The incidence of new *H. pylori* infections among adults in the Western world is less than 0.5% per year; the higher prevalence of infection among the elderly thus reflects a birth cohort effect with higher infection rates in the past, The active elimination of *H. pylori* from the population and improved hygiene and housing conditions have resulted in a lower infection rate in children, which is reflected in the age distribution of this lifelong-colonizing bacterium more than half of the world's entire population is known to be infected with *H. pylori,* it is generally acquired during the first 5 years of life.[24]the proportion of infection of *H. pylori* acquired by children ranges from 30 to 50%, whereas it reaches a limit of over 90% during adulthood in developing countries. In developed countries, the prevalence of the infection in children is low (1.2-12.2%) compared with developing countries where *H. pylori* is the most frequently isolated bacteria in a 10-year-old,This may be explained by the poor hygiene and sanitation, low socioeconomic status, and overcrowded conditions, which increase the risk of infection.[24]

2.6 Transmission and Sources of Infection

H. pylori have been detected in saliva, vomits, gastric refluxate, and feces, but there is no conclusive evidence for predominant transmission via any of these products, This may be due to the fact that most research on transmission has focused on adults, It appeared that there was no clear increased risk for being a carrier of *H. pylori* among dentists, gastroenterologists, nurses, partners of an *H. pylori*-positive spouse, or visitors to a clinic for sexually transmitted disease, As a result of these and other investigations, it is generally believed that acquisition mostly occurs in early childhood, most likely from close family members, premastication of food by the parent is an uncertain risk factor for transmission of *H. pylori*, Childhood crowding in and outside the family are all positively associated with *H. pylori* prevalence, whereas among adults crowding appears less

important, with the exception of certain circumstances, such as among army recruits, Several studies have reported the presence of *H. pylori* DNA in environmental water sources, but this probably reflects contamination with either naked DNA or dead *H. pylori* organisms. *H. pylori* being successfully cultured from water, but this involved wastewater and as such may well represent fecal contamination of the water source, Spread via fecal contaminants is supported by the occurrence of *H. pylori* infections among institutionalized young people during outbreaks of gastroenteritis, Other possible sources include contaminated food, as *H. pylori* may survive briefly on refrigerated food.[25]Coupled with the extreme sensitivity of *H. pylori* to atmospheric oxygen pressure, lack of nutrients, and temperatures outside the 34°C to 40°C range, direct person-to-person transmission remains the most likely transmission route.[26]

2.7 Bacterial factors

These include factors that enhance mucosal colonization and factors that mediate tissue injury.

2.7.1 Colonization factors

2.7.1.1 Flagella

the possession of spiral shaped, uni-polar, sheathed flagella ,Allows the organism to move rapidly from the lumen of the stomach, where the *pH* is low through the mucus layer to an area where *pH* is near neutral to permit optimal growth.[27]

2.7.1.2 Urease

H. pylori have a great capacity for urease production, probably more than almost all other bacterial species, Urease hydrolyses urea to produce ammonia (NH_3) and carbon dioxide (CO_2). The presence of NH_3 reduces the acidity of the stomach; which may be necessary for providing a congenial environment for *H. pylori*. Ure1, a *pH*-gated channel helps to regulate the production of urea Possession of urease however, may not be absolutely

essential for colonization because urease negative *H. pylori* have been cultured from patients with duodenal ulcer.[28]

2.7.1.3 Adherence factors

H. pylori have tissue tropism for the gastric epithelium it possesses fibrillaradhesins, located on its surface which binds closely to the carbohydrate receptors on the mucosal cell leading to the formation of an adherence pedestal, the best-characterized of these adhesins is BabA, which is a 78-kD outer-membrane protein that binds to the fucosylated Lewis B blood group antigen, BabA is relevant in *H. pylori* associated disease and may influence disease severity, although the results of several studies are contradictory, This property prevents the organism from being shed during cell and mucus turnover.[29]

2.7.2 Factors mediating tissue injury

2.7.2.1 lipopolyscaaride

They are glycolipids found in the cell envelope of gram negative bacteria of which *H. pylori* is one, Lipopolysaccharides are endotoxins which stimulate the release of cytokines through their lipid component, they also interfere with gastric epithelial cell–laminin interaction which may lead to loss of mucosal integrity, inhibit mucin synthesis, and stimulate pepsinogen secretion.[30]

2.7.2.2 Leukocyte recruitment and activating factors

These are soluble surface proteins with chemotactic properties produced by the organism. They help to recruit monocytes and neutrophils to the lamina propria and to activate these inflammatory cells.[31]

2.7.2.3Vacuolating Cytotoxin (VacA)

It is a protein that induces vacuole formation in eukaryotic cells. It is encoded by the *vacA* gene, all strains of *H. pylori* possess the *vacA* gene, but only about 50% express the mature protein. Antibodies to VacA can be used

to detect VacA-producing *H. pylori* strains, VacA is an exotoxin, which inserts itself into the epithelial cell membrane to form a hexameric anion-selective, voltage-dependent channel through which bicarbonate and organic anions can be released,it also acts on the host mitochondrial membrane to induce apoptosis leading to release of cytochrome C from the intermembrane space.[32]

2.7.2.4 Cytotoxin-Associated Antigen (CagA)

It is a highly antigenic protein encoded by the *cagA gen*e that is part of the cag pathogenicity island (*Cag*PaI), The presence of *Cag*PaI is associated with a more prominent tissue inflammatory response than is seen with strains lacking it, This increase in inflammation is associated with an increased risk of developing a symptomatic outcome of the infection, especially PUD and gastric adenocarcinoma, antibodies to CagA can be used to detect CagA-producing *H. pylori* strains, The *cag*PaI encodes a type IV secretory apparatus that injects CagA into mammalian cells , where it triggers cytokine production, *Cag*PaI-positive *H. pylori* also induces apoptosis via the mitochondrial pathway Apoptosis of epithelial cells compromises epithelial barrier which protect the epithelium against luminal acid and pepsin.[32]

2.7.2.5 Outer Membrane Inflammatory Protein (OipA)

It is possessed by most strains with CagA. It acts synergistically with CagA to produce a more intense inflammatory response.[33]

2.7.2.6 Heat shock proteins

These are highly antigenic heat shock proteins known as HspA and HspB. Their role in the pathogenesis of the infection is still not fully known, it has been observed that, even in the absence of VacA and CagA, *H. pylori* can sensitize human gastric epithelial cells and enhance susceptibility to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) mediated apoptosis ,the infection chronicity is sustained through suppression of *H.*

pylori specific memory CD4+ T-cell responses by antigen specific CD4+ CD25 high regulatory T cells.[34]

2.8 Pathogenesis of Infection

2.8.1 Disease spectrum

It has been demonstrated that *H. pylori* is involved in the pathogenesis of several diseases, these include

2.8.1.1Gastritis

This could occur in form of either acute or chronic gastritis, *H. pylori*causes gastritis Acute infection is typically manifested as a transient mild illness characterized by epigastric pain, nausea, histological finding of neutrophilic gastritis and a transient hypochlorhydria, it is not known how often acute infection clears spontaneously. However, studies in children suggest that spontaneous loss of infection may be common.[34]Acute *H. pylori* infection is diagnosed by the presence of a positive UBT and negative IgG anti–*H. pylori* antibodies ,Chronic gastritis often presents in form of chronic active, non-atrophic superficial antral gastritis, with a picture of focal epithelial cell damage This is usually asymptomatic, although it may be associated with PUD, Chronic atrophic gastritis resulting from progression of the non-atrophic chronic gastritis may also occur in a smaller percentage of patients with gastritis. [35]

2.8.1.2Peptic ulcer disease

An individual infected with *H. pylori* has an estimated lifetime risk of about 10-20% for the development of PUD, This is at least 3-4 folds higher than in non-infected subjects. *H. pylori* infection can be diagnosed in 90-100% of duodenal ulcer (DU) patients and in 60-100% of gastric ulcer (GU) patients, the gastritis induced by *H. pylori* can progress to ulceration of the mucosa, Apoptosis of epithelial cells and subsequent compromise in the mucosal protective barrier exposes gastric mucosa to the direct assault of luminal acid

and pepsin Occlusion of mucosal end-arteries due to impaired fibrinolytic activity may contribute to the focal nature of PUD.[36]

Patients with DU have been known, before the discovery of *H pylori*, secrete about twice as much acid as controls because they have twice as many parietal cells, acid hyper secretion in DU is virtually always due to *H. pylori* infection because secretion returns to normal after the infection is eradicated, The predominantly antral gastritis in DU diminishes the number of somatostatin-producing cells in the antrum, This leads to a reduction in production of somatostatin, reduced somatostatin-mediated inhibition of gastrin release from the G cells,the increased acid secretion may, on its own increase the risk of duodenal ulceration or may induce gastric metaplasia in the duodenum, which becomes colonized by *H. pylori*, then inflamed (duodenitis), and finally ulcerated, Successful eradication of *H. pylori* leads to PUD healing and less frequent recurrence of the ulcer.[37]

2.8.1.3 Gastric adenocarcinoma

H. pylori has been implicated as the strongest risk factor in the pathogenesis of gastric adenocarcinoma, especially the distal type, and it has been classified as a class I (or definite) carcinogen by the WHO, the pathogenesis of gastric cancer includes a sequence of events that begins with *H. pylori*-induced chronic superficial gastritis, progressing towards atrophic gastritis, intestinal metaplasia, dysplasia and eventually gastric cancer, This sequence takes decades to complete, several aspects of the inflammatory milieu that have been implicated as carcinogens include increased oxidative stress and the formation of oxygen-free radicals leading to DNA damage, increased pro-inflammatory cytokine production such as IL-1B and TNF which stimulate greater cell turnover and reduced apoptosis, and potential for faulty or incomplete DNA repair ,bacterial virulence has been shown to be an important factor in carcinogenesis, For instance patients infected with CagA-

positive strains have been shown to have a higher risk of developing gastric carcinoma than those infected with CagA-negative *H. pylori* strains.[38]

H. pylori-infected cells also express some factors similar to those commonly implicated in carcinogenesis e.g. matrix metalloproteinase-7 (MMP-7) which has been found to be up-regulated in colorectal cancer, MMP-7 is important in the normal and pathological remodelling of epithelial-matrix interactions and is up-regulated in gastric cancer too, It plays an important role in promoting tissue invasion and metastasis of cancer cells. This up-regulation is also dependent on the *cag*-PaI, Although *H. pylori* is now thought to account for 80% or more of gastric cancers, it is noteworthy that only 3% of infected patients progress to gastric cancer.[38]

2.8.1.4 MALT lymphoma

The molecular pathogenesis of MALT lymphoma is incompletely understood but seems to also involve strain-specific *H. pylori* factors, as well as host genetic factors, such as polymorphisms in the promoters of inflammatory cytokines such as IL-1B and TNF. It is believed that *H. pylori* infection leads to the formation of *H. pylori*-reactive T cells, which then cause polyclonal B-cell proliferations.[39] a monoclonal B-cell tumor emerges in the proliferating B cells, probably as a result of accumulation of mutations in growth-regulatory genes, Some studies have implicated CagA in the development MALT lymphoma via impairment of p53-dependent apoptosis, eradication of *H. pylori* "cures" the lymphoma by removing antigenic stimulus for T cells.[39]

2.8.1.5 Functional dyspepsia

The prevalence of *H. pylori* is generally high in patients with dyspepsia irrespective of the subgroup, The implication of *H. pylori* in the pathogenesis of ulcer dyspepsia is well established but there are dissenting views on the role it plays in the pathogenesis of functional dyspepsia, While some studies

showed association between *H. pylori* infection and the clinical diagnosis of functional dyspepsia .[40]

2.8.1.6 Extra-gastroduodenal diseases

H. pylori has also been suggested to be causally related to several extra-gastro duodenal diseases ,these associations are generally weak because they were not obtained from randomized controlled studies.[41]

2.8.1.7 *H. pylori*-Associated Pathogenesis

The primary disorder, which occurs after colonization with *H. pylori*, is chronic active gastritis, This condition can be observed in all *H. pylori*-positive subjects, The intragastric distribution and severity of this chronic inflammatory process depend on a variety of factors, such as characteristics of the colonizing strain, host genetics and immune response, diet, and the level of acid production, *H. pylori*-induced ulcer disease, gastric cancer, and lymphoma are all complications of this chronic inflammation; ulcer disease and gastric cancer in particular occur in those individuals and at those sites with the most severe inflammation Understanding of these factors is thus crucial for the recognition of the role of *H. pylori* in the etiology of upper gastrointestinal pathology.[42]

2.9 Signs and symptoms

Up to 85% of people infected with *H. pylori* never experience symptoms or complications. Acute infection may appear as an acute gastritis with abdominal pain or nausea., where this develops into chronic gastritis, the symptoms, if present, are often those of nonulcerdyspepsia: stomach pains, nausea, bloating, belching, and sometimes vomiting or black stool.[43]

Individuals infected with *H. pylori* have a 10 to 20% lifetime risk of developing pepticulcers and a 1 to 2% risk of acquiring stomach cancer Inflammation of the pyloric antrum is more likely to lead to duodenal ulcers, while inflammation of the corpus (body of the stomach) is more likely to lead to gastric ulcers and gastric carcinoma ,however, *H. pylori* possibly

plays a role only in the first stage that leads to common chronic inflammation, but not in further stages leading to carcinogenesis.[44]

H. pylori have been associated with colorectal polyps and colorectal cancer, It may also be associated with eye disease.[45]

Pain typically occurs when the stomach is empty, between meals, and in the early morning hours, but it can also occur at other times. Less common ulcer symptoms include nausea, vomiting, and loss of appetite ,Bleeding can also occur; prolonged bleeding may cause anemia leading to weakness and fatigue, if bleeding is heavy, hematemesis, hematochezia, or melena may occur.[46]

2.10 Genes Involved In Virulence And Pathogenesis

Study of the *H. pylori* genome is centered on attempts to understand pathogenesis, the ability of this organism to cause disease, about 29% of the loci have a colonization defect when mutated, two of sequenced strains have an around 40-kb-long Cag pathogenicityisl and (a common gene sequence believed responsible for pathogenesis) that contains over 40 genes. This pathogenicity island is usually absent from *H. pylori* strains isolated from humans who are carriers of *H. pylori*, but remain asymptomatic.[47]

2.11 Cag pathogenicity island

The pathogenicity of *H. pylori* may be increased by genes of the *cag* pathogenicity island; about 50-70% of *H. pylori* strains in Western countries carry it, Western people infected with strains carrying the *cag* PAI have a stronger inflammatory response in the stomach and are at a greater risk of developing peptic ulcers or stomach cancer than those infected with strains lacking the island. Following attachment of *H. pylori* to stomach epithelial cells, the type IV secretion system expressed by the *cag* PAI "injects" the inflammation-inducing agent, peptidoglycan, from their own cell walls into the epithelial cells. The injected peptidoglycan is recognized by the

cytoplasmic pattern recognition receptor (immune sensor) Nod1, which then stimulates expression of cytokines that promote inflammation.[48]

The type-IV secretion apparatus also injects the *cag* PAI-encoded protein CagA into the stomach's epithelial cells, where it disrupts the cytoskeleton, adherence to adjacent cells, intracellular signaling, cellpolarity, and other cellular activities ,once inside the cell, the CagA protein is phosphorylated on tyrosine residues by a host cell membrane-associated tyrosine kinase (TK), CagA then allosterically activates protein tyrosine phosphatase/proto-oncogeneShp2,pathogenic strains of *H. pylori* have been shown to activate the epidermal growth factor receptor (EGFR), a membrane protein with a TK domain, Activation of the EGFR by *H. pylori* is associated with altered signal transduction and gene expression in host epithelial cells that may contribute to pathogenesis, A C-terminal region of the CagA protein (amino acids 873–1002) has also been suggested to be able to regulate host cell gene transcription, independent of protein tyrosine phosphorylation.[49]

2.12Immune Response to*H. Pylori* Infection

Immune responses to *H. pylori* infection have been studied in twenty adult volunteers experimentally infected with *H. pylori*, Gastric biopsies performed 2 wk after infection showed infiltration of lymphocytes and monocytes, along with significantly increased expression of IL-1, IL-8, and IL-6 in the gastric antrum, Anti-*H. Pylori* immunoglobulin IgM and IgG responses were detected in the serum of infected individualsin addition, 4 wk after infection the numbers of gastric $CD4^+$ and $CD8^+$ T cells were increased compared to preinfection levels, these data provide evidence that gastric and systemic immune responses develops within a short period of time after *H. pylori* infection, Gastric mucosal biopsies from humans persistently infected with *H. pylori* reveal an increased infiltration of various types of leukocytes compared to biopsies from uninfected humans, Lymphocytes (T and B cells), monocytes, eosinophils, macrophages, neutrophils, mast cells and dendritic

cells are usually present.[50]B cells and $CD4^+$ T cells together with dendritic cells (DC) sometimes organize into lymphoid folliclesreflecting ongoing antigen presentation and chronic immune responses. *H. pylori*-specific $CD4^+$ T cells are detectable in the gastric mucosa and peripheral blood of infected-individuals but not uninfected humans, Levels of cytokines [interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), IL-1, IL-6, IL-7, IL-8, IL-10, and IL-18) are increased in the stomach of *H. pylori*-infected humans compared to uninfected humans,IL-4 has not been detected in the gastric mucosa of most *H. pylori*-infected individuals, therefore, it has been concluded that *H. pylori* infection leads to a T helper cell (Th)1-polarized response, *H. pylori* infection has also been associated with upregulation of IL-17A expression in the gastric mucosa.[51]IL-17A is the most widely studied member of the IL-17 family of cytokines (IL-17A-F), and is produced by Th17 $CD4^+$ T cells as well as other subsets of immune cells, Extracellular bacterial and fungal infections elicit strong IL-17A responses that stimulate stromal and epithelial cells to release pro-inflammatory cytokines and chemokines, *e.g*., TNF-α, IL-1β, IL-6, which recruit neutrophils, macrophages and lymphocytes to the site of infection, Furthermore, it has been described that *H. pylori* infection also leads to the generation of regulatory T cells (Treg),Depletion of Treg through injection of anti-CD25 antibodies to mice before *H.pylori* infection promoted gastritis and reduced bacterial load, very elegant studies originated from the group of PD Smith clearly showed that in children, *H. pylori* infection is associated with low Th17 and Th1 responses, high Treg response and reduced gastritis as compared with adults, suggesting that *H. pylori* specific Treg play key roles in bacterial persistence, associated with cellular responses, a humoral immune response is elicited in nearly all *H. pylori*-infected humans, Serum IgA and IgG antibodies in chronically infected persons are directed toward many different *H. pylori* antigens, A local antibody response directed toward *H. pylori* antigens is also detectable with

chronic *H. pylori* infection, *H. pylori* infection induces autoantibodies reactive with gastric epithelial cells, which could drive gastritis, These autoantibodies could be directly cytolytic to epithelial cells through activation of complement, inducing apoptosis or triggering an antibody-dependent cellular cytotoxicity reaction leading to the tissue destruction.[52]

T helper (Th) cell subsets and their signature cytokines especially IFN-γ, contribute to anti-bacterial response, but at the mean time sustaining chronic inflammatory responses in the site of infection.[53]

The role of Th cell mentioned in Figure 2.1.

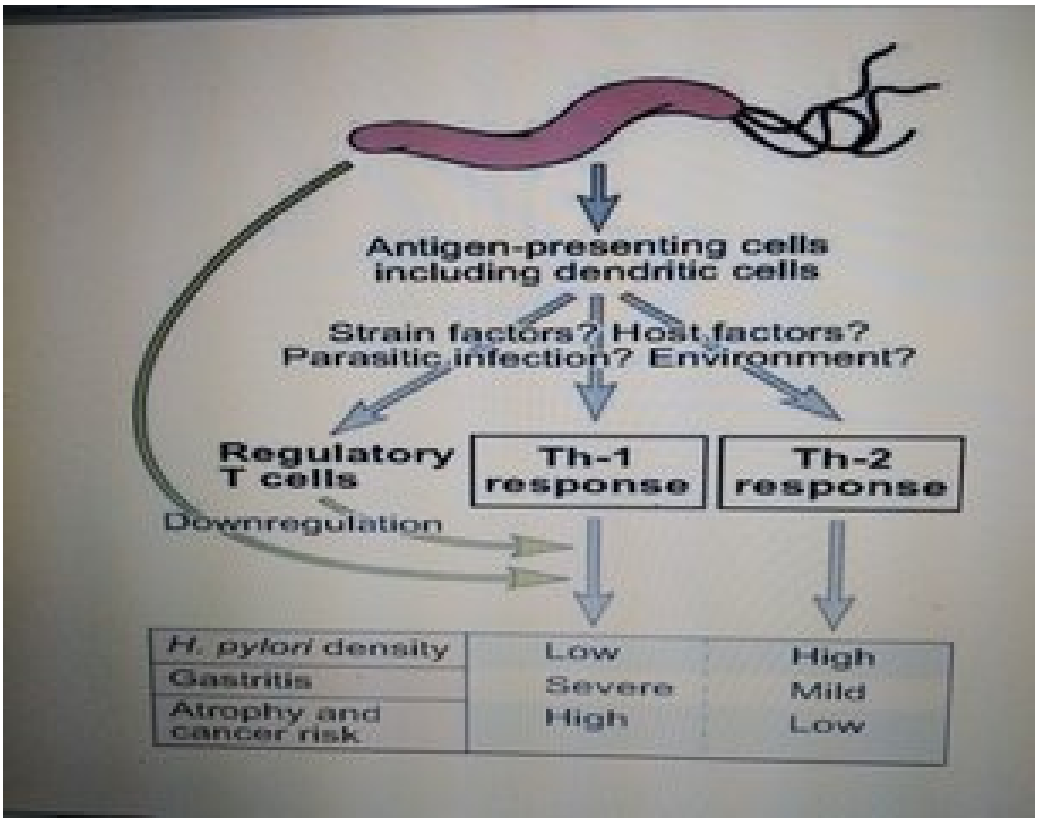

Figure 2.1: The central role of the Thelper cell response to *H. pylori* infection.[53]

2.13 Diagnosis of *H.pylori*

In view of the importance of *H. pylori* in the etiopathogenesis of major gastrointestinal and extra-gastrointestinal diseases, it is pertinent to look into cost-effective and reliable diagnostic tests for early detection and therapy. *Helicobacter pylori* diagnostic tests can be broadly classified into invasive and non-invasive tests' Invasive tests require endoscopic gastro-duodenal biopsy samples while the non-invasive tests do not.(54)

Despite the good sensitivity and specificity of most of the commonly used tests, determination of gold standard has been difficult because none of them is perfect, A combination of at least two tests is commonly used as gold standard, though some researchers now use UBT as their gold standard, it is not uncommon to use at least one invasive test whenever two tests are used as gold standard.(55)

2.13.1 Invasive tests

2.13.1.1 Histology

Multiple levels of each biopsy are routinely stained with haematoxylin and eosin (H&E) and with a special stain such as Warthin-Starry silver, Giemsa, or Cresyl-fast violet, the standard H&E stain is used to determine histological chronic or chronic active inflammation (gastritis) but could also demonstrate *H. pylori* if a large number of the organism arepresent,atrophy and intestinal metaplasia can also be assessed. Small numbers of bacteria are better detected by the special stains, An important advantage of histology is that in addition to the historical record provided, sections from biopsies (or even additional sections) can be examined in the future, thedrawbacks of the test include high observer-dependency, relatively long waiting time for result, requirement of specialized skills for performance and relatively high cost.(56)

2.13.1.2 Rapid urease test (RUT)

The urease enzyme which is produced by *H. pylori* is utilized in performing this test, Gastric biopsy is placed in a medium that contains urea and a *pH* indicator, The urease breaks down the urea to produce ammonia that increases the *pH* of the medium which leads to a color change, the specificity and sensitivity of the test are greater than 90%, but false-positive results do occur,It can be performed and read within 1 to 24 hour depending on the make. Its comparative advantage to histology lies in its rapidity, simplicity and inexpensiveness; but it cannot be used to evaluate gastritis.[57]

2.13.1.3 Culture

This is done under stringent conditions. Endoscopy biopsy must be transported to the laboratory at 4°C within 24 hrs or at-70°C for a longer period. Fresh selective and non-selective media are needed to culture the organism. After introduction of the specimen into the culture medium, the plates are inspected for about 10 days, due to the focal nature of inflammatory lesions produced by *H. pylori*, multiple biopsies are usually taken from the gastric antrum and corpus to increase the yield of the test The specificity of the test is 100% while the sensitivity is slightly less, a major advantage of the test is that, pure growth of the organism can be obtained for proper identification and detailed studies e.g. antibiotics sensitivity when there is failure of the second line drugs, strain typing, genetic studies ,the disadvantages of the test include longer duration for result availability, high cost and the stringent condition needed for transportation to the laboratory.[58]

2.13.1.4 Polymerase chain reaction (PCR)

This is a molecular technique which amplifies fragment of a gene specific for the *H. pylori* e.g. *vacA* and *cagA* gene sequences, 16SrNA, 23SrRNA, and ureC are targeted, The biopsy is lyses to liberate the DNA Specifically designed primers and polymerase enzyme are used to amplify gene,

amplification is done in 30-40 cycles at different temperatures with each cycle. This allows for denaturation, annealing and elongation, The amplified products are thereafter identified by electrophoresis, as a result of the development of string-capsule test device, gastric sample for PCR can now be obtained without having to biopsy the stomach PCR has a sensitivity and specificity that are well above 90%, It can be used to analyze bacterial genotypes, study pattern of antibiotic resistance and *H. pylori* transmission within families and the community, The main disadvantages are that it is expensive, and the procedure requires technical expertise to perform.[59]

2.13.1.5 DNA-Enzyme immunoassay

This is a form of PCR where the PCR amplicons are detected by calorimetric method. It is ELISA-based and involves the use of coated micro wells, this method is more rapid than the standard PCR and result can be obtained within a few hours.[60]

2.13.1.6 Fluorescent in situ hybridization (FISH)

This is another molecular test for diagnosing *H. pylori* that is particularly useful in detection of *H. pylori* clarithromycin resistance/sensitivity.[61]

2.13.2 Non-invasive tests

2.13.2.1 Serology

Chronic *H. pylori* infection elicits a circulating antibody response that can be quantitatively measured by serological assay technique like enzyme-linked immunosorbent assay (ELISA), though tests for IgG, IgA and IgM antibodies can be done, only IgG antibody test is reliable, It involves the use of serum or plasma, and lately tests on whole blood.[62] micro well coated with *H. pylori* antigen is exposed to *H. pylori* antibody (in serum, plasma or whole blood) in the presence of an indicator, The color change resulting from the antigen-antibody reaction is read visually or with the use of a spectrophotometer, other serological methods that can be used include immunoblot, flow microsphere immunofluorescence and chromatographic

tests. Because of its easy availability, affordability, and simplicity, it is commonly used in prevalence studies of *H. pylori* ,its major drawback is its poor discriminatory power between current infection and previous exposure, since it may still be positive several months after *H. pylori* eradication, It is therefore generally not useful in confirming cure after antimicrobial therapy but it is useful for the initial diagnosis of *H. pylori* infection and epidemiological surveys.(63)

2.13.2.2 Urea breath test (UBT)

This is an indirect method of detecting the presence of *H. pylori* in the stomach premised on the ability of *H. pylori* to produce the urease enzyme. Urea labeled with either ^{13}C or ^{14}C is ingested by the patient, if urease is present in the stomach as a result of *H. pylori* infection, labeled CO^2 will be split off and absorbed into the circulation, where its presence can be determined by analysis of expired breath by means of a spectrometer. The result is expressed in delta/mil,^{13}C UBT is preferred to ^{14}C UBT especially in children and pregnant women because it is stable and non-radioactive ,UBT is now being considered as the gold standard by some researchers, it is the non-invasive test with the highest sensitivity and specificity (>95%) and is the preferred means of evaluating the success of antimicrobial therapy in clinical practice, it is not as expensive as endoscopy, portable and cheaper spectrometers are now available thereby eliminating the need to send collected air samples to a central spectrometer, there is the possibility of false positive results when there is bacterial overgrowth of urease-producing organisms, recent use of antibiotics, bismuth preparations or acid suppression therapy, due to their effect on the colony size of *H. pylori*, can produce false negative results.(64)

2.13.2.3 Stool antigen test (SAT)

The test is based on the detection of *H. pylori* antigen in the stool *Helicobacter pylori* adhering to gastric epithelium in infected persons appear

in their stool as a consequence of the normal shedding of the epithelium, this means that the test is a direct test of active infection which gives it an advantage over serology, It is an enzyme immunoassay test which is available in both polyclonal and monoclonal forms, the monoclonal immunoassay is newer and more sensitive and specific than the polyclonal assay ,and may be considered as an alternative to UBT in the initial diagnosis of patients with dyspepsia who do not require immediate endoscopy.[65] SAT is simple and relatively cheap, It can be carried out in most routine laboratories, It is slightly less reliable when used soon after the end of *H. pylori* eradication therapy, It is now generally recommended to wait for about 12 weeks to reliably confirm eradication, its diagnostic accuracy is impaired by gastrointestinal bleeding, a major drawback is related to the inconvenience of stool handling ,additional non-invasive tests that are yet to be recommended for routine clinical diagnoses of *H. pylori* include stool PCR, Urine antibody test and Saliva antibody test.[66]

2.13.3 Calculating of specificity and sensitivity

Sensitivity

The ability of a test to correctly classify an individual as diseased.

Sensitivity = a/a+c

a= true positive /true positive +false negative = probability of being test positive when disease present.

Specificity

The ability of test to correctly classify an individual as disease free.

Specificity = d/ d+b

d=true negative / true negative +false positive

= probability of being test negative when disease absent.

Positive predictive value (ppv)

It is the percentage of patient with apositive test who actually has the disease.

PPV=a/a+b

PPV= true positive /true positive +false positive

Negative predictive value (NPV)

It is the percentage of patient with negative test who do not have the disease.

NPV =d/d+c

d= true negative / true negative +false negative.[67]

2.14 Treatment

The optimal treatment for *H. pylori* activity is used in bismuth-based quadruple therapy and seems almost totally maintains high eradication rates, independent of antibiotic resistance.[68]

H.pylori eradication protocols, once *H. pylori* is detected in a person with a peptic ulcer, the normal procedure is to eradicate it and allow the ulcer to heal. The standard first-line therapy is a one-week "triple therapy" consisting of proton pump inhibitors such as omeprazole and the antibiotics clarithromycin and amoxicillin, Variations of the triple therapy have been developed over the years, such as using a different proton pump inhibitor, as with pantoprazole or rabeprazole, or replacing amoxicillin with metronidazole for people who are allergic to penicillin. In areas with higher rates of clarithromycin resistance, other options are recommended. Such a therapy has revolutionized the treatment of peptic ulcers and has made a cure to the disease possible. Previously, the only option was symptom control using antacids, H_2-antagonists or proton pump inhibitors alone, an increasing number of infected individuals are found to harbor antibiotic-resistant bacteria. This results in initial treatment failure and requires additional rounds of antibiotic therapy or alternative strategies, such as a quadruple therapy, which adds a bismuthcolloid, such as bismuth subsalicylate. For the treatment of clarithromycin-resistant strains of *H. pylori*, the use of levofloxacin. [69]

2.15. Previous studies

Study at the University Hospital of Kiel for stool antigen test was enrolled in the study between 2002 and 2003[70].

Study at Department of Gastroenterology, University of São Paulo School of Medicine, São Paulo, SP, Brazil ,2004. [71]

Study at Tabriz University of Medical Sciences Dermatology Clinics from October 2011 to January 2011.Patients.[72]

Study done by Ahmad Kumo Bello in Department of Medicine, Ahmad Bello University Teaching Hospital, P. M. B. 06, Nigeria[73].

Study at, Department of Microbiology, Immunology and Infectious Diseases (KMB, WAA, GAB), College of Medicine and Medical Sciences, Arabian Gulf University, Kingdom of Bahrain and Department of Medical Research (GAB), University of Udine.[74]

Study done by Nogdallaat Sudan University College of Medical Laboratory Science conducted at Faisal special hospital, Fedail hospital and Omdurman military hospitalduring the period from April to December 2018.[75]

Chapter three

Materials and Method

3. Material and Methods

3.1 Study design

This is a descriptive cross sectional study.

3.2 Study duration

Study was conducted from January 2018 – April 2019.

3.3 Study area

The study was done in Shendi city.

3.4 Study population

Patients suspected with upper gastrodudoneal disease were included in the study after ethical consent obtained.

3.5Inclusion criteria

Patients with upper gastrodudoneal symptoms be fore treatment were included.

3.6 Exclusion criteria

Patients with upper gastrodudoneal diseases under treatment and immunocompromized patient were excluded.

3.7 Sample size and sampling:

The sample size calculated according to the below equation

$$Ss=\frac{z2*(p) * (1-p)}{C2}$$

Ss =sample size

z=z value (1.96 for 95% confidence level)

p= percentage picking a choice expressed as decimal 0.5 for sample size needed)

C= Confidence interval, expressed as decimal (.04 = ± 4)

A total of (100) sampleshave been chosenaccording to the available facility and cost.

3.8 Permission and Ethical Considerations

After Approval by Ethical Committee of Medical Laboratory Sciences College –Shendi University According to research ethics, permission was obtained from all participants informed with research objectives and then stool samples were collected (Appendix II) .

3.9 Stool Sampling

Stool sample was collected using sterile stool container, about 2 g of stool was put in eppendorf tube and freezing in (-20°C) for DNA extraction, the remaining one testing by ICT stool test.

3.10 Material

For ICT stool test:(Stool container& ICT device).

For PCR: (Eppendorf tube, DNA template, Tag polymerase, primers, deoxynuclutidetriphosphate, and buffer solution).

3.11 Data Collection Tools

Data was collected using self-administrated pre-coded questionnaire which specifically designed to obtain both demographic and clinical data for study for study volunteer that helped in study (Appendix I).

3.12.H.pyloriantigen detection by ICT method

3.12.1Procedure

Specimen was collected and then removed the test device from the foil and was holed upright and carefully braked off the tip of collection device then squeezed 2 drops of sample in the sample well of the cassette and the result was readied after 10 min, positive result confirmed by present of red line wear as negative result by absent of red line.

3.12.2 DNA Extraction

3.12.2.1 Procedure

Stool sample was weighted about (200 mg) in a 2 ml micro centrifuge tube and was placed the tube on ice then added 1.4 ml Buffer ASL to each stool sample, vortex continuously for 1 min or until the stool sample is thoroughly

homogenized, and heated the suspension for 5 min at 70°C then was vortex for 15s and centrifuge sample at full speed for 1 min to pellet stool particles then pipettes1.2 ml of the supernatant into a new 2 ml microcentrifuge tube and discard the pellet and 1 inhibited Tablet was added to each sample and vortex immediately and continuously for 1 min or until the tablet is completely suspended then incubated suspension for 1 minat room temperature to allow inhibitors to adsorb to the Inhibit EX matrix, centrifuge sample at full speed for 3 min to pellet inhibitors bound to Inhibit EXmatrix, then all the supernatant was pipette into a new 1.5ml microcentrifuge tube anddiscarded the pellet, centrifuge the sample at full speed for 3 min then pipettes 15µl proteinase K into a new 1.5 ml micro centrifuge tube containing proteinase K then added 200µl Buffer AL and vortex for 15s then was incubated at 70°C for 10 min and 200µl of ethanol (100%) was added to the lysate, and mix by vortexing then labeled the lid of a new QIAamp spin column placed in a 2 ml collection tube and carefully apply the complete lysateand closed the cap and centrifuge at full speed for 1 min then placed the QIAamp spin column in a new 2 ml collection tube, and discard the tube containing the filtrate then carefully opened the QIAamp spin column and add 500µl Buffer AW1, and close the cap and centrifuge at full speed for 1min. Placed the QIAamp spin column in a new 2mlcollection tube, and discarded the collection tube containing the filtrate and discard the old collection tube with the filtrate, centrifuge at full speed for 1min, then transfer the QIAamp spin column into a new labeled 1.5 ml micro centrifuge tube and carefully opened the QIAamp spin column and pipettes 200µlbuffer Al directly onto the QIAamp membrane and closed the cap and incubate for 1min at room temperature, then centrifuge at full speed for 1 min to elute DNA[76].

3.12.3PCR for *H.pylori*

3.12.3.1 Procedure

PCR was based on the DNA sequence of a species-specific protein antigen ofprimary amplification reaction was performed by a protocol.The reaction was followed by a semi nested PCR developed in laboratory with the upstream primer (5′-TGGCGTGTCTATTGACAGCGAGC-3′) of the primary PCR and the nested primer (5′-TGATCACTGCATGTCTTACTTTCATGTTTTT-3′).

Reactions were performed with a volume of 50µl with TRIO-Thermo block (MB Minerva biolab) for thermal cycling. Primary PCR product (0.5µl) was added to a reaction mixture consisting of the four deoxynucleotidesat 100 µM each, 0.1 µM each primer, and 0.5 U of the *Tag* DNA polymerase, in a standard PCR incubation buffer (10 mMTris-HCl [pH 8.3], 50 mMKCl, 2.5 mM $MgCl_2$, 0.001% gelatin).

Amplification in the semi nested PCR consisted of an initial denaturation step at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 68°C for 1 min, and extension at 72°C for 45 s. After the last cycle, the mixture was incubated at 72°C for 5 min. The amplification product was analyzed by electrophoresis on a 1.5% agarose gel by a standard protocol, and a band at 209 bp was considered a positive PCR result.[77]

3.12.4 PCR amplification using the cagA primer

3.12.4.1 Procedure

PCR was carried out using the primer set cagA-F(5′-CAA TGA ATT TTT GAT CCG GG-3′) and cagA-R (5′-GAT AAC AGG CAA GCT TTT GAG GGA3′). The 25 µl reaction mixture consisted of 1x PCR buffer, 1.5Mm magnesium chloride, 200µM of each d NTP, 20pmol of each primer and 1U Tag DNA polymerase. An initial denaturation at 94c for 5min and followed by 40 cycles of denaturation at 94c for 1 min and annealing at 54c for 1min and extention at 72c for 1 min this was followed by a finalextention of 72c

for 5 min. A 5µl of the PCR product were separated on 1.5% Agarose gel with TBE buffer (Tris Boric Acid)and 100 bp ladder was used as DNA molecular weight standard and stained with 0.15% Ethidiumbromide, then the product was visualized by using UV gel documentation system, PCR product size 349 bp[78].

3.13Data analysis.

The collected data code in master sheet and proceed for analysis using SPSS version (22) used (P.value, Stander Deviation, Mean)

Chapter four

Results

4.Results

Demographic and clinical data

A total of (100) stool samples were collected from patients suspected with uppergastrodudoneal disease, 60/100 (60%) were males while 40/100(40%) were females with mean of age (37.2 ±11.8yrs) which Indicated in table (4.1).

In regarding to distribution of Age, the patient categorized into two Groups, the first one from (15-40 years) 23/100were male while 15/100Were female and the other group from (41-65years) 37/100 of them Were Male and 25/100were female as listed in table (4.2).

Regarding patient's symptoms, 29/100 presented with abdominal pain, 25/100 with vomiting,18/100with nausea, 6/100 with Combined Symptom, showed in table (4.3).

The frequency of *H. Pylori* infection among study population detected by ICT & Seminested PCR techniques were89/100(89%) and65/100 65% Respectively as listed in table (4.4).with product size of 209bp Mentioned in Fig (4.1).

Concerning relationship between gender and presence of *H. Pylori* Infection, there are no statically Correlation significant With (P.Value) observed as mentioned in table (4.5). At the Same time with Age Showed Strong statically correlation significant with (p.value) as showed in table (4.6).

Detection of stool antigen test, had shown sensitivity of 93.8% and 63.6% for specificity with 68.5% for positive predictive value and 63.6 % For Negative predictive value as mentioned in table (4.7) using PCR as Golden Standard.

correlation of ICT and semi nested PCR with clinical symptoms tabulated in table (4.8).

Frequency of CagAgene among study population by technique (PCR)

The frequency of *CagA* gene among Infected Patient Was 58.5% (38/65) using PCR Listed in Table(4.9):with Product Size 349 bp as Shown in Fig (4.2).

Concerning relationship Between age and presence of CagA gene, there are no Statically correlation significant With (P.Value 0.532) observed as mentioned in table (4.10). at the same time with gender showed strong Statically correlation significant with (p.value0.834) as showed in table (4.11).

Correlation of infection and CagA gene and clinical symptoms listed in Table (4.12).

Table (4.1):Distribution of study population according to gender

Gender	Frequency	Percent %
Male	60	60%
Female	40	40%
Total	100	100%

Table (4.2): Distribution of gender according to age group

Age group	Gender		Total
	Male	Female	
15 – 40	23%	15%	38%
41 – 65	37%	25%	62%
Total	60%	40%	100%

Table (4.3):Distribution of symptoms among study population

Total	Symptoms
29%	Abdominal pain
25%	Vomiting
22%	Heart pain
18%	Nausea
6%	Combination of symptoms
100%	**Total**

Table (4.4): Frequency of *H.Pylori* infection among study population using(ICT&PCR)techniques

Technique		Result
PCR	ICT	
65%	89%	Positive
35%	11%	Negative
100%	100%	Total

High positive result detected by ICT stool antigen in comparison to PCR resulting from false positive detection by ICT.

Fig (4.1): Detection of *H.pylori* DNA in stool samples by semi nested PCR and analysis of the amplification product (209 bp) byagarose gel electrophoresis and ethidium bromide staining. L (ladder marker 100 bp), Lane1 (Negative control) Lane 2 (Positive control). Lanes (4,5 and 8) positive samples. And Lanes (6, 7, 9 and 10) negative samples,

Table (4.5): Relationship between gender groups and presence of *H. Pylori* infection

Gender	ICT test		PCR for *H.pylori*	
	Positive	Negative	Positive	Negative
Male	53%	07%	22%	22%
Female	36%	04%	43%	13%
P. value	0.794		0.466	

Table (4.6): Correlation between ages and *H. Pylori* infection

Age group	ICT		PCR for *H. Pylori*	
	Positive	Negative	Positive	Negative
15-40	31%	07%	38%	16%
41-65	58%	04%	27%	19%
P.value	0.027		0.001	

Table (4.7): Specificity &Sensitivity of ICT Using PCR as Golden Test

PCR			
Negative	**Positive**		
28^{B}	61^{A}	Positive	ICT
7^{D}	4^{C}	Negative	

True positive (A) False positive (B)

False negative (C) True negative (D)

SENSETIVITY = A/A+C*100

SPECIFITY = D/D+B*100

Positive predictive value = A/A+B*100

Negative predictive value = D/D+C*100

Sensitivity = 93.8%Specificity =63.6%

Positive predictive value =68.5%Negative predictive value =63.6%

Table (4.8): Correlation of ICT and Seminested PCR with clinical symptoms

Symptoms	ICT		PCR	
	Positive	Negative	Positive	Negative
Abdominal pain	25	4	20	9
Vomiting	22	3	15	10
Heart pain	19	3	12	7
Nausea	16	2	12	6
Combination of symptoms	6	0	6	0
Total	100		100	
P.value	.472		.005	

Table (4.9): Frequency of CagAgene among study population by technique (PCR)

Techniques	Positive	Negative	Total	Percent%
PCR	38	62	100	100%

Fig (4.2) Detection of *cagA* gene in stool samples by PCR and analysis of the amplification product (349 bp) in agarose gel electrophoresis and ethidium bromide staining lines: L 100 bp ladder marker, (1)negative control, (2) positive control & (4-7) infected patients

Table (4.10): Correlation of CagA gene among Age group

Total	CagA		Age Group	
	Positive	Negative		
38	16	22	15 – 40	
62	22	40	41 – 65	
100	38	62	Total	
0.532			P.value	

Table (4.11):Correlation of CagA gene Among Gender group

Total	Cag A		Gender group	
	Positive	Negative		
60	22	38		Female
40	16	24		Male
100	38	62	Total	
0.834			P.Value	

Table (4.12): Correlation of infection and CagA gene and clinical symptoms

Symptoms	CagA	
	Positive	Negative
Abdominal pain	14	15
Vomiting	8	17
Heart pain	3	19
Nausea	10	8
Combination of symptoms	6	0
Total	100	
P.value	.472	

Chapter Five

Discussion

Conclusion

Recommendations

5. Discussion

H. pylori is one of the most common chronic infections of humans and have a worldwide distribution. More than 50% of the world's populations are colonized by H. *Pylori*.[79]

The prevalence of *H. Pylori* infection varies widely according to geographical area, patient's age and socioeconomically status and the rates of infection range between70-90% in developing countries and 25-50% in developed.[80]However, there are no reliable data on this infection in Shendi. This study was performed to detect the frequency of *H. Pylori* among 100 patients present with upper gastroduodeneal symptoms from outpatient clinic in Shendi, 60 (60%) males and 40 (40%)female, the age between (15- 65yrs) with mean of (37.2±11.8yrs), also to detect the presence of *CagA* gene, the most familial virulent gene for H. *Pylori* pathogen city.

In this study patient's symptoms, 29/100 presented with abdominal pain, 25/100 with Vomiting,18/100 with Nausea, 6/100 with combined symptom. The frequency of *H. Pylori* among patients show to be 89% and 65% by ICT and PCR respectively, used PCR as gold standard. In this study the rate of *H. pylori* infection was nearly to study in eastern Sudan 80%.[81] alsoconsistent with prevalence in Africa (70.1%) Nigeria (87.7%)[82]. and high in compared with U.S.A and Australia which have a low prevalence of *H. pylori* (35.6% and 24.6% respectively).[82]*H. pylori* has been detected in individuals of all ages throughout the world and its prevalence ranges between 20%-80%.[83]

However, if concentration of antigen becomes low, false negativity may also be reported. Perri et al.[84]compared the performance of antigen detection vs UBT in 458 dyspeptic patient and reported discrepancy in 8% of the case. They suggested that antigen detection was less accurate.[84]Despite all the above observation on performance of antigen detection *H. pylori* in stool, it has certain disadvantage: antigen excretion may vary over the time period

and antigen may degrade while passing through intestine.[85] Cut off titer, though difficult to decide but crucial to reach the conclusion by using antigen detection technique. However, stool antigen detection using monoclonal antibody has been recommended as it gives equivalent diagnosis accuracy.[86]

The advantage of antigen detection test is to evaluate the eradication of *H. pylori* infection. This indicates PCR assay with nested or seminested primers is more sensitive and specific for detecting *H. pylori* DNA in stool sample than ICT and used as a gold standard for detecting infection and even used to follow up after treatment. [87]

PCR is regarded as a highly sensitive method to detect DNA of *H. pylori* from different clinical samples.[88]and provide useful information concerning the presence of genes encoding specific virulence factors and antibiotic resistance.[89] Both sensitivity and specificity of nested PCR has been reported to be 100%.[90]in contrast, the sensitivity and specificity of fecal antigen have been found to be 67%-100% and 61%-100%.Moreover, PCR based techniques have been very successfully used in specimens of stool .[91]

In regard to correlation of presence of infection and age, our study show highly statistically significant with (P=0.001), On the other hand there is no statistically significant with gender P.value (0.466). Our result agree with study done by *Inelmen*in University Hospital of Kiel[62]., and disagree with study done by *Saleh* in Tabriz University of Medical Sciences[64]. Concerning gender, the study revealed that the most patients are male indicating that more affected with *H.pylori*, gender is one of risk factor for H *.pylori* infection.[92]

In our study ICT show (93.8%) sensitivity and (63.6%) specificity with(68.5%) positive predictive value and(63.6%) for negative predictive value different to study done in Sudan by Mohammed Shams Alfalah.[93]

,ICT show (100%) Sensitivity, (96.1%)Specificity, positive predictive value (93.3%), and (100%) for negative predictive value[93], another study concluded by Vaira et al.[94] was observed the sensitivity of 94.1% and specificity of 91.8%.

Regarding detection the present of *H.pylori* and correlation of ICT and semi nested PCR with Symptoms, there was no statistical correlation with (P.value 0.472) and with Significant Statistical Correlation with (P.value 0.005) .

Regarding *CagA* gene, our study present 58% (35/65) this agree with study done by *Sicinschi*.[95]*H. pylori* strains containing the CagA gene are known to cause more extensive inflammation in the stomach mucosa and antibodies against *CagA* persist long after eradication.[96]High prevalence of cagA gene found in U.S.A represent (85%), (93%)in Nigeria, and(96%)in Indian population.

In this study there are no statistical correlation between present of CagA gene and age, gender, symptoms with (P.value0.532, 0.834,0.472) respectively this agree with study done by Nogdalla at Sudan University of Science & Technology. [75]

Lastly Helicobacter pylori releases cytotoxin associated gene A(CagA) is more virulent and involved in the formation of cholesterol patches in arteries, induction of autoimmune disorder, and release of immune mediated response.[97]

So that properly managed to prevent chronic complication.

Conclusion

On the basis of this study we concluded that:

High rate of *H. pylori* infection and *cagA* gene was found among study population.

Seminested PCR is a specific and sensitive method for detecting *H. pylori* DNA in stool samples in compare with ICT.

Significant correlation showed between *H.pylori* infection and age, while no statistical significant correlation with gender was observed.

Recommendations

- Another broader study in this region assessing the association of different demographic and lifestyle factors is needed.
- Stool test used for the screening diagnosis of *H. pylori* infection.
- PCR with nested primers recommended for confirmation detection of *Helicobacter pylori* infection.
- A new generation of stool antigen kits must be developed using monoclonal antibodies to give comparable accuracy for the test.
- Future study with large sample sizeto detect the differ virulence factors using advancing techniques.
- Using other samples like biopsy.

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Appendices

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Appendix I

بسم الله الرحمن الرحيم

Shendi university

College of post graduate studies

Questionnaire about Detection of Helicobacter pylori and CagA gene in stool samples from patients suspected with upper gastroduodeneal diseases in Shendi City

Name ……NO( )

Age …….. year

( ) Gender : male ( ) female

Symptom

( ) Heart pain ( ) Vomiting Abdominal pain ( )

( ) other Nausea ( )

'Duration of symptoms

( ) more than month ( ) 1 – 4 week ( ) 4 -7 day

Other disease

( )Diabetes mellitus ( ) cancer ( ) cardiac disease

Previously use the treatment of helicobacter pylori

( ) No Yes( )

Appendix II

إقـــرار بالموافقـــة

الاسم :----------

العمر :---------- العنوان :----------

أوافق بمحض إرادتي بالمشاركة في البحث العلمي المتعلق بالكشف عن بكتريا هيليكوباكتر بيلوري والجين المرتبط السام للخلايا في عينات البراز من المرضي المشتبه بهم بالإصابة بمرض المعدة والأمعاء العلوي في مدينه شندي.

أمل عبد الحفيظ سعيد محمد علي

بعد أن شرح لي بأنه لا يترتب عليه أي أذى جسدي أو نفسي واعلم أن المشاركة في هذا البحث لن تؤثر بأي حال من الأحوال في الرعاية الطبية التي أتلقاها كما أنه يحق لي بدون إبداء أسباب الانسحاب من هذا البحث في أي مرحلة من مراحله

البحث بإشراف:

د. هاديه عباس الطيب احمد

التوقيع : ---------- التاريخ