Sudan University of Science and Technology

College of Graduate Studies

Molecular Characterization of *Helicobacter pylori* Virulence Genes and Antimicrobial Resistance Genes in Gastric Biopsies among Symptomatic Patients, Khartoum State-Sudan

التوصيف الجزيئي لجينات عوامل الضراوة والجينات المقاومة للمضادات الحيوية للبكتريا الحلزونية في خزعات المعدة بين المرضى الذين تظهر عليهم الأعراض، ولاية الخرطوم-السودان

A thesis Submitted for Fulfillment of Requirements for the Ph.D. Degree in Medical Laboratory Science (Microbiology)

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2022

DECLARATION

I declare that this thesis is submitted to Sudan University of Science and Technology, College of Graduate Studies for the degree of doctor of philosophy and has not been previously submitted by me for this degree at this university or any other universities or institutes.

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الآية

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قال تعالى :

(أَمَّنْ هُوَ قَانِتٌ آنَاءَ اللَّيْلِ سَاجِدًا وَقَائِمًا يَحْذَرُ الْآخِرَةَ وَيَرْجُو رَحْمَةَ رَبِّهِ قُلْ هَلْ يَسْتَوِي الَّذِينَ يَعْلَمُونَ وَالَّذِينَ لَا يَعْلَمُونَ إِنَّمَا يَتَذَكَّرُ أُولُو الْأَلْبَابِ)

صدق الله العظيم

سورة الزمر - آية 9

DEDICATION

This work is completely dedicated to

my respectful parents
and
beloved husband

without whose constant support this thesis was not possible

Maram

Acknowledgments

Firstly, thanks to ALMIGHTY ALLAH for helping me to complete this work. I would like to thank my supervisor Prof. Yousif Fadalla Hamedelnil for his dedicated support and guidance. I also would like to thank my co –supervisor Dr. Hisham Nouraldayem Altayb for providing encouragement and was always willing and enthusiastic to assist in any way he could throughout the research project.

Finally, many thanks to all participants that took part in the study and enabled this research to be possible.

Abstract

Helicobacter pylori is a gastrointestinal bacterium that causes peptic ulcer and stomach cancer in about half of the world's population. The outcome of *H. pylori*-related disorders is influenced by many virulence factors. Clarithromycin- resistant *H. pylori* strains represent a worldwide health problem. This is descriptive cross sectional study aimed to detect the frequency of *H. pylori*, the virulence factors of *H. pylori*, and clarithromycin resistant associated mutations in Sudanese patients with gastritis symptoms. Also to study molecular characterization of *H. pylori* virulence genes and antimicrobial resistance genes by PCR and sequencing.

Gastric biopsies were obtained from 384 patients with gastritis symptoms from different hospitals in Khartoum State from December 2018 to January 2021, their ages ranged from 14 to 88 years and mean age of 43 years. Out of them, 209 (54.4%) were males, while 175 (45.6%) were females. All gastric biopsies were subjected to polymerase chain reaction (PCR) to detect *H. pylori 16S rRNA* gene, virulence genes (*cagA*, *cagE*, *vacA*, *iceA1*, and *dupA*), and Clarithromycin resistance genes. Allele-specific PCR and DNA sequencing were used to screen for the presence of A2142G and A2143G point mutations.

From 384 patients, two hundred sixty-nine (70.0%) patients were diagnosed with gastritis which was found to be the most common endoscopic findings, thirty-eight (9.9%) as a gastric ulcer, twenty-eight (7.3%) as a duodenal ulcer, sixteen (4.2%) as esophagitis and thirty-three (8.6%) were of normal mucosa.

H. pylori was detected in 28.4% (109/384) specimens by PCR using specific *H. pylori 16S rRNA*, there was no significant association between the presence of *H. pylori*, socio-demographic data (gender, age groups, and geographical distribution), and clinical outcome among study population.

The positive specimens were genotyped using PCR targeting *cagA*, *cagE*, *vacA*, *dupA*, and *iceA1* genes. All of strains were *vacA* positive 100% (109/109) followed by *dupA* 44.0% (48/109), *cagA* 38.5% (42/109), *cagE* gene 37.6% (41/109), and *iceA1* gene was detected in only 18.3% (20/109). The *vacA s1/m1* 70% (77/109) was the most prevalent *vacA* subtype. There was no significant difference between the presence of *H. pylori* virulence genes in regards to gender with different age groups. Although there was no significant association of *H. pylori cagA*, *cagE*, *iceA1*, and *vacA s/m* status according to endoscopic findings, the *dupA* gene was significantly associated with the clinical outcome (*p*. value of 0.016).

Allele-specific PCR detected the variant A2142G in the 9/53 (~ 17%) specimen, while A2143G mutation was not found in any specimen. The DNA sequencing revealed the presence of mutations associated with clarithromycin-resistance in 36% (9/25) of samples; the A2142G was present in one sample, A2143G in 5 samples, and T2182C in 4 samples. Additionally, another point mutation (C2195T) was detected in 3 samples.

The study conclude *H. pylori* virulence genes were extremely prevalent and diverse among Sudanese gastritis patients. *H. pylori dupA* gene was associated with the clinical outcome. A high frequency of mutations associated with clarithromycin resistance using DNA sequencing of the *23S rRNA* gene's V domain. This information should be taken into consideration to avoid eradication therapy failure.

المستخلص

جرثومة المعدة هي بكتيريا معوية معدية تسبب قرحة الجهاز الهضمي وسرطان المعدة في حوالي نصف سكان العالم. نتائج الإضطرابات المرتبطة بجرثومة المعدة تتأثر بالعديد من عوامل الضراوة. تمثل سلالات جرثومة المعدة المقاومة للكلاريثروميسين مشكلة صحية عالمية. هدفت هذه الدراسة الوصفية المقطعية إلى الكشف عن إنتشار بكتيريا جرثومة المعدة وعوامل الضراوة والطفرات المصاحبة لمقاومة كلاريثروميسين في المرضى السودانيين الذين يعانون من أعراض التهاب المعدة. أيضا لدراسة التوصيف الجزيئي لجينات عوامل الضراوة والجينات المقاومة للمضادات الحيوية بواسطة تفاعل البلمرة التسلسلي و التسلسل الجيني.

أخذت خزعات المعدة من 384 مريضاً لديه أعراض التهاب الجهاز الهضمي في مستشفيات مختلفة بولاية الخرطوم من ديسمبر 2018 إلى يناير 2021، تراوحت أعمارهم بين 14 إلى 88 عاماً ومتوسط أعمارهم 43 سنة. من بينهم 209 (54.4٪) ذكور، 175 (45.6٪) إناث. خضعت جميع خزعات المعدة لفحص تفاعل البلمرة التسلسلي للكشف عن جين جرثومة المعدة16S rRNA وجينات الضراوة (cagA، cagE، vacA، iceA، و dupA) وجينات مقاومة كلاريثروميسين. تم استخدام تفاعل البلمرة التسلسلي الأليلي الخاص و التسلسل الجيني للكشف عن وجود طفرات نقطية A2142G و A2143G .

من بين 384 مريضًا، شخصت مائتان وتسعة وستين (70.0٪) من المرضى بإلتهاب المعدة الذي وجد أنه أكثر نتائج المنظار الداخلي شيوعًا، وثمانية وثلاثون مريضًا (9.9٪) قرحة معدة، وثمانية وعشرون مريضًا (7.3٪) قرحة الإثني عشر، وإلتهاب المريء في ستة عشر (4.2٪) مريضًا و ثلاثة وثلاثون مريضًا (8.6٪) كانت من الغشاء المخاطي الطبيعي.

تم اكتشاف جرثومة المعدة في 28.4٪ (384/109) عينة بواسطة تفاعل البلمرة التسلسلي باستخدام جرثومة المعدة 16S rRNA، لم يكن هناك إرتباط إحصائي بين وجود البكتريا الحلزونية، والبيانات الاجتماعية (الجنس، الفئات العمرية، والتوزيع الجغرافي)، والنتائج السريرية بين مجتمع الدراسة.

أجري التنميط الجيني للعينات الإيجابية باستخدام تفاعل البلمرة التسلسلي لجينات cagA و cagE و vacA و dupA و iceA1 . كانت جميع السلالات تحمل vacA بنسبة 100٪ (109/109) متبوعة بـ dupA 44.0 (48/109)٪، cagA جين38.5 (42/109)٪ ، cagE جين37.6 (41/109) ٪ ، وتم إكتشاف جين iceA1 فقط في 18.3٪ (109/20). كان vacA s1 / m1 70 ٪ (109/77) هو النوع الفرعي الأكثر انتشارًا. لم يكن هناك فرق إحصائي بين وجود جينات الضراوة للبكتريا الحلزونية فيما يتعلق بالجنس مع مختلف الفئات العمرية. على الرغم من عدم وجود إرتباط إحصائي بين الجينات cagA و cagE و iceA1 و vacA s / m وفقًا لنتائج المنظار الداخلي. إرتبط جين dupA بشكل كبير مع النتيجة السريرية (*p*. value of 0.016).

إكتشف تفاعل البلمرة التسلسلي الأليلي الخاص المتغير A2142G في 53/9 (17٪ تقريبًا) عينة ، بينما لم يتم العثور على طفرة A2143G في أي عينة. كشف التسلسل الجيني عن وجود طفرات مرتبطة بمقاومة الكلاريثروميسين في 36٪ (25/9) من العينات ؛ كان A2142G موجودًا في عينة واحدة ، A2143G في 5 عينات و T2182C في 4 عينات. بالإضافة إلى ذلك ، تم اكتشاف طفرة نقطية أخرى (C2195T) في 3 عينات.

خلصت الدراسة الي أن جينات ضراوة جرثومة المعدة منتشرة للغاية ومتنوعة بين المرضى السودانيين الذين يعانون من التهاب المعدة. إرتبط جين dupA لجرثومة المعدة بالنتيجة السريرية. هنالك تكرار

مرتفع للطفرات المرتبطة بمقاومة كلاريثروميسين بإستخدام تسلسل الجيني للمجال V للجين23S rRNA.

يجب أن تؤخذ هذه المعلومات في الاعتبار لتجنب فشل الإبادة بالعلاج.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

GC	Gastric Cancer
MALT	Mucosa-Associated lymphoid tissue
VacA	Vacuolating Cytotoxin A
CagA	Cytotoxic-associated gene A
Cag E	Cytotoxin associated gene E
DupA	Duodenal ulcer promoting gene A
IceA	Induced by contact with epithelium A
BabA	Blood group antigen binding Adhesion
H and E	Hematoxylin and Eosin
CLO	Campylobacter – Like Organism
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked Immunosorbent Assay
IgG	Immunoglobulin G
PCR	Polymerase Chain Reaction
PBS	Phosphate Buffer saline
PPIs	Proton Pump Inhibitors
SPSS	Statistical Package for Social Science
UBT	Urea Breathe Test

CHAPTER ONE

1 INTRODUCTION

1.1 Introduction

Helicobacter pylori (*H. pylori*) is one of the most important human pathogenic microorganisms with single polar, multiple flagellum and spirally curved Gram-negative bacilli belongs to the micro aerobic bacteria, with a high infection rate and a strong association with stomach and duodenal diseases (Canwat, 2021; Liu *et al.*, 2021; Xu *et al.*, 2021). *H. pylori* has a significant correlation with chronic gastritis (gastric mucosal atrophy and erosion), peptic ulcer, MALT (mucosa-associated lymphoid tissue) lymphoma and gastric cancer (GC) (Liu *et al.*, 2021), which is the second leading cause of cancer death worldwide and is closely related to the host genetic, environment and virulent strains of *H. pylori* (Oktem-Okullu *et al.*, 2020; Chen *et al.*, 2021; Liu *et al.*, 2021). The World Health Organization's International Agency for Research on Cancer (IARC) has classified *H. pylori* as a type I (definite) carcinogen since 1994 (Chen *et al.*, 2021).

H. pylori infection is a global health problem. The prevalence rate of *H. pylori* differs between the developed countries that have low prevalence (20% to 50%), while in developing countries that have a high prevalence, the infection rate of middle-aged people has reached 80%. The fecal–oral and the oral–oral routes are considered as the main transmission routes of *H. pylori* (Ansari and Yamaoka, 2019; Oktem-Okullu *et al.*, 2020; Chen *et al.*, 2021). Children under 5 years of age represent a key population for transmission of *H. pylori*. However, the infection rate is positively correlated with age and is a cumulative process. Among individuals aged ≥ 19 years, the infection rate can reach 75% (Oktem-Okullu *et al.*, 2020; Xu *et al.*, 2021).

Several virulence factors of *H. pylori* such as secretion systems, cytotoxins and flagella are contributed to the pathogenicity. Urease is an important one and is critical for bacterial colonization of the human gastric mucosa (De la Cruz *et al.*, 2017). Some researchers hypothesized that "there are differences in the *in vivo* expression of *H. pylori* virulence-associated genes in patients with different clinical manifestations, which might result from the response to physical or chemical differences in the gastric environment or perhaps even be related causally to the development of disease and clinical outcomes " (Avilés-Jiménez *et al.*, 2012; Ansari and Yamaoka, 2019; Hedayati and Salavati, 2021; Muñoz-Ramirez *et al.*, 2021).

The most important virulence genes include vacuolating cytotoxin A (*vacA*), cytotoxin associated protein A (*cagA*), cytotoxin associated gene E (*cagE*), induced by contact with epithelium A (*iceA*), duodenal ulcer promoting gene A (*dupA*), and blood group antigen-binding adhesin (*babA*) have been identified (El-Shenawy *et al.*, 2017; Liu *et al.*, 2021). *CagA* and *VacA* are the two important determinants of *H. pylori* toxicity, inducing the damage of epithelial cells and chronic inflammation, which may ultimately lead to GC (El-Shenawy *et al.*, 2017; Liu *et al.*, 2021).

Diagnosing *H. pylori* infection is sometimes difficult, but accurate diagnosis is essential for the effective treatment and management of infections. It can be diagnosed by invasive (requiring endoscopy and biopsy which include, culture, histological examination, and rapid urease test, CLO (Campylobacter like organism) test, smear examination, and molecular studies) or noninvasive techniques (including serology, respiratory urea breath test, or the detection of fecal antigen) (Kalali *et al.*, 2015; Mawlood *et al.*, 2019). Endoscopy is warranted for dyspeptic patients with accompanying alarm symptoms such as weight loss, persistent vomiting, gastrointestinal bleeding, and abdominal mass or iron-deficiency anaemia. In addition, endoscopy is recommended for patients with new onset dyspepsia above the age of 45 (European guidelines) or 55 (United States guidelines). *H. pylori* infection can be detected in endoscopic gastric biopsy samples by several methods (Smith *et al.*, 2014). The first described method for diagnosis of *H. pylori* infection was the histological techniques which detect the presence of typical spiral motile bacteria accompanied by inflammatory reaction in the histopathological sections of stomach that routinely stained with Giemsa or hematoxylin and eosin (H and E) (Patel *et al.*, 2014; Kalali *et al.*, 2015). *H. pylori* culture is highly specific with lower sensitivity and is not a routine procedure in initial diagnosis because requires a microaerophilic atmosphere and takes a long time. Cultured live *H. pylori* is used for diagnostic approaches and for the detection of antibiotic resistance if treatment failure is suspected (Patel *et al.*, 2014; Kalali *et al.*, 2015; Nevoa *et al.*, 2017).

Molecular methods like Polymerase Chain Reaction (PCR) which is highly specific and sensitive for the diagnosis of *H. pylori* from gastric biopsy, saliva and stool specimen, as well as for detection of virulence and drug resistance genes (Kalali *et al.*, 2015; Ogaya *et al.*, 2015; Nevoa *et al.*, 2017). Recently, next-generation sequencing (NGS) has been applied to clarify the evolution and pathogenicity of *H. pylori*, as well as to identify its novel virulence

factors. Another interesting practical application is the detection of genomic changes related to drug resistance through a comparison of the genomes of wild-type strains and of those that survive antibiotic treatment. Using next-generation sequencing, which able to detect potential mutations throughout the genome of *H. pylori* and therefore identify novel mutations if they exist (Binh *et al.*, 2015; van Vliet and Kusters, 2015).

For the treatment of *H. pylori*, multiple antibiotic regimens have been evaluated including triple therapy (using a proton pump inhibitor (PPI) such as omeprazole combined with clarithromycin and amoxicillin or metronidazole), sequential therapy, quadruple therapy, and levofloxacin-based triple therapy. Antimicrobial susceptibility testing is the best approach for the selection of the treatment therapy method (Sahara *et al.*, 2018; Oktem-Okullu *et al.*, 2020). However, standard triple therapy for *H. pylori* infection has limited efficacy and the reinfection rate is relatively high, because of side effects, poor compliance of patients to therapy, antibiotic overuse and, more importantly, the development of antimicrobial resistance is the major cause of treatment failure (Bıcak *et al.*, 2017; Xu *et al.*, 2021). The fluoroquinolone group of antibiotics (levofloxacin-containing triple therapy) or bismuth-containing quadruple therapy is considered an alternative and is often recommended following failure with first-line therapy (Bińkowska *et al.*, 2018). Antibiotic resistance of *H. pylori* to many of these antibiotics is increasing, with various levels of resistance noted. The rates of resistance are higher in developing countries than in developed countries. However, most of the antibiotic resistance mechanisms described to date are as a result of point mutations on the bacterial chromosome, a consequence of a significantly high mutation rate in *H. pylori* (Gemilyan *et al.*, 2019; Zhang *et al.*, 2021). Clarithromycin (CLR) is one of the most widely used antimicrobials in *H. pylori* eradication regimens which binds to the peptidyltransferase region of the bacterial 23S ribosomal ribonucleic acid (rRNA) and inhibits protein synthesis (Kocsmár *et al.*, 2021). However, CLR Resistance is most commonly caused by point mutations of the (rRNA) gene, the main component of the 50S subunit (Mahmoudi *et al.*, 2017; Hua *et al.*, 2018).

In the Sudan according to published data, a study by Hamid and Eldaif estimated the prevalence of *H. pylori* infection to be 80% among patients with symptoms of gastritis, 56% with duodenal ulcer, while 60% with duodenitis and 16% apparently look normal (Hamid and Eldaif, 2014). Another study in Eastern Sudan showed that high prevalence of *H. pylori* infection was estimated to be 80% among patients with gastritis and Barrett's oesophagus

(Abdallah *et al.*, 2014). Study was conducted by Abdalaziz *et al.*, 2013, to determine the association between *H. pylori* infection and stomach cancer using PCR technique, indicated that *H. pylori* infection is associated with stomach adenocarcinoma (Abdalaziz *et al.*, 2013). In another study 81 gastric biopsies were screened to determine the presence of *H. pylori*. Their results showed higher prevalence of *H. pylori* in patients with gastritis (22.2% yielded positive culture results) (Mamoun *et al.*, 2015). A study conducted by Osman *et al.*, 2016, on 68 patients with symptoms of gastritis attending the Modern Medical Center (Khartoum) were investigated serologically for *H. pylori* infection, using the immunochromatographic assay (ICA) test. A control group of 62 healthy individuals were similarly investigated as a comparative group. *H. pylori* reactivity among gastritis patients was higher (63.2%) than that of the healthy control group (45.2%) (Osman *et al.*, 2016).

1.2 Rationale

The genetic factors have a significant impact on the clinical outcome and anatomical distribution of *H. pylori* infection, so different genotypes of *H. pylori* produce different virulence factors and polymorphisms in several genes are considered to increase the risk for the development of GC (Huang *et al.*, 2016; Reshetnyak and Reshetnyak, 2017; Jonaitis *et al.*, 2021; Zeyaullah *et al.*, 2021). In Sudan and probably many of the third world countries the cost of diagnosis plays a major role, rather than the accuracy of the diagnostic method. Hence, diagnosis of *H. pylori* infections is largely based on serology, detection of stool antigen and rarely endoscopy and culture. The aims of this study is to investigate *H. pylori* infection in Sudan and determine the possible association between *H. pylori* virulence genes and their relationships with clinical outcomes.

The eradication of *H. pylori* may reduce the risk of gastric cancer, however, clarithromycin is drug of choice for treatment of *H. pylori* infections in many countries, but there are increased reports of recurrences of infection due to increasing prevalence of *H. pylori* resistant strains and this affects eradication of *H. pylori* infection, and may lead to serious problems for the treatment of *H. pylori*-related disorders (Suzuki and Mori, 2018; Argueta *et al.*, 2021; Kuo *et al.*, 2021). Knowledge about the mechanisms of resistance may help in use new strategies of antibiotic combinations, with the aim of improving treatment success and outcomes. The aim of this study was to determine the H. pylori resistance to clarithromycin in Sudanese patients with gastritis symptoms.

1.3 Objectives

1.3.1 General objective

To study the virulence genes and antimicrobial resistance genes of *Helicobacter pylori* in gastric biopsy among symptomatic patients in Khartoum State.

1.3.2 Specific objectives

1. To detect *H. pylori 16S rRNA* gene from gastric biopsy specimens using conventional PCR methods.
2. To detect the presence of *H. pylori vacA, cagA, cagE, iceA,* and *dupA* genes using Polymerase Chain Reaction (PCR) technique.
3. To detect *H. pylori* clarithromycin resistant genes by PCR and DNA sequencing.
4. To correlate between the virulence genes of the bacteria and the occurrence of *H. pylori* associated diseases.
5. To compare the isolated *Helicobacter pylori* sequences with *Helicobacter pylori* reference strains located in Gene bank.
6. To determine phylogenetic relationship among the isolated *Helicobacter pylori* and other *Helicobacter pylori* in Gene bank.

CHAPTER TWO

2 LITERATURE REVIEW

2.1 Historical background

The accumulated scientific data can confirm that *H. pylori* infection is important in the mechanism of Peptic Ulcer Disease (PUD) development. *H. pylori* was first reported in 1875 when Bottcher and Letulle observed it on the margins of peptic ulcers. The bacterium spiral-shaped Gram-negative rods organisms resembling *Campylobacter* spp. were found in patients with type B gastritis and did not grow in the artificial nutrient media that were known at that time, and this accidental discovery was long forgotten (Reshetnyak and Reshetnyak, 2017). In 1980, Australian pathologist Robin Warren together with Barry Marshall isolated *H. pylori* from human gastric mucosal biopsy specimens and cultured it in artificial nutrient media. Marshall demonstrated the role of *H. pylori* infection in the development of gastrointestinal diseases in 1983. He drank a culture of the bacterium to prove the etiological role of *H. pylori* in the development of antral gastritis. Thereafter, he developed *H. pylori*-associated antral gastritis (Roesler *et al.*, 2014). After that, many researchers concentrated on the study of *H. pylori*, this organism was originally classified as *Campylobacter* but was subsequently reclassified as a new genus, *Helicobacter* (Murray *et al.*, 2020). There has been gradually increasing evidence that duodenal ulcers and duodenitis are also associated with *H. pylori* infection. In 2005, Warren and Marshall received the Nobel Prize in Physiology or Medicine for the discovery of *H. pylori* pathogenicity and rekindled interest in the study of this microorganism. Since then, the association of *H. pylori* with digestive system diseases has been the subject of much research attention (Reshetnyak and Reshetnyak, 2017; Murray *et al.*, 2020).

2.2 Definition

Helicobacter pylori is a flagellate Gram-negative, S-shaped or slightly spirally curved shaped micro- aerophilic bacterium belong to Epsilon proteobacteria, found on the luminal surface of the gastric epithelium that demonstrates bluntly rounded ends in gastric biopsy specimens (Roesler *et al.*, 2014). *H. pylori* is 2.5–5.0 mm long and 0.5–1.0 mm wide, with four to six polar-sheated flagella, which are essential for bacterial motility (Roesler *et al.*, 2014).

2.3 Classification

The genus *Helicobacter* (helix and bacteria) is heterogeneous. Majority of *H. pylori* colonize mammalian stomach or intestines. The *Helicobacter* genus now includes at least 26 formally

named species, and more that are still being studied. Some of them were previously known by other names (Reshetnyak and Reshetnyak, 2017).

Humans have been found to have only 11 *Helicobacter* species: *H. pylori*, *H. heilmannii*, (formerly known as *Gastrospirillum hominis*) and *H. felis* in the GM, *H. cinaedi*, *H. westmeadii*, *H. canadensis*, *H. fennelliae*, *H. canis*, *H. pullorum*, and *H. rappini* (formerly known as *"Flexispira rappini"*) in the small intestinal mucosa (Tille, 2014; Reshetnyak and Reshetnyak, 2017). Some *Helicobacter* species have been isolated from the human hepatobiliary system: *H. pylori* from the liver, *H. bilis*, *H. pullorum*, and *H. rappini* from the bile ducts. *H. pylori* is the best known bacterium. *H. pylori* includes several strains. *H. pylori* strains isolated from unrelated humans exhibit a high level of genetic diversity (Reshetnyak and Reshetnyak, 2017). Currently, 35 species have been characterized according to sequence analysis of their *16S rRNA* genes, but this taxonomy is changing rapidly (Murray *et al.*, 2020).

2.4 Physiology and Antigenic structure

Thirty-seven degrees Celsius and pH 4.0-6.0 are the most favorable conditions for the life, growth, and reproduction of *H. pylori*; although, the species also survives at pH 2.5. *H. pylori in vivo* and under optimum *in vitro* conditions exists as an S-shaped bacterium with 1 to 3 turns, 0.5 μm × 5 μm in length (Reshetnyak and Reshetnyak, 2017). *Helicobacter pylori* were motile with a rapid corkscrew-like or slower wave-like motion due to the presence of bipolar tuft of 10 to 14 sheathed flagella (van Duynhoven and Jonge, 2001). The flagellum of *H. pylori* is 30 nm in diameter, consisting of an internal filament approximately 12 nm in diameter surrounded by a sheath, the outer membrane of which is continuous with the outer membrane of the cell. Unipolar flagella are essential for the unique motility of *H. pylori*. Remarkably, the unipolar flagella of *H. pylori* are driven by one of the largest flagellar motors found in bacteria (Reshetnyak and Reshetnyak, 2017). The flagellar motor provides higher torque needed by the bacterium to navigate the viscous environment of the human stomach. Thin sections of *H. pylori* reveal the typical cell wall detail of a Gram-negative bacterium that consists of outer and inner, or plasma, membranes separated by the periplasm of approximately 30 nm thickness 9 to 11 periplasmic fibers or electron-dense glycocalyx or capsule-like layer (Reshetnyak and Reshetnyak, 2017).

H. pylori is mainly present as a spiral-shaped form in human gastric biopsy specimens. On aging, the bacterial cells lose their typical spiral-shaped form and convert to coccoid ones.

When influenced by adverse factors (temperature or pH changes, prolonged fasting when cultivated, or use of antibacterial drugs), non-spore-forming microorganisms can be transformed into a latent coccoid form. The ability of *H. pylori* to transform from the spiral shaped form to the coccoid form is one of its most important, but not exclusive properties. Through the course of evolution, *H. pylori* has evolved special adaptive mechanisms and acquired vital physiological properties allowing it to survive extreme situations in the human organism, when cultivated, and to survive in the external environment (Rajesh and Rattan, 2008; Reshetnyak and Reshetnyak, 2017).

Helicobacter pylori produce an abundance of urease enzyme. These properties are believed to be important for survival in gastric acids and rapid movement through the viscous mucus layer towards a neutral pH environment. Most *H. pylori* are catalase and oxidase-positive and do not ferment or oxidize carbohydrates, although they can metabolize amino acids by fermentative pathways (Murray *et al.*, 2020). Lipopolysaccharide (LPS), consisting of lipid A, core oligosaccharide, and an O side chain, is present in the outer membrane. *H. pylori* lipid A has low endotoxin activity compared with other Gram-negative bacteria, and the O side chain is antigenically similar to the Lewis blood group antigens, which may protect the bacteria from immune clearance (Murray *et al.*, 2020) .

Electron dense granule bodies have been observed in *H. pylori*, and these granules are mainly polyphosphate granules, localized to three different regions: the cytoplasm, the flagella pole and the cell membrane, it may serve as a source of energy (Wahab *et al.*, 2015).

H. pylori is microaerophilic in nature, depending on culture conditions, it requires 5-10% oxygen and 5 to 10% carbon dioxide for optimum growth in a culture medium. It grows well at a temperature of 30-37°C all these requirements are fulfilled in the gastrointestinal tract of mammals (Wang *et al.*, 2015). Numerous studies have shown that *H. pylori* uses both aerobic respiration and fermentation pathways. Complete genome sequencing and studies of *H. pylori* metabolism and physiology indicate that *H. pylori* uses glucose as its primary energy and carbon source by the Entner-Doudoroff and pentose phosphate pathways (Park *et al.*, 2016).

H. pylori anaerobically produces lactate and acetate from pyruvate or aerobically produces acetate or CO_2. *H. pylori* metabolizes pyruvate by the anaerobic mixed acid fermentation pathway, accumulating alanine, lactate, acetate, formate, and succinate. It also uses the tricarboxylic acid cycle, which appears to be a noncyclic, branched pathway characteristic of

anaerobic metabolism that produces succinate in the reductive dicarboxylic acid branch and α-ketoglutarate in the oxidative tricarboxylic acid branch. *H. pylori* constitutively expresses the aerobic respiratory chain with a *cbb*3-type cytochrome c oxidase as the terminal oxidase. Whole genome analysis of two *H. pylori* strains revealed the presence of genes encoding components of the membrane-embedded F0 proton channel and the catalytic F1 complex, suggesting that *H. pylori* produces a significant portion of its ATP by aerobic respiration. In addition, *H. pylori* uses anaerobic respiration utilizing H_2 as an electron donor (Park *et al.*, 2016).

2.5 Virulence genes

As a highly heterogeneous bacterium virulence of *H. pylori* varies geographically. Due to the research in the literature, it is clearly explained that *H. pylori* virulence factors have a very important effect on both bacterial pathogenicity and treatment outcome. Vacuolating cytotoxin gene A (*vacA*), cytotoxin-associated gene A (*cagA*), outer inflammatory protein A (*oipA*), the blood group antigen-binding adhesin gene A (*babA*), neutrophil-activating protein A (*napA*), the putative neuraminyllactose-binding hemagglutinin homolog (*hpaA*), duodenal ulcer promoting gene A (*dupA*), urease A (*ureA*), and urease B (*ureB*) are the most important virulence genes of this bacterium that take the role in the invasion, adhesion, and colonization of *H. pylori* strains in gastric epithelial cells (Oktem-Okullu *et al.*, 2020).

2.5.1 Cytotoxin-associated gene A (*cagA*)

One important virulence factor of *H. pylori* is the *cagA* gene was the first virulence factor detected in *H. pylori* strains (Kadi *et al.*, 2014). The *cag*PAI pathogenicity island (*cag*PAI) is an approximately 40-kb locus composed of about 27 to 31 genes, many of which are responsible for the synthesis of a type IV secretion system (T4SS) that injects the *cagA* oncoprotein into host epithelial cells. Translocated *cagA* alters the cell-cell junctions, motility, and cytoskeleton arrangement and induces a proinflammatory and antiapoptotic gene expression profile via activation of the NF-_B transcription factor. In addition to the *cag*PAI, other genes are relevant for colonization, persistence, and damage to the gastric mucosa, as they encode adhesins (BabA and SabA), proteins that protect *H. pylori* from the oxidative burst (KatA, NapA, and arginase), and the pore-forming cytotoxin *VacA*, which induces *in vitro* epithelial cell vacuolation, inhibition of T cell activation and proliferation, and apoptosis (Avilés-Jiménez *et al.*, 2012; Hammond *et al.*, 2015). One of the *cag*-PAI genes is *cagE*, located in the right half of the *cag*-PAI, have suggested that this gene is a more

accurate marker of an intact *cag*-PAI than other *cag* genes. Another gene that codifies proteins of a type IV secretion systems *virB11*. This gene is located in the left half of the *cag*-PAI. The *VirB11* protein has a ring-shaped structure composed of six monomeric units. It is important for the transportation of protein complexes and exhibits ATPase activity (Lima *et al.*, 2010). Several studies have described an association between *H. pylori cagE* and gastritis, duodenal ulcer, and peptic ulcer disease. Only a few studies have described an association with gastric cancer, but the number of cases included has been small and therefore the test results have often been combined with those of other diseases. In contrast to studies of *cagE*, those related to the gene *vir*B11 are more frequently in vitro, with rare reports in vivo (Lima *et al.*, 2010).

The sequence of the second repeat region of *cagA* was found to differ considerably between East-Asian-type-*cagA* and Westerntype- *cagA*. Each *cagA* is assigned to a sequence type consisting of the names of the EPIYA segments in its sequence (that is, ABC, ABCC, or ABCCC for Western-type- and ABD for East-Asian-type-*cagA*). East-Asian-type-*cagA* has a higher binding affinity for the Src homology-2 domain containing phosphatase 2 (SHP2), resulting in a higher risk of peptic ulcer and/or gastric cancer than Western-type *cagA* (Miftahussurur *et al.*, 2015; Hashinaga *et al.*, 2016; Yuan *et al.*, 2017). The pre-EPIYA region of *cagA*, located about 300-bp upstream of the first EPIYA motif, has also been investigated. Alignment of these sequences revealed that a 39-bp deletion was present in most strains isolated from East Asia, but was absent in most strains from Western countries (no deletion) (Yong *et al.*, 2015; Boonyanugomol *et al.*, 2018).

2.5.2 Vacuolating cytotoxin gene A (*vacA*)

The *vacA* gene, encoding the vacuolating toxin, is considered an important virulence factor and it is present in all strains (Marie, 2012). *VacA* is an *H. pylori* toxin with multiple cellular effects in different host cell types. However, there is significant variation among strains in their capacity to induce cell vacuolization. This variation is attributed to the genetic structural diversity of the *vacA* gene that can assume different polymorphic rearrangements. The initial studies on *vacA* detected two main polymorphic regions; the signal (s)- and the middle (m)-regions. The s- region assumes two forms *s1* or *s2* allele and the m-region encoded the *vacA* *m1* or *m2* allele (Fernández Tilapa *et al.*, 2011). The *vacA* type *s1* strains appear to be more active than *s2* strains and are found more frequently in ulcer disease. The *vacA* *m1* type strains are associated with greater gastric epithelial damage than *m2* strains (Marie, 2012;

Dabiri *et al.*, 2017). The combination of s- and m-region allelic types determines the production of the cytotoxin and is associated with pathogenicity of *H. pylori* strains. *vacA s1/m1* strains produce a large amount of toxin, *s1/ m2* strains produce moderate amounts and *s2/m2* strains produce very little or no toxin (El-Shenawy *et al.*, 2017; Boonyanugomol *et al.*, 2018).

2.5.3 The induced by contact with epithelium A (*iceA*) gene

The *iceA* gene was identified in the *H. pylori* isolated from PUD (Peptic Ulcer Disease) and gastritis patients. There are at least two alleles of *iceA*, *iceA1*, and *iceA2*. The relationship between *H. pylori iceA* and clinical outcomes is controversial. Some studies have suggested that *iceA* (*iceA1*/*iceA2*) may be significantly associated with diseases of digestive system, whereas others showed contrary findings (Huang *et al.*, 2016).

The expression of *iceA1* was upregulated on contact between *H. pylori* and human epithelial cells, and the *iceA1* genotype was associated with enhanced mucosal interleukin (IL)-8 expression and acute antral inflammation (Miftahussurur *et al.*, 2015; Dabiri *et al.*, 2017).

2.5.4 The duodenal ulcer promoting gene (*dupA*)

The *dupA* gene, the first genetic factor of *H. pylori* to be characterized, was reported to be associated with a differential susceptibility to duodenal ulcer (DU) and gastric cancer (Miftahussurur *et al.*, 2015; Dabiri *et al.*, 2017). *DupA* gene was the first putative disease specific marker whose association was described using *H. pylori* strains obtained in both Asian (Japan and Korea) and Western (Colombia) regions (Roesler *et al.*, 2014). This gene increases the survival of the microorganism at low pH values (Reshetnyak and Reshetnyak, 2017).

H. pylori DU promoting gene (*dupA*), located in the plasticity region of *H. pylori* genome, has been initially described as a risk marker for DU development and a protective factor against GC. The *dupA* gene encompasses two continuous sequences, *jhp0917* and *jhp0918*, as described in strain J99. The *jhp0917* gene encodes a protein of 475 amino acids but lacks a region homologous to the C-terminus of *virB4*, whereas *jhp0918* gene encodes a product of 140 amino acids that is homologous to the missing *virB4* region. Originally, it was reported that the presence of *jhp0917– jhp0918* (*dupA* gene) was a marker for the development of DU disease, but some studies demonstrated that this gene can also be associated with GC development. The function of *dupA* gene is not fully understood. It is possible that it acts in combination with other *vir* homologues in the plasticity region to form a type IV secretion

system similar to the *cag*PAI. In addition, it has been associated with increased IL-8 production from the antral gastric mucosa in vivo as well as from gastric epithelial cells in vitro. The gene presence is thought to be also involved in DNA uptake/DNA transfer and protein transfer, and in vitro experiments using *dupA*-deleted and complemented mutants, showing that the absence of *dupA* gene was associated with increased susceptibility to low pH (Roesler *et al.*, 2014).

2.6 Epidemiology and pathogenesis

Helicobacter pylori's primary habitat is the human gastric mucosa. The organism is distributed worldwide. Although acquired early in life in underdeveloped countries and persists life-long in the absence of antibiotic treatment, the exact mode of transmission is unknown (Kadi *et al.*, 2014). It can be vertical (parents to children) or horizontal modes of transmission (environmental contamination) (Bıcak *et al.*, 2017). An oral-oral, fecal-oral, poorly disinfected endoscopes and a common environmental source have been proposed as possible routes of transmission, with familial transmission associated with *H. pylori* infections based on the intrafamilial clustering observed in some studies. Research studies suggest mother-to-child transmission as the most probable cause of intrafamilial spread (Tille, 2014). Children are often infected by a strain, which is a genetic fingerprint identical to that of their parents, and they maintain this genotype even after moving to a different environment (Roesler *et al.*, 2014). In this regard, the practice of good hygiene and improved living conditions become an essential factor in reducing the rate of transmission of the infection (Ofori *et al.*, 2019). A close relationship between an increased prevalence of *H. pylori* infections in a specific geographic area with the particular characteristics of the infecting strain, the number of household members (crowded family) and/or the extension of the breastfeeding period of individuals have been proposed. Also, a strong link between poor socioeconomic (sanitary) conditions and increased *H. pylori* infection in the Latin-American population has been observed (Bıcak *et al.*, 2017; Paredes-Osses *et al.*, 2017).

The finding of strain-specific genes from the comparison of sequenced *H. pylori* strains demonstrates the high diversity of *H. pylori* genome and this high level of genetic diversity can be an important factor in its adaptation to the host stomach and also for the clinical outcome of infection, an aspect that remains unclear. However, it is thought to involve an interplay among the virulence of infecting strains, host genetics, and environmental factors

and experience with other bacterial pathogens suggests that *H. pylori*-specific factors may influence the microorganism's pathogenicity (Roesler *et al.*, 2014; Osman *et al.*, 2016).

Colonization with *H. pylori* invariably leads to histological evidence of gastritis (that is, infiltration of neutrophils and mononuclear cells into the gastric mucosa). The acute phase of gastritis is characterized by a feeling of fullness, nausea, vomiting, and hypochlorhydria (decreased acid production in the stomach). The ulcers develop at the sites of intense inflammation, commonly involving the junction between the corpus and antrum (gastric ulcer) or the proximal duodenum (duodenal ulcer). *H. pylori* is responsible for 85% of gastric ulcers and 95% of duodenal ulcers. Recognition of the role of *H. pylori* has dramatically changed the treatment and prognosis of peptic ulcer disease (Murray *et al.*, 2020).

H. pylori is able to cause severe clinical outcomes such as duodenal and gastric ulcers, and is classified as a carcinogen causing gastric adenocarcinoma and mucosa-associated lymphoid tissue (MALT) lymphoma (Amilon *et al.*, 2015). These more severe clinical outcomes present as ulcers in approximately 10-15 % of all infected individuals, and as gastric cancer in an additional 1-3 %. The incidence rates of these diseases vary world-wide with e.g., considerably higher incidence of gastric cancer in East Asia, Central America and South America. What leads to this divergence in clinical outcome is not entirely known, but both host genetics modulating the immune response towards the infection, as well as bacterial genetics and environmental factors such as smoking and high intake of salt has been shown to play a role (Thorell *et al.*, 2016). Due to poor correlation between symptoms and disease, many of gastric cancer cases are detected lately when the disease is rooted and become incurable (Kadi *et al.*, 2014).

H. pylori is also closely related to some hematological systemic disorders and several autoimmune diseases, including hypoferric anemia, idiopathic thrombocytopenic purpura (ITP), Sjögren syndrome, Graves'disease, and autoimmune pancreatitis. As a result of this association with autoimmune diseases, we hypothesized that *H. pylori* might induce systemic immunological changes (Menoni *et al.*, 2013). Recently, it was demonstrated that it is involved in extragastric diseases such as atherosclerosis, ischemic heart disease, immunologic dysfunction, migraines and pediatric growth retardation (Wang *et al.*, 2015). The majority of the *H. pylori*-infected population remains asymptomatic, and few individuals may develop gastric cancer (Yong *et al.*, 2015; Boonyanugomol *et al.*, 2018).

2.7 Laboratory diagnosis

2.7.1 Non-invasive tests

2.7.1.1 Urea Breath Test (UBT)

The urea breath test is based on the presence of urease enzyme in live *H pylori* which breaks down urea into ammonia and carbon dioxide after ingestion of urea labeled with either ^{13}C or ^{14}C, breath samples are collected for up to 30 minutes by exhaling into a carbon dioxide-trapping agent (Best *et al.*, 2018). The UBT is easy to perform and highly accurate with a specificity and sensitivity of 95% (Smith *et al.*, 2014).

The urea breath test is performed by the clinician or the clinician's assistant. The thresholds used include the percentage of carbon recovered during the collection time or counts per minute. Threshold levels above 4% or 5% are commonly used to diagnose *H pylori* infection. A wide range of threshold counts per minute, ranging from more than 25 counts per minute to 1000 counts per minute, have been used for diagnosis of *H pylori* infection (Best *et al.*, 2018).

Although the 13C-urea breath test shows high sensitivity for adults, its sensitivity is lower for children and patients at the beginning of the disease. Additionally, it requires the use of gas chromatography and mass spectroscopy, which increase its cost (Allahverdiyev *et al.*, 2015). UBT is used to evaluate eradication therapy after giving anti *H. pylori* regimens. The sensitivity is quite good in post therapy and it may be explained by the fact that the UBT gives positive results when other biopsy based tests fail. It is sensitive enough in detecting the infection even in cases of moderate colonization or patchy distribution of *H. pylori*. However, false positive results due to the presence of other urease producing microorganisms are sometimes expected as it has been established that *H. pylori* is not the only bacteria colonizing stomach. However, proton pump inhibitors anti *H. pylori* drugs (PPIs and antibiotics) may produce false-negative results. Further, metabolically inactive coccoid form of *H. pylori* present in the stomach will not give the positive UBT. The other advantage is that UBT could be also used in pediatric patients (Patel *et al.*, 2014).

2.7.1.2 Serology

Immunoblot analysis of serum from *H. pylori* infected patients has revealed that there are several antigens capable of inducing immune response. Some of the prominent antigens include surface and secretary antigens lipopolysaccharides (LPS), *cagA*, different urease components, heat shock proteins, catalase *etc*. Basically *H. pylori* infection is a chronic

condition and therefore IgG response predominates. It is really difficult to pin point acute *H. pylori* and IgM response has rarely been reported Although, IgG response primarily occurs at mucosal surfaces, it can be detected in all elevated in majority of the patients (Patel *et al.*, 2014).

There is significant variability in the accuracy of serology test kits, but validated commercial kits with an accuracy of over 90%, 85% sensitivity and 80% specificity are available (Smith *et al.*, 2014), but are considered insufficient for follow-up of the treatment (Allahverdiyev *et al.*, 2015). However, in difficult situations, where bacterial density is low due to gastric atrophy or therapy using PPI and/or antibiotics, serological methods are useful (Patel *et al.*, 2014).

There are three main methods for these tests: the enzyme-linked immunosorbent assay (ELISA) test, latex agglutination tests, and Western blotting. Of these, ELISA is the most commonly used method. Total immunoglobulin, immunoglobulin subtypes, and antibody response to specific antigens can all be tested. Since they do not require any special equipment, they can be easily performed. However, serology may be positive because of the presence of active infection at the time of the test, previous infection, or because of non-specific cross-reacting antibodies (Best *et al.*, 2018).

2.7.1.3 Fecal antigen test

The *H. pylori* stool antigen test, which is a practicable serological method for detection of the antigen before and after treatment (Allahverdiyev *et al.*, 2015), which displaying specificity and sensitivity of at least 95% (Smith *et al.*, 2014). These tests use monoclonal and polyclonal antibodies to detect the presence of *H pylori* antigen in stools and active *H pylori* infection can be diagnosed. Several thresholds have been used for other tests, for example, an optical density of ≥ 0.15, ≥ 0.16, and ≥ 0.19 have all been used as thresholds for diagnosis of *H pylori* using monoclonal antibodies for stool antigen tests (Best *et al.*, 2018). The risk of false negative results can be obtained from patients that use PPI medications and bismuth derivatives (Allahverdiyev *et al.*, 2015).

2.7.1.4 Polymerase chain reaction (PCR)

While *H. pylori* culture allows an evaluation of antibiotic resistance irrespective of the intrinsic mechanism involved, *H. pylori* is a fastidious bacterium and culture is time-consuming and often difficult with sensitivity values of culture from gastric biopsies as low as 55%-73%. Molecular testing, PCR and real-time PCR are the most frequently used

methods for *H. pylori* offers an attractive alternative to culture and allows for molecular genetics identification of *H. pylori* directly from biopsy samples in addition to culture material (Tonkic *et al.*, 2012). PCR based techniques have been very successfully used in specimens of gastric biopsy, stool, and saliva (Patel *et al.*, 2014).

The targets of these PCR methods include the *16S rRNA* gene, the random chromosome sequence, the 26-kDa species-specific antigen (SSA) genes, the urease (*ureA*) gene, and the *glmM* (*ureC*) gene (Córdova Espinoza *et al.*, 2011). The highly conserved *16S rRNA* gene in bacteria exhibits sequences which are shared by different species of *Helicobacter*, thus compromising its relevant diagnosis of *H. pylori* (Patel *et al.*, 2014). As such, it provides the opportunity for rapid analysis, enabling same-day diagnosis. Molecular testing has been recommended to detect *H. pylori* and both clarithromycin and quinolone resistance when standard culture and susceptibility testing are not possible (Tonkic *et al.*, 2012; Smith *et al.*, 2014).

Several issues are to be considered when choosing a molecular assay for *H. pylori* antimicrobial susceptibility testing, including cost, local expertise in molecular diagnosis, the equipment available and the sensitivity and specificity of the test. Molecular-based techniques are highly accurate in detecting minimal traces of antibiotic resistant *H. pylori* strains, even in small tissue samples. Moreover, these tools are accurate in detecting the co-existence of *H. pylori* strains susceptible and resistant to the same antibiotic within the same patient sample, known as hetero-resistance. This is potentially important given that in one recent study molecular-based tests indicated that in relation to genetic clarithromycin resistance, *H. pylori* infection was cured less frequently in patients with pure resistant strains (46%) than those infected with hetero-resistant strains (78.5%) or susceptible strains (94.5%) (Smith *et al.*, 2014).

Despite the numerous advantages of PCR-based techniques, there are still some challenges regarding its appropriate application in diagnostics. While correct sampling and preparation of samples prior to assay is a common issue in diagnostic assay, sensitivity and specificity of PCR-based assay are strongly dependent on the design of the method. For instance, due to the extraordinary variability of *H. pylori* genome, the selection of the target gene and PCR primer pairs dramatically influence specificity and sensitivity of the test (Kalali *et al.*, 2015). As the whole genome of most known pathogens including many *H. pylori* strains has been successfully sequenced in the last decade, it is very important to design and select the PCR

primers based on a comprehensive bioinformatics analysis of relevant genomes. Here, it is mandatory to design primers according to genomic sequences that are highly conserved in all *H. pylori* strains (Kalali *et al.*, 2015).

There are many modifications of the PCR technology for increasing the sensitivity of detection. The use of nested or semi nested PCR has been suggested using internal primer targeting conserved gene (heat shock protein; *Hsp60*) and increasing specificity and sensitivity up to 100% (Patel *et al.*, 2014).

2.7.2 Invasive tests

2.7.2.1 Endoscopic examination

Endoscopy is often performed to screen for gastric cancer and other diseases. It is also useful for the detailed examination of various epigastric symptoms, positive barium meal studies for gastric diseases, and abnormal serum pepsinogen levels (Shichijo *et al.*, 2017) Additionally, endoscopy is helpful in diagnosing *H. pylori* infection; atrophy, diffuse redness, mucosal swelling, enlarged folds, and nodularity being representative findings for *H. pylori*-positive gastritis. In contrast, a regular arrangement of collecting venules and fundic gland polyps are characteristic of H. *pylori*-negative gastric mucosa, whereas map-like redness is characteristic of gastric mucosa from which *H. pylori* has been eradicated (Kato *et al.*, 2013). A precise endoscopic diagnosis of *H. pylori*-positivity should trigger confirmation by various tests such as measurement of blood or urine anti-*H. pylori* IgG and fecal antigen, urease breath, or rapid urease tests, followed by eradication therapy for the prevention of gastric cancer and other diseases (Shichijo *et al.*, 2019). However, a diagnosis based on endoscopic findings requires training, is time-consuming and subjective, and may result in false-positive and false-negative results depending on the skill of the endoscopist. Patients were placed in the left lateral decubitus position after a 6-8 h fast and upper endoscopy was performed with a typical forward-viewing endoscope. Topical lidocaine was used to anesthetize the patient's oropharynx. Under direct vision, the esophagus was intubated and subsequently, the esophagus, stomach and duodenum were examined. By retroverting the tip of the gastroscope, the gastric fundus could also be viewed (Shichijo *et al.*, 2017).

Chronic gastritis has usually been diagnosed by histological examination. It has long been believed that endoscopic findings correlate poorly with histopathological findings of chronic gastritis. The Sydney System based on this concept was divided into the histological division and endoscopic division. However, recent advances in endoscopy have resulted in

improvement in the diagnosis of chronic gastritis without the need for histological assessment of biopsied specimens. Various endoscopic features associated with gastric inflammation that often accompanies structural mucosal changes are assessed. Endoscopic findings that correspond to histological changes have gradually become clear by using not only white light endoscopy but also image-enhanced endoscopy (IEE). The Kyoto classification of endoscopic gastritis is a novel classification system established in Japan. In this classification system, nineteen endoscopic findings related to gastritis are characterized according to topography and infectious condition of *H. pylori* (Kato *et al.*, 2013).

2.7.2.2 Rapid urease test (RUT)

For routine clinical practice, rapid urease test (RUT) is the most useful invasive test for the diagnosis of *H. pylori* infection because it is inexpensive, rapid, easy to perform, highly specific (has more than 90% specificity and accuracy), and widely available (Tonkic *et al.*, 2012; Nishizawa and Suzuki, 2014; Allahverdiyev *et al.*, 2015). A positive RUT is sufficient to initiate eradication treatment (Tonkic *et al.*, 2012)**.** The sensitivity of RUT is influenced by the bacterial density and the forms (spiral or coccoid) of bacteria present in the biopsy. The minimum of 10^4 organisms per biopsy piece are required for a positive RUT result but a good proportion of patients may harbor lower densities than this. Further, low density of the bacterium post treatment, bleeding patients and patients taking H2- receptor antagonists or proton pump inhibitors may also adversely affect the performance of test (Patel *et al.*, 2014). In addition, this method may also return false-positive results when urease-positive bacteria are present in the specimen. For these reasons, the CLO test has lower sensitivity (Allahverdiyev *et al.*, 2015).

The rapid urease-test for *Campylobacter*-like organisms (CLO) involves placing the biopsy specimen in a solution of urea and pH-sensitive dye (e.g. phenolphthalein) mixed into a gel that contains urea. When a biopsy specimen from a patient who has *H. pylori* infection is tested, bacterial urease hydrolyzes urea into ammonia, water and carbon dioxide and an increase in pH. It is a rapid test; results indicated by the colour of the medium are available immediately (change from yellow to pink or red), often within 20 minutes and always within 24 hours. When two biopsy specimens are tested sensitivity and specificity for the urease test are high (Smith *et al.*, 2014).

Several commercial urease tests, including gel-based tests (CLO test, Hp Fast), paper-based tests (PyloriTek, ProntoDry) and liquid-based tests (UFT300, EndoscHp) are available now,

and different commercial RUTs have different reaction time to provide results. CLO test usually takes 24 h to obtain accurate result, whereas PyloriTek takes 1 h and UFT 300 takes 5 min to provide more rapid results. Reading the urease tests earlier than recommended time may lead to false negative results (Nishizawa and Suzuki, 2014).

2.7.2.3 Histopathology

Histology is considered to be the first and gold standard in the direct diagnosis of *H. pylori* gastritis and has been reported to be the most sensitive method. Histopathology provides a permanent record of the nature and grading of a patient's gastritis as well as detecting *H. pylori* (Allahverdiyev *et al.*, 2015). Several stains like Giemsa, acridine orange, Warthine Starry, Hp silver stain, Dieterle, Giminez, McMullen; and immunostaining are used to detect *H. pylori* (Patel *et al.*, 2014). The presence of typical spiral motile bacteria accompanied by inflammatory reaction in the histopathological sections of stomach was the first described method used for the diagnosis of the *H. pylori* (Kalali *et al.*, 2015). However, the accuracy of the histo-pathological diagnosis of *H. pylori* always depends on the number and the location of collected biopsy materials. Biopsy specimens were collected at five sites according to the Sydney System recommendation (Tonkic *et al.*, 2012; Allahverdiyev *et al.*, 2015).

The biopsy specimens were stained with hematoxylin-eosin (HE staining) as well as conventional Giemsa staining and the presence or absence of *H. pylori* infection was microscopically investigated. Moreover, the possible presence of other bacterial species with a similar morphology to *H. pylori* in the stomach can be another source of error which negatively affects the accuracy of the test. In addition, treatment with proton pump inhibitors (PPI) or antibiotics prior to sampling may transform the shape of *H. pylori* to a coccoid form (Kalali *et al.*, 2015). However, histopathologic detection requires expert pathologists for the accurate examination of the samples (Kato *et al.*, 2013). When the microscopic diagnosis of *H. pylori* infection was doubtful, the preparation was subjected to immunostaining and *in situ* hybridization to increase sensitivity and specificity to make a definite diagnosis (Tonkic *et al.*, 2012; Allahverdiyev *et al.*, 2015). However, fluorescent *in situ* hybridization is specific for detection of *H. pylori* in histological preparations can be done irrespective of the shape of bacteria. Fluorescent labeled oligonucleotide probes are used in this method targeting the *16S rRNA* and *23S rRNA* genes. This method is the fastest and takes 3 h to detect *H. pylori*. *In situ* hybridization and immunochemical methods can also be used to detect virulence factors and

location of strains in the gastric mucosa. This method being independent of morphological identification usually does not have individual biasness (Patel *et al.*, 2014).

2.7.2.4 Culture and Antimicrobial Susceptibility Testing

Culture and antimicrobial susceptibility testing guidelines have been outlined by the European *Helicobacter* Study Group (EHSG) (Smith *et al.*, 2014). *H. pylori* culture and antibiotic susceptibility testing carried out in an effort to predict antibiotic treatment outcome and guide clinicians in their choice of therapy and should be performed if primary resistance to clarithromycin exceeds 20% in a given geographical area. Furthermore, after the first eradication failure, culture should be considered in all regions before providing second-line treatment (Tonkic *et al.*, 2012). The best specimens for isolation of *H. pylori* are biopsy samples obtained during endoscopy (Patel *et al.*, 2014). As use of PPIs or antimicrobials inhibits the growth of *H. pylori* and reduces the chances of successful culture, patients should avoid taking PPIs for at least 2 weeks and antimicrobials for 4 weeks prior to endoscopy. Biopsy specimens should be transported and processed for culture as soon as possible, ideally within 6 h. If processing is delayed refrigeration is recommended (Smith *et al.*, 2014). Therefore, it is advisable to collect multiple biopsy specimens and recommended to take at least two biopsy specimens from the antrum and one each specimens from the anterior and posterior corpus. It has been observed that the corpus may be the only site which remains positive naturally or sometimes due to consumption of antisecretory drugs (Patel *et al.*, 2014). The cultivation of *H. pylori* from gastric biopsies taken from patients is the most specific and most sensitive. However, cultivation of *H. pylori* requires specific agar and special atmospheric conditions, which hinder its routine use as a diagnostic method (Allahverdiyev *et al.*, 2015).

The commonly used media include Pylori agar, Skirrow agar, Columbia blood agar, Brucella agar, Brain heart infusion or Trypticase soy agar, supplemented with sheep or horse blood. The agar plates are usually incubated in a microaerobic environment (80%-90% N2, 5%-10% CO2, 5%-10% O2) at 35 to 37°C for at least 5-7 days because *H. pylori* has been considered a microaerophile, although colonies are usually visible at 3-5 days. Antimicrobial supplements (vancomycin, 10 mg/L; amphotericin B, 10 mg/L and cefsulodin or trimethoprim, 5 mg/L) are recommended for selective media to facilitate primary isolation, to inhibit overgrowth with contaminating bacteria and fungi (Patel *et al.*, 2014). The presence of *H. pylori* should be confirmed by the Gram stain, and positive oxidase, urease and catalase

tests (Allahverdiyev *et al.*, 2015; Kalali *et al.*, 2015). Moreover, it is possible that the bacterium goes into a viable form that cannot be cultured (coccoid form) which leads to false negative results (Kalali *et al.*, 2015).

As per Koch's postulates culture is considered to be the most specific way to establish the *H. pylori* infection. The sensitivity of isolation the bacterium has been reported to vary greatly among laboratories because it is very fastidious in nature. Even the experienced laboratories recover the organism from only 50% to 70% of actually infected biopsies. Recovery from stool, saliva, and vomitus is very difficult because of the presence of other commensal flora comprising other organisms hampering the growth of *H. pylori*. Bacteriological culture is a tedious, time-consuming procedure, and unnecessary for the routine diagnosis of *H. pylori* infection because other noninvasive tests will detect evidence of the organism in majority of the patients. Culture allows testing of the antibiotic sensitivity of *H. pylori* to choose the appropriate agent/s for eradication (Patel *et al.*, 2014).

Fresh cultures (48-72 h growth) at an inoculums concentration of McFarland 3 should be used for *H. pylori* culture-based antimicrobial susceptibility testing. Culture medium manufactured specifically for antimicrobial susceptibility testing (e.g., Mueller Hinton agar; Oxoid, Basingstoke, United Kingdom) should be used and the depth of the agar should be kept consistent across tests (Smith *et al.*, 2014).

Several assays are commercially available to test the antimicrobials commonly used to treat *H. pylori*. The disc diffusion method (Oxoid) involves placing an antibiotic coated disc directly onto the agar plate inoculated with *H. pylori* and determining the zone of bacterial growth inhibition. This cost-effective method is widely used for antimicrobial susceptibility testing for a variety of microorganisms, but disc diffusion criteria for antimicrobial susceptibility testing for *H. pylori* have not to date been defined by the European Committee on Antimicrobial Susceptibility Testing (EUCAST). Both EUCAST and the British Society for Antimicrobial Chemotherapy recommend E test strips for *H. pylori* culture-based antimicrobial susceptibility testing. E tests are plastic strips calibrated with a predefined concentration gradient of antibiotic and enable the quantitative determination of the minimum inhibitory concentration (MIC) of an antimicrobial agent required to inhibit bacterial growth. The MIC can be read directly from the scale printed on the strip at the point where the edge of the inhibition ellipse of bacterial culture intersects with the strip. E test MIC results may be validated subsequently using agar dilution approaches. When performing antimicrobial

susceptibility testing, quality control tests involving reference strains with known susceptibility or resistance should be included (Smith *et al.*, 2014).

2.8 Treatment

Treatment for *H. pylori* is recommended in all symptomatic individuals. Eradication of *H. pylori* infection provides a long-term cure for both duodenal and gastric ulcers in the majority of patients whose ulcers are not associated with non-steroidal anti-inflammatory drug use. In addition, evidence suggests that *H. pylori* eradication reduces the development of atrophic gastritis and the risk of cancer progression in infected individuals without premalignant gastric lesions. Furthermore, eradication of infection leads to regression of most localized gastric MALT lymphomas (Smith *et al.*, 2014).

Currently, numerous antibiotic-based therapies are available. However, these therapies have several inherent problems, including the appearance of resistance to the antibiotics used and associated adverse effects, the risk of re-infection and the high cost of antibiotic therapy (Ayala *et al.*, 2014). Because *H. pylori* localizes on the acidic surface of the gastric mucosa, an acid suppressant (generally a proton pump inhibitor, PPI) like omeprazole, lansoprazole, pantoprazole, rabeprazole, or esomeprazole is required for maintaining a constant pH and facilitating bacterial replication to increase the efficacy of antibiotics (Arslan *et al.*, 2017; Bińkowska *et al.*, 2018).

In general, several international guidelines for treating patients diagnosed with *H. pylori* infections are consistent with the use of triple therapy as the first-line treatment (Ayala *et al.*, 2014). A triple therapy regimen is applied including usually a proton pump inhibitor (PPI) combined the antibiotics clarithromycin and amoxicillin taken twice daily for 7-14 days. Metronidazole is used instead of amoxicillin in patients with a penicillin allergy (Smith *et al.*, 2014; Abadi, 2017; Arslan *et al.*, 2017; Demiray-Gürbüz *et al.*, 2017; Sahara *et al.*, 2018). Clarithromycin-based therapies were considered to be the best tolerated and safest therapies; however in developing countries increasingly antimicrobial resistance, mainly to metronidazole and clarithromycin, is observed (Ogata *et al.*, 2014). Clarithromycin resistance rate has increased from 7% to 35% during the last years, thus a reduced rate of bacterial elimination is not surprising (Abadi, 2017).

Clarithromycin, a member of the macrolide family, is still the most potent antibiotic used in *H. pylori* eradication treatment protocols. Clarithromycin binds to the 50S ribosomal subunit in the 23S rRNA and the consequent inhibition of protein synthesis results in a

bacteriostatic effect (Demiray-Gürbüz *et al.*, 2017; Bińkowska *et al.*, 2018; Kocsmár *et al.*, 2021). Clarithromycin resistance (Cla-res) fundamentally depends on specific point mutations of certain codons in the peptidyl transferase region of the *23S rRNA*, which are able to negatively influence the affinity of clarithromycin for the bacterial ribosome. Moreover, there is an efflux pump mechanism, but it is able to effectively excrete clarithromycin only in *23S rRNA* mutant strains, suggesting that it is not an independent resistance system but acts synergistically with mutational resistance (Kocsmár *et al.*, 2021). Resistance to clarithromycin is associated with three main point mutations at positions A to G at 2142, 2143 and A to C at 2142 of the *23S rRNA* gene. All antibiotic resistance mechanisms in *H. pylori* seem to be chromosomally mediated. Novel technologies that include in situ hybridization for clarithromycin resistance on gastric biopsies are excellent options if culture is not possible (Demiray-Gürbüz *et al.*, 2017).

Metronidazole resistance is caused by failure of enzymatic reduction, which prevents the production of antibacterial metabolites. This resistance mechanism has been elucidated with mutations of genes encoding certain electron transport proteins operative in the reduction process (Lee *et al.*, 2018).

Resistance to metronidazole is predominantly associated with mutations *rdxA* (*hp0954*), a gene encoding an oxygen-insensitive NAD(P)H nitroreductase. Mutations in two additional genes, *frxA* (*hp0642*) and *frxB* (*hp1508*, encoding a ferredoxin-like enzyme), both of which encode NAD(P)H-flavin oxidoreductases, have been shown to enhance *H. pylori* resistance when found along with *rdxA* gene mutations (Binh *et al.*, 2015; Eng *et al.*, 2015). However, the precise mechanism of metronidazole resistance is still debated, given that metronidazole resistance may also arise in *H. pylori* with mutations in the *frxA* gene only. In addition, it is unclear whether other mutations in genes outside *rdxA* or *frxA* are associated with metronidazole resistance (Binh *et al.*, 2015).

To overcome antimicrobial resistance seen in *H. pylori* treatment, new antibiotics and different drug combinations have been evaluated (Oktem-Okullu *et al.*, 2020). Typically, in second-line therapy at least one antibiotic should be altered from what was administered as first-line treatment. After several reports of failures in first-line therapy, 10 days of bismuth-containing quadruple therapy (PPI, bismuth, tetracycline, and amoxicillin) or levofloxacin-containing triple therapy as second-line treatment was increasingly recommended (Abadi, 2017; Arslan *et al.*, 2017). As bismuth salts are not available in every country, non-bismuth

quadruple therapies, namely sequential therapy (5 d PPI and amoxicillin; 5 d PPI with clarithromycin and metronidazole) or concomitant therapy (PPI with amoxicillin, metronidazole and clarithromycin) may be prescribed as alternative therapies (Smith *et al.*, 2014).

Following treatment, eradication of *H. pylori* should be confirmed by the UBT, stool antigen test or by endoscopy if required. As antibodies persist for months following infection, serology testing is not recommended for eradication confirmation (Smith *et al.*, 2014).

Following failure of initial therapy, a PPI with amoxicillin and levofloxacin is recommended. As non-compliance may lead to treatment failure, adherence should be strongly emphasised for subsequent therapies. If third-line treatment is required, a number of studies support quinolone or rifabutin-based regimens but treatment should only be prescribed following antimicrobial susceptibility testing where possible (Smith *et al.*, 2014; Abadi, 2017; Arslan *et al.*, 2017).

CHAPTER THREE

3 MATERIALS AND METHODS

3.1 Study design

This study is descriptive, cross sectional hospital based study.

3.2 Study area

This study was conducted in Khartoum State, Sudan, in selected hospitals (Omdurman Medical Military Hospital, National Ribat Hospital, Ibn Sina Hospital, Fedail specialize Hospital, and Al Faisal specialize Hospital).

3.3 Study population and duration

Male and female patients from all age groups who subjected for gastric biopsy through Oesophago-Gastro-Duodenoscopy (OGD) units were enrolled in this study in period between December 2018 to January 2021.

3.4 Inclusion criteria

Gastric tissue specimens were collected by physicians from patients (both gender of different age groups) undergoing endoscopic examination and suffering from dyspepsia and other gastritis-related symptoms.

3.5 Exclusion criteria

Patients who had received antibiotics, proton pump inhibitors, H2 blockers, or colloidal bismuth sulfate within the previous two months of endoscopy, patients with a history of gastric resection, and patients with complicated peptic ulcer disease, i.e., hemorrhage, were excluded.

3.6 Ethical considerations

Ethical approval was passed and reviewed by Ethical and Scientific Committee of Medical Laboratory Science College, Sudan University of Science and Technology and Ministry of Health Research Department, Khartoum State and from Hospitals; which insure that all ethical consideration for conducting the research in a way that protects patient's confidentiality and privacy. Verbal and written informed consent was obtained from all study subjects and they were told that they have right to voluntary and they can withdraw at any time without any deprivation. All participants were informed about the objectives and procedure of study.

3.7 Sampling

3.7.1 Sample size

Three hundred and eighty-four gastric biopsies were collected from patients with gastritis symptoms. The sample size was calculated according to the following equation:

$N= t^2 * P (1-p) / M^2$

N= sample size.

t= confidence level at (95%).

P= prevalence of the problem (50 %).

M= margin of error at (5%).

3.7.2 Sampling technique

This study was based on non-probability convenience sampling technique.

3.8 Data collection

Socio-demographic and clinical data were collected by clinician through a data collection form.

3.9 Experimental Work

3.9.1 Specimen collection

Biopsies of gastric tissue were collected by clinician from the corpus or the antrum or corpus and antrum together of the patient's stomach.

3.9.2 Specimen Transportation

Fresh biopsies of gastric tissue were placed immediately and transported in tubes containing saline to research lab. College of Medial Laboratory Science.

3.9.3 Genomic DNA extraction

DNA extraction of gastric biopsies was performed using the guanidine chloride method as described by Abd Al Rahem and Elhag (Abd Al Rahem and Elhag, 2018). Biopsies were grounded by sterile blades and tips and then washed with phosphate buffer saline (PBS). Two ml of lysis buffer were added, followed by 10 µl of proteinase K, 1 ml of guanidine chloride, and 300 µl of ammonium (NH_4) acetate, then vortexed and incubated at 65°C for 2 hrs. The mixture was cooled to room temperature, and then 2 ml of pre-cooled chloroform was applied, vortexed, and centrifuged for 5 min at 3000 rpm. The upper layer of the mixture was moved to a new tube, and 10 ml of absolute cold ethanol were added, shaken, and held for 2 hrs or overnight at -20 °C. The tube was then centrifuged for 15–20 min at 3000 rpm, the supernatant was carefully removed, and the tube was inverted for 5 min on tissue paper. The

pellet was washed with 70% ethanol, centrifuged for 5 min at 3000 rpm. The supernatant was poured away, allowing the pellet to dry for 10 min. Then re-suspended into 50 µl of distilled water, briefly vortexed, and held overnight at -20 °C. The extracted DNA was stored at -80 °C until use.

3.9.4 PCR analysis

PCR assays were performed for detection of *H. pylori 16S rRNA* gene, virulence genes (*cagA*, *cagE*, *vacA*, *iceA1*, and *dupA*), and *H. pylori* resistance genes. The primers sequences, sizes, and conditions of PCR amplifications were used as indicated in Table 3.1. The primers were dissolved according to manufacturer guidelines to prepare 10 pmol/µl. Conventional PCR assay was performed according to the manufacturer's instructions, in a volume of 25 µl of reaction mixture containing 5 µl of ready to use master mix (Taq DNA polymerase, dNTPs and $MgCl_2$) (iNtRON Biotechnology, Seongnam, Korea), 2 µl of DNA template, 1 µl of forward (F) primer, 1 µl of reverse (R) primer and 16µl Distilled water (D.W). For each batch of PCR assay, distilled water instead of the genomic DNA templates was used as a negative control. The reaction mixtures were cycled in an automated thermalcycler (CLASSIC K960, China).

3.9.4.1 Detection of *H. pylori 16S rRNA* gene

PCR was performed on extracted DNA using *H. pylori 16S rRNA* specific PCR (''Hp16s") (Table 3.1). The following cycling conditions were used: initial denaturation at 94 °C for 3 min, 35 cycles of denaturation at 94°C for 30 sec, annealing for 30s at 53°C, extension at 72 °C for 45 s, and a final extension at 72 °C for 5 min (Ye, 2004).

3.9.4.2 PCR analysis for virulence genes

PCR was performed to detect *cagA* and *cagE* genes according to Tomasini *et al.* under the following cycling conditions: 94°C for 3 min, 35 cycles of 94°C for 30 sec, 53°C for 30 sec and 72°C for 45 sec with final extension at 72°C for 5min (Tomasini *et al.*, 2003). PCR condition for the *vacA* gene 95°C for 5 min, 37 cycles of 95°C for 1 min, 60°C for 1 min and 72°C for 1 min with final extension at 72°C for 5min (El-Shenawy *et al.*, 2017). For the *iceA1* gene 95°C for 5 min, 37 cycles of 95°C for 1 min, 55°C for 50 sec and 72°C for 1 min with final extension at 72°C for 5min (El-Shenawy *et al.*, 2017). For the *dupA* gene 94°C for 1 min, 35 cycles of 94°C for 30 sec, 54°C for 30 sec and 72°C for 45 sec with final extension at 72°C for 5 min (Lu *et al.*, 2005). The primers used are listed in Table 3.1.

3.9.4.3 Detection of *23S rRNA* gene conferring clarithromycin resistance

Allele-specific PCR was used for the detection of A2142G and A2143G point mutations using four primers called FP-1, RP-1, RP2142G, and FP2143G (Table 3.1). When the strain is wild type (wt), neither RP2142G nor FP2143G anneals with the template and polymerase chain reaction (PCR) amplification proceeds between FP-1 and RP-1, resulting in a 320 bp amplicon. In the case of the presence of A2142G mutation, the PCR amplification primarily takes place between FP-1 and RP2142 G, which results in an amplicon of 238 bp. Similarly, in the case of the A2143G mutation, the PCR amplification goes between FP2143G and RP-1, resulting in an amplicon of 118 bp (Furuta *et al.*, 2007). The primers were dissolved according to manufacturer guidelines to prepare 10 pmol/µl. Amplification of allele-specific was as follows: initial denaturation at 95 °C for 5 min followed by 35 cycles of denaturation at 95 °C for 15 s, annealing at 60.5 °C for 20s, and extension at 68 °C for the 30s and a final extension of 2 min at 68 °C (Furuta *et al.*, 2007).

3.9.5 Visualization of amplicons

After amplification, 5 µl of the product was run in electrophoreses on a 1.5% agarose gel containing Ethidium bromide (0.5µg/ml), then visualized under an ultraviolet illuminator (UVitec – UK) and photographed. A 100-bp and 50-bp DNA ladder was used as a size marker (iNtRON biotechnology. Korea). A negative control (DNA-free) was included in each PCR test.

3.9.6 DNA sequencing

A total of 10 PCR amplified products for *16S rRNA* gene and 25 PCR amplified products of *23S rRNA* were sent for sequencing (by BGI, business, China) for both strands of PCR products.

3.9.7 Bioinformatics analysis

3.9.7.1 Sequences similarity and alignment

The sequences obtained in this study were identified by searching data bases using BLAST sequence analysis tools (http:/www.ncbi nlm.nih.gov/BLAST/). The sequences were compared using nucleotide-nucleotide BLAST (blastn) with default setting except the sequences were not filtered for low complexity. Species were identified based on the highest similarity score (100%) with reference database sequence. Highly similar sequences were retrieved from NCBI and subjected to multiple sequence alignment using the BioEdit software to compare between wild and mutant genes.

The sequences of *16S rRNA* gene were compared with the *16S rRNA* reference obtained from NCBI database (NR_114587.1 *Helicobacter pylori* strain ATCC 43504 16S ribosomal RNA and KF297892.1 *Helicobacter pylori* isolate Hp1 16S ribosomal RNA gene) and submitted to GenBank with accession numbers (Table 3.2).

The sequences of *23S rRNA* gene were compared with the *23S rRNA* reference (U27270) (Hall, 1999) and submitted to GenBank with accession numbers (Table 3.3).

3.9.7.2 Phylogenetic tree

Phylogenetic tree of *H. pylori 16S rRNA* gene and their evolutionary relationship with well-characterized reference strains obtained from NCBI database (NR_041808.1, NR_119304.1, NR_114587.1, MN326691.1, KC819620.1, KF297893.1, and KF297892.1) was constructed by the neighbor-joining method with the Bootstrap test of phylogeny in Molecular Evolutionary Genetics Analysis (MEGA) program, version 7. Bootstrap resembling strategy and reconstruction were carried out 100 times to confirm the reliability of the phylogene.

Table 3. 1 Primers used for amplification of *H. pylori 16S rRNA*, virulence genes (*cagA*, *cagE*, *vacA*, *iceA1*, and *dupA*), and *H. pylori* resistance genes.

Primer name	Primer sequence (5′-3′)	Amplicon size (bp)	Reference
16S rRNA	F ´ GCTAAGAGATCAGCCTATGTCC R´ TGGCAATCAGCGTCAGGTAAT	532	(Ye, 2004)
cagA	F ´ ATAATGCTAAATTAGACAACTTGAGCGA R´ AGAAACAAAAGCAATACGATCATTC	128	(Tomasini *et al.*, 2003)
cagE	F ´ TTGAAAACTTCAAGGATAGGATAGAGC R´ GCCTAGCGTAATATCACCATTACCC	508	(Tomasini *et al.*, 2003)
vacA (*s1/s2*) *vacA* (*m1/m2*)	F ´ ATGGAAATACAACAAACACAC R´ CTGCTTGAATGCGCCAAAC F ´ CAATCTGTCCAATCAAGCGAG R´ GCGTCTAAATAATTCCAAGG	259/286 570/642	(El-Shenawy *et al.*, 2017)
dupA *(jhp0917)*	F ´ TGGTTTCTACTGACAGAGCGC R´ AACACGCTGACAGGACAATCTCCC	307	(Lu *et al.*, 2005)
iceA1	F ´ GTGTTTTTAACCAAAGTATC R´ CTATAGCCASTYTCTTTGCA	246	(El-Shenawy *et al.*, 2017)
FP-1 *RP-1* *RP2142G* *FP2143G*	TCGAAGGTTAAGAGGATGCGTCAGTC GACTCCATAAGAGCCAAAGCCCTTAC AGTAAAGGTCCACGGGGTATTCC CCGCGGCAAGACAGAGA	320 238 118	(Furuta *et al.*, 2007)

Table 3. 2 GenBank accession numbers for *16S rRNA* nucleotide sequences

	label	**Accession numbers**
SUB283711	F13	MN431461
SUB283711	D3	MN431462
SUB283711	D10	MN431463
SUB283711	D38	MN431464
SUB283711	M10	MN431465
SUB283711	M12	MN431466
SUB283711	M13	MN431467

Table 3. 3 GenBank accession numbers for *23S rRNA* nucleotide sequences

	label	**Accession numbers**
SUB6244332	B13	MN396405
SUB6244332	D3	MN396406
SUB6244332	D4	MN396407
SUB6244332	D8	MN396408
SUB6244332	D19	MN396409
SUB6244332	D20	MN396410
SUB6244332	D31	MN396411
SUB6244332	D33	MN396412
SUB6244332	D34	MN396413
SUB6244332	D35	MN396414
SUB6244332	F16	MN396415
SUB6244332	F21	MN396416
SUB6244332	F25	MN396417
SUB6244332	K2	MN396418
SUB6244332	K37	MN396419
SUB6244332	K47	MN396420
SUB6244332	M1	MN396421
SUB6244332	M10	MN396422
SUB6244332	M11	MN396423
SUB6244332	M12	MN396424
SUB6244332	M113	MN396425
SUB6244332	M14	MN396426
SUB6244332	M15	MN396427
SUB6244332	M42	MN396428
SUB6244332	C5	MN396429

3.10 Data analysis

Statistical analysis was done using IBM Statistical Package for Social Sciences (SPSS) software version 20.0. Data were presented in forms of tables and figures. Frequencies, mean and standard deviation were calculated. Chi-square test was done for the analysis of categorical variables. A *p*-value of <0.05 was considered statistically significant.

CHAPTER FOUR

4 RESULTS

A total of 384 patients who subjected for gastric biopsy through Oesophago-Gastro-Duodenoscopy (OGD) were participated in this study, their ages ranged from 14 to 88 years and mean age of 43 years ±16.39 (SD). Out of them, 209 (54.4%) were males while 175 (45.6%) were females.

Patients enrolled in the study were divided into three age groups: youth 14-24 years 36 (9.4%), adults 25-64 years 300 (78.1%), and seniors 65 years and older 48 (12.5%) as shown on table (4.1). Two hundred sixty-nine (70.1%) specimens were collected from Khartoum locality, and one hundred and fifteen (29.9%) specimens were collected from Omdurman locality.

According to endoscopic findings by a physician, two hundred sixty-nine (70.0%) patients were diagnosed as gastritis, thirty-eight (9.9%) as a gastric ulcer (G. ulcer), twenty-eight (7.3%) as a duodenal ulcer (D. ulcer), sixteen (4.2%) as esophagitis and thirty-three (8.6%) were of normal finding as shown in figure (4.1).

Out of 384 specimens investigated for the presence of *H. pylori* using primer targeting specific *16S rRNA* gene by PCR. *H. pylori* were positive in 109 (28.4%) specimens while 275 (71.6%) were negative (figure 4.2).

PCR testing for *H. pylori* virulence genes from tissue biopsy showed that 38.5% (42/109) of tested *H. pylori* were *cagA* positive, 61.5% (67/109) were *cagA* negative (figure 4.3). For *cagE* gene 37.6% (41/109) were *cagE* positive, 62.4% (68/109) were *cagE* negative (figure 4.4). Regarding v*acA* status (genotyping), *vacA* gene was positive in 100% (109/109) samples, the tested DNA samples were surveyed for *s* (signal) and *m* (middle) regions of *vacA* gene by multiple sets of primers targeted (*s1* and *s2* alleles of *s* region, and *m1*, *m2* alleles of *m* region) (figure 4.5). The *vacAs1* gene was identified in *H. pylori* strains from 85/109 (78.0%), *vacAs2* was identified from 24/109 (22.0%), *vacAm1* was detected in 88/109 (80.7%), and *vacAm2* was detected in 21/109 (19.3%). The *vacA s1/m1* genotype was identified in seventy-seven (70.0%) isolates, the *vacA s1/m2* genotype was identified in only eight (7.3%) isolates, the *vacA s2/m1* was identified in twelve (11.0%) isolates, while the *vacA s2/m2* genotype was identified in twelve (11.0%).

The *dupA* genotype was detected in 48/109 (44.0%) samples (figure 4.6), while the *iceA1* gene was detected in only 20 (18.3%) samples (figure 4.7).

In this study, there was no significant association between the presence of *H. pylori* and gender of study population with *p*. value of 0.597 (Table 4.2). Among different patient's age groups, there was no significant association with the presence of *H. pylori* with *p*. value of 0.833 (Table 4.3). According to geographical distribution of study population, there was no significant association with the presence of *H. pylori*, with *p*. value of 0.407 (Table 4.4).

Regarding the gender and the different age groups, overall, there was no significant difference in distribution of *H. pylori* virulence genes (*cagA*, *cagE*, *vacA*, *dupA*, and *iceA1*) between males and females with different ages (Table 4.5 and Table 4.6).

A physician performed an endoscopic examination on all patients to evaluate clinical outcomes, which were classified as normal gastric mucosa, gastritis, gastric ulcer (GU), esophagitis, and duodenal ulcers (DU). There is no significant association between the presence of *H. pylori* and endoscopic findings (*p*. value= 0.129) (Table 4.7). Although no significant association of *H. pylori cagA*, *cagE*, *iceA1*, and *vacA s/m* genes status with the endoscopic findings (*p*. value= 0.422, 0.768, 0.482, and 0.576 respectively), the *dupA* gene was significantly associated with the clinical outcome (*p*. value of 0.016) as shown on table (4.8).

Out of the 109 *16S rRNA* positive samples, 53 (48.6%) were infected with the wild type (wt) *23S rRNA* strains of *H. pylori* by conventional PCR (figure 4.8). Allele-specific PCR was used for the detection of A2142G and A2143G point mutations; the A2142G point mutation was detected in 9/53 (~17%) specimens with band length 238bp. whereas the second mutation (A2143G) was not detected in all samples.

DNA sequencing was performed for all positive *23S rRNA* strains of *H. pylori*. From twenty-five successfully sequenced samples, 12 samples exhibited different types of mutations at *23S rRNA* gene, 9 (36%) samples showed mutations associated with clarithromycin resistance. And three samples reported with a mutation (C2195T) have no association with clarithromycin resistance. From the mutation associated with clarithromycin resistance, one sample showed the presence of A2142G point mutation, and the A2143G was found in 5 samples. Two other mutations (T2182C and C2195T) were detected in 4 and 3 samples, respectively. The A2142G was detected in one sample labeled D20, A2143G mutation detected in 5 samples D19, D33, K2, K37, and M14, T2182C mutation was detected in samples F11, K37, M11, and C5. The C2195T was detected in 3 samples D3, D4, and D34 (figure 4.9).

The Phylogenetic tree analysis was performed to compare the genetic distances and evolutionary lineage for 7 isolates with well-characterized reference isolates from Genbank. The analysis showed that the seven strains similar to the Hungary group reference strains.as shown in figure (4.10).

Table 4. 1 Distribution of the patients according to age groups

Age groups in years	Frequency (No)	Percent (%)
14-24	36	9.4
25-64	300	78.1
≥ 65	48	12.5
Total	384	100.0

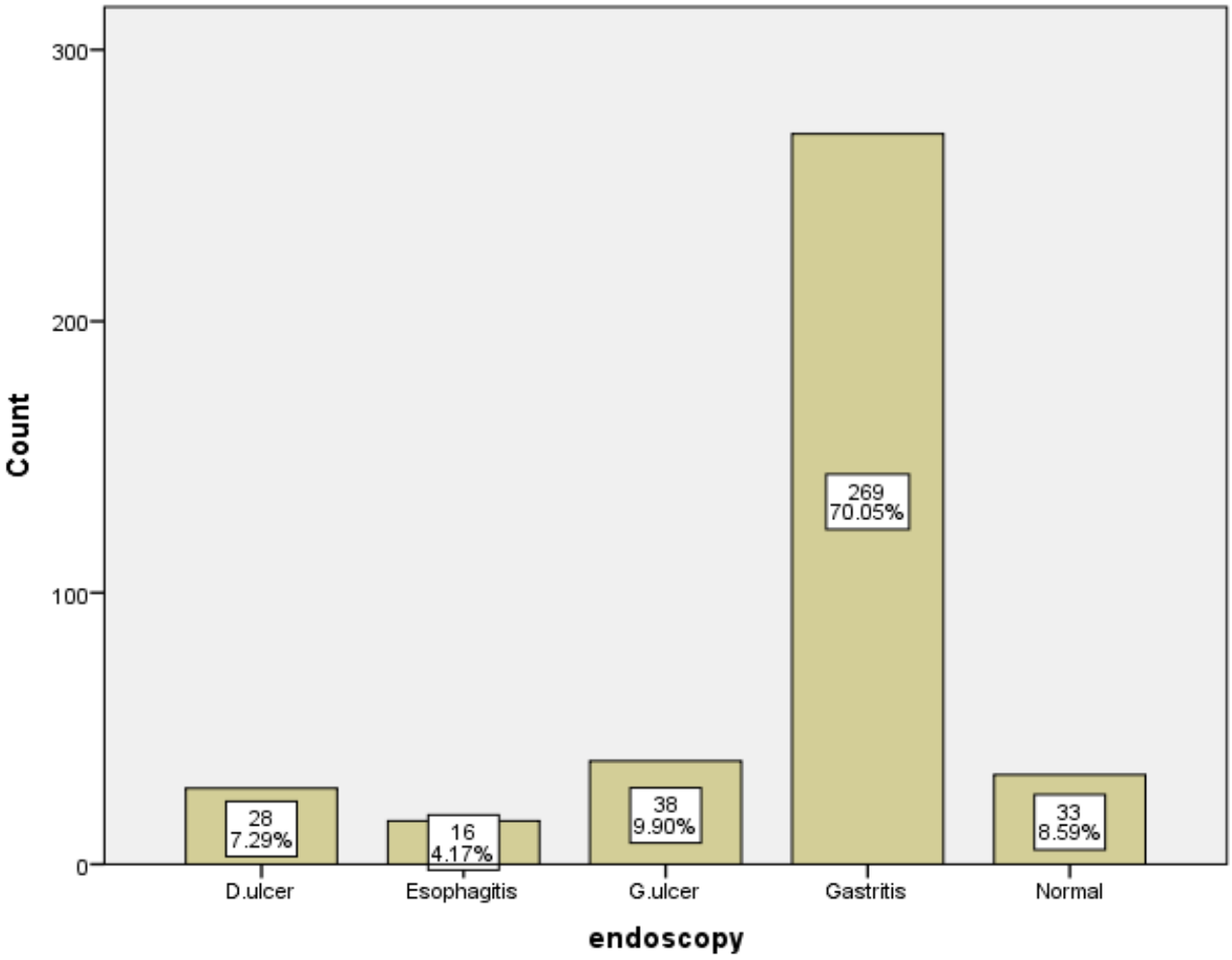

Figure 4. 1 Endoscopic findings of patients

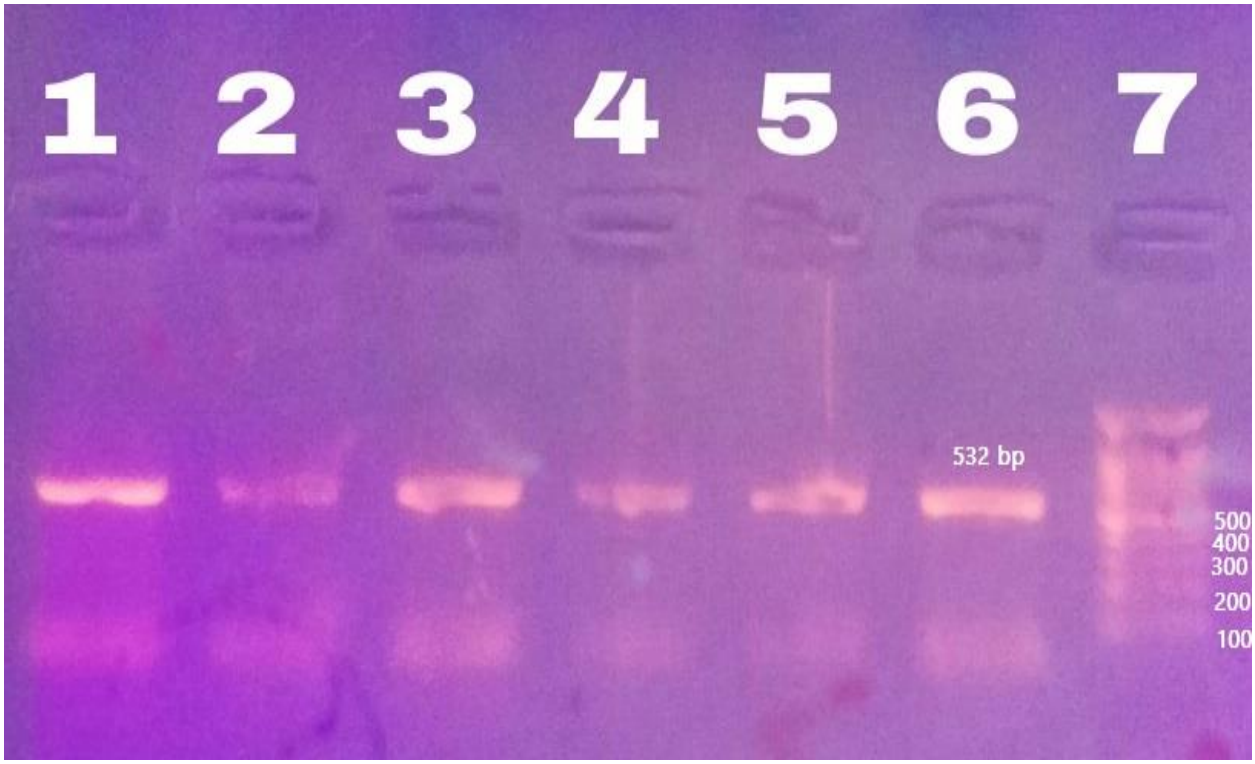

Figure 4. 2 PCR amplification of *H. pylori 16S rRNA* gene. Lane 7 marker (100-1500 bp), lane 6 positive control, lanes 1-5 contain positive samples (532 bp).

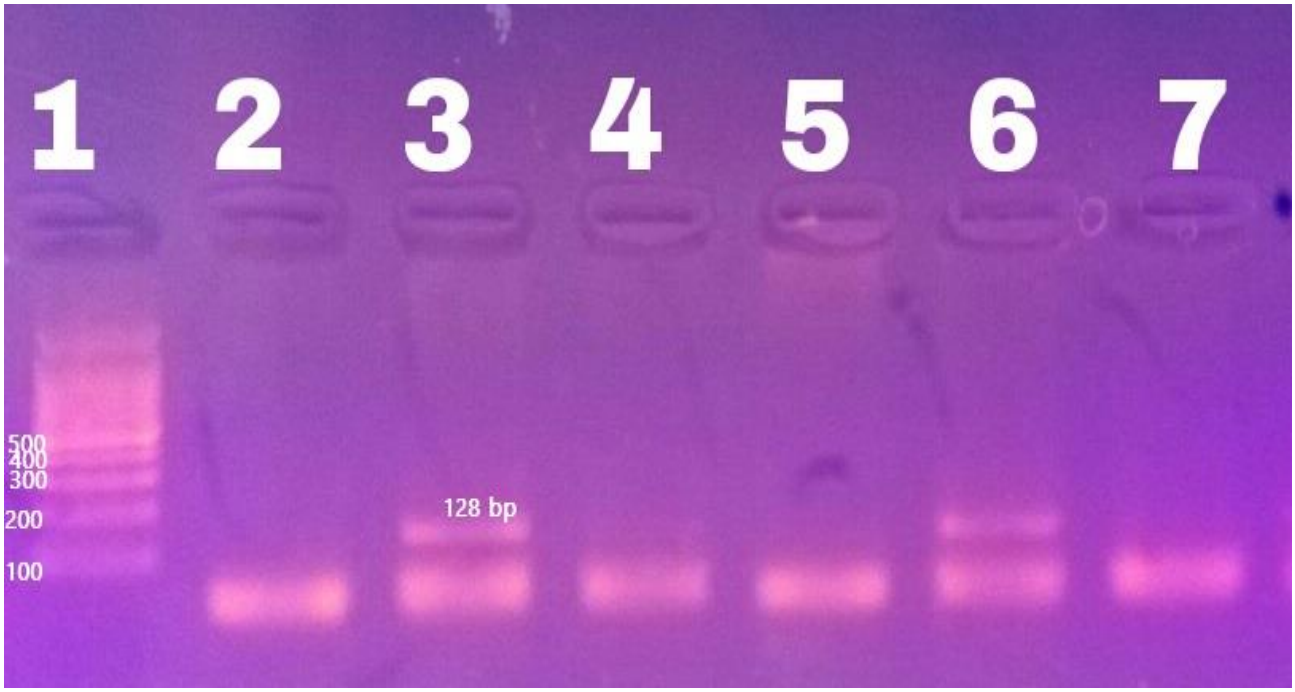

Figure 4. 3 PCR amplification of *H. pylori cagA* gene.

lane 1 marker (100 bp), lanes 3 and 6 contain amplicons of *cagA* gene (128 bp).

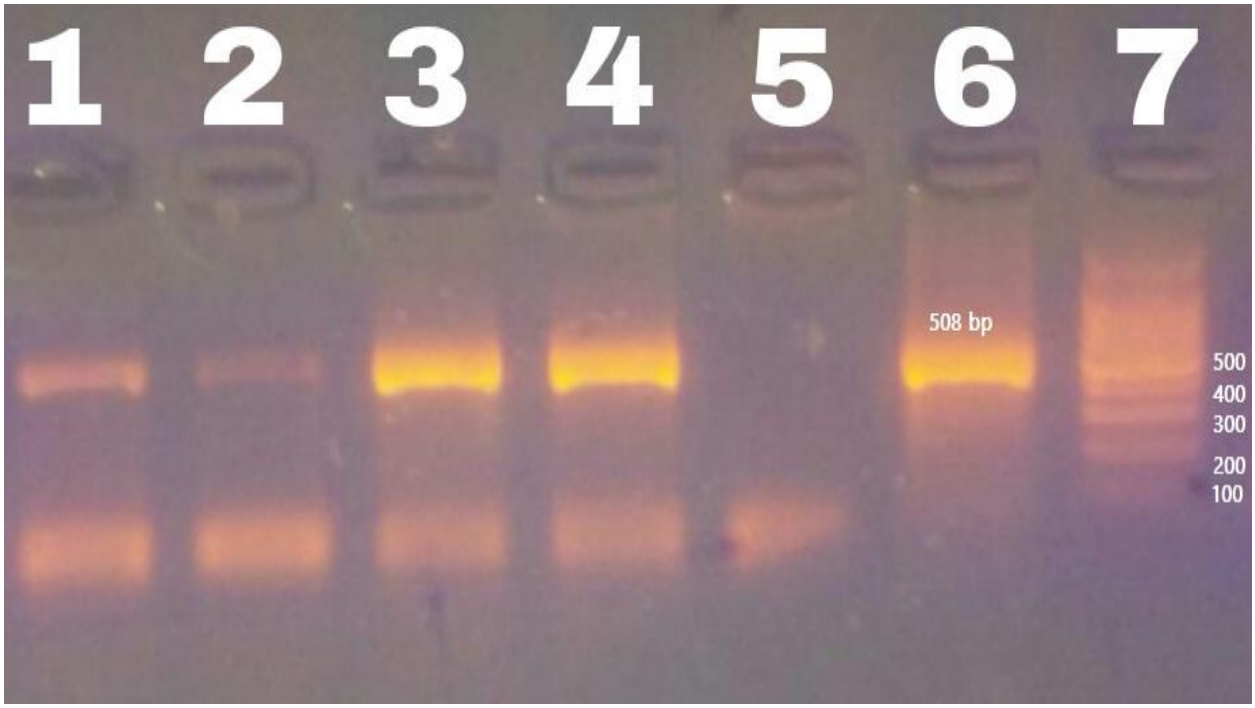

Figure 4. 4 PCR amplification of *H. pylori cag E* gene.

Lane 7 marker (100 bp), lanes 1-4 and 6 contain amplicons of cag E gene (508 bp).

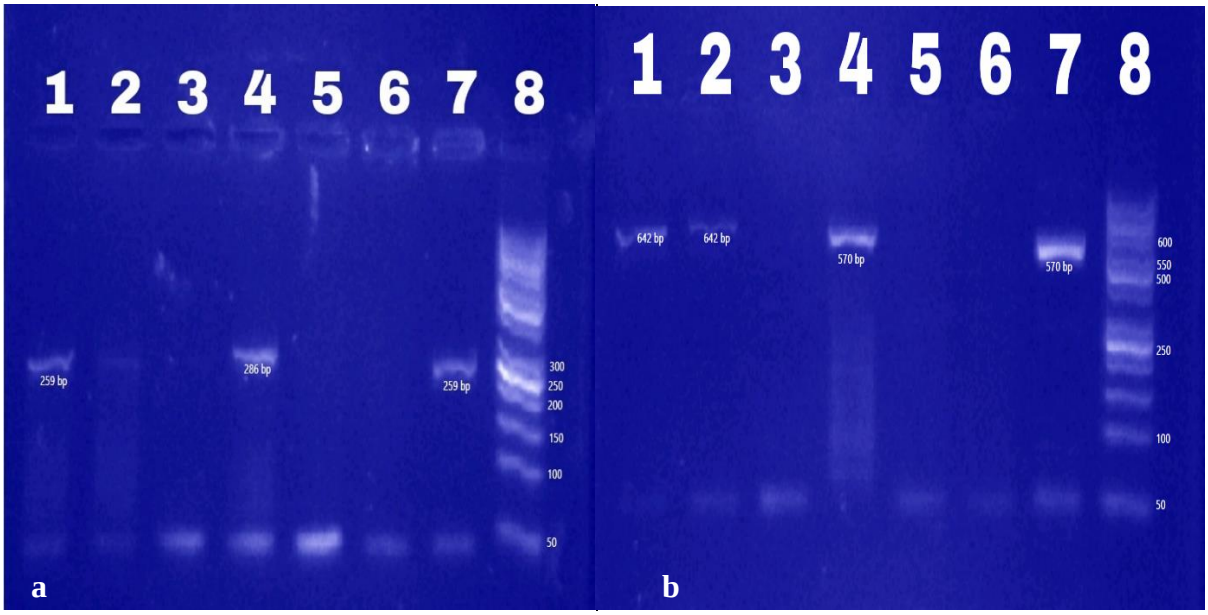

Figure 4. 5 PCR amplification of *H. pylori vacA* gene.

a. *vacA s* gene. Lane 8 marker (50 bp), lanes 1 and 7 contain amplicons of *vacA s1* (259 bp), lane 4 contains amplicons of *vacA s2* (286 bp). **b.** *vacA m* gene. Lane 8 marker (50 bp), lanes 4 and 7 contain amplicons of *vacA m1* (570 bp), lanes 1 and 2 contain amplicons of *vacA m2* (642 bp).

Figure 4. 6 PCR amplification of *H. pylori dupA* gene.

Lane 1 marker (100 bp), lanes 2, 4-8 contain amplicons of *dupA* gene (307 bp).

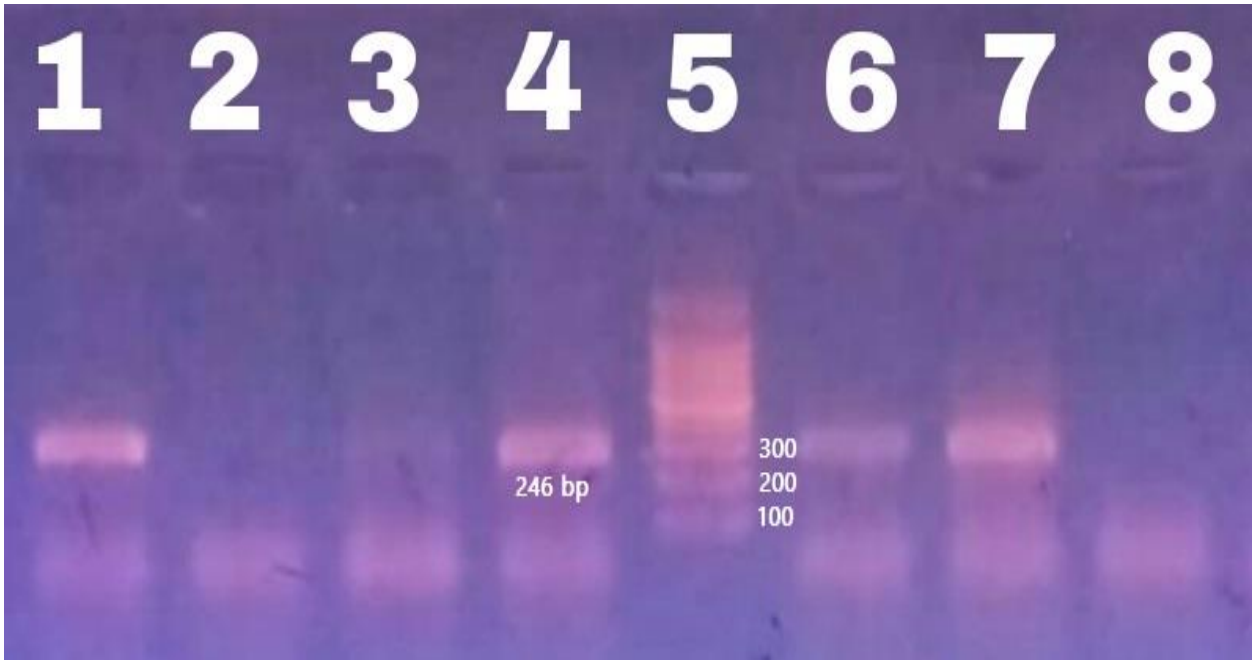

Figure 4. 7 PCR amplification of *H. pylori iceA1* gene.

Lane 5 marker (100 bp), lanes 1, 4, 6, and 7 contain amplicons of *iceA1* gene (246 bp)

Table 4. 2 Association between the presence of *H. pylori* and gender.

Gender	*16S rRNA* status N (%)		
	positive	Negative	*p*. value
Male	57 (14.8%)	152 (39.6%)	0.597
Female	52 (13.5%)	123 (32.0%)	
Total	109 (28.4%)	275 (71.6%)	

Table 4. 3 Association between the presence of *H. pylori* and age groups.

Age groups in years	*16S rRNA* status N (%)		
	positive	Negative	*p*. value
14-24	11 (2.9%)	25 (6.5%)	0.833
25-64	86 (22.4%)	214 (55.7%)	
≥ 65	12 (3.1%)	36 (9.4%)	
Total	109 (28.4%)	275 (71.6%)	

Table 4. 4 Association between the presence of *H. pylori* and residence.

Residence	***16S rRNA* status N (%)**		
	positive	**Negative**	***p*. value**
Khartoum	73 (19.0%)	196 (51.0%)	0.407
Omdurman	36 (9.4%)	79 (20.6%)	
Total	109 (28.4%)	275 (71.6%)	

Table 4. 5 Association between the presence of *H. pylori* virulence genes and gender.

Genotype/ Allele		Gender		*p*. value
		Male N (%)	Female N (%)	
vacA	*s1*	44 (40.4)	41 (37.6)	0.835
	s2	13 (11.9)	11 (10.1)	
	m1	47 (43.1)	41 (37.6)	0.633
	m2	10 (9.2)	11 (10.1)	
	s1/m1	41 (37.6)	36 (33.0)	0.818
	s1/m2	3 (2.8)	5 (4.6)	
	s2/m1	7 (6.4)	5 (4.6)	
	s2/m2	6 (5.5)	6 (5.5)	
cagA	+ve	23 (21.1)	19 (17.4)	0.683
	-ve	34 (31.2)	33 (30.3)	
cagE	+ve	25 (22.9)	16 (14.7)	0.159
	-ve	32 (29.4)	36 (33.0)	
dupA	+ve	24 (22.0)	24 (22.0)	0.671
	-ve	33 (30.3)	28 (25.7)	
iceA1	+ve	8 (7.3)	12 (11.0)	0.223
	-ve	49 (45.0)	40 (36.7)	
Total		209 (54.4)	175 (45.6)	

Table 4. 6 Association between the presence of *H. pylori* virulence genes and age groups.

Genotype/ Allele		Age groups			*p*. value
		14-24 N (%)	25-64 N (%)	≥ 65 N (%)	
vacA	*s1*	8 (7.3)	67 (61.5)	10 (9.2)	0.828
	s2	3 (2.8)	19 (17.4)	2 (1.8)	
	m1	9 (8.3)	68 (62.4)	11 (10.1)	0.582
	m2	2 (1.8)	18 (16.5)	1 (0.9)	
	s1/m1	7 (6.4)	61 (56.0)	9 (8.3)	0.823
	s1/m2	1 (0.9)	6 (5.5)	1 (0.9)	
	s2/m1	2 (1.8)	8 (7.3)	2 (1.8)	
	s2/m2	1 (0.9)	11 (10.1)	0 (0.0)	
cagA	+ve	4 (3.7)	33 (30.3)	5 (4.6)	0.964
	-ve	7 (6.4)	53 (48.6)	7 (6.4)	
cagE	+ve	4 (3.7)	34 (31.2)	3 (2.8)	0.620
	-ve	7 (6.4)	52 (47.7)	9 (8.3)	
dupA	+ve	5 (4.6)	36 (33.0)	7 (6.4)	0.557
	-ve	6 (5.5)	50 (45.9)	5 (4.6)	
iceA1	+ve	4 (3.7)	15 (13.8)	1 (0.9)	0.199
	-ve	7 (6.4)	71 (65.1)	11 (10.1)	
Total		11 (10.1)	86 (78.9)	12 (11.0)	

Table 4. 7 Association between the presence of *H. pylori* infection and endoscopic findings.

Genotype/ Allele		Diagnosis					*p.* value
		D.ulcer N (%)	Esophagitis N (%)	G.ulcer N (%)	Gastritis N (%)	Normal N (%)	
16S rRNA	+ve	13 (3.4)	3 (0.8)	12 (3.1)	75 (19.5)	6 (1.6)	0.129
	-ve	15 (3.9)	13 (3.4)	26 (6.8)	194 (50.5)	27 (7.0)	
Total		28 (7.3)	16 (4.2)	38 (9.9)	269 (70.1)	33 (8.6)	

Table 4. 8 Association between the presence of *H. pylori* virulence genes and endoscopic findings.

Genotype/ Allele		Diagnosis					*p.* value
		D.ulcer N (%)	Esophagitis N (%)	G.ulcer N (%)	Gastritis N (%)	Normal N (%)	
vacA	*s1*	10 (9.2)	3 (2.8)	10 (9.2)	56 (51.4)	6 (5.5)	0.520
	s2	3 (2.8)	0 (0.0)	2 (1.8)	19 (17.4)	0 (0.0)	
	m1	12 (11.0)	2 (1.8)	10 (9.2)	59 (54.1)	5 (4.6)	0.775
	m2	1 (0.9)	1 (0.9)	2 (1.8)	16 (14.7)	1 (0.9)	
	s1/m1	10 (9.2)	2 (1.8)	10 (9.2)	50 (45.9)	5 (4.6)	0.576
	s1/m2	0 (0.0)	1 (0.9)	0 (0.0)	6 (5.5)	1 (0.9)	
	s2/m1	2 (1.8)	0 (0.0)	0 (0.0)	10 (9.2)	0 (0.0)	
	s2/m2	1 (0.9)	0 (0.0)	2 (1.8)	9 (8.3)	0 (0.0)	
cagA	+ve	6 (5.5)	2 (1.8)	2 (1.8)	30 (27.5)	2 (1.8)	0.422
	-ve	7 (6.4)	1 (0.9)	10 (9.2)	45 (41.3)	4 (3.7)	
cagE	+ve	4 (3.7)	2 (1.8)	4 (3.7)	28 (25.7)	3 (2.8)	0.768
	-ve	9 (8.3)	1 (0.9)	8 (7.3)	47 (43.1)	3 (2.8)	
dupA	+ve	8 (7.3)	0 (0.0)	8 (7.3)	27 (24.8)	5 (4.6)	0.016
	-ve	5 (4.6)	3 (2.8)	4 (3.7)	48 (44.0)	1 (0.9)	
iceA1	+ve	1 (0.9)	0 (0.0)	4 (3.7)	14 (12.8)	1 (0.9)	0.482
	-ve	12 (11.0)	3 (2.8)	8 (7.3)	61 (56.0)	5 (4.6)	
Total		13 (11.9)	3 (2.8)	12 (11.0)	75 (68.8)	6 (5.5)	

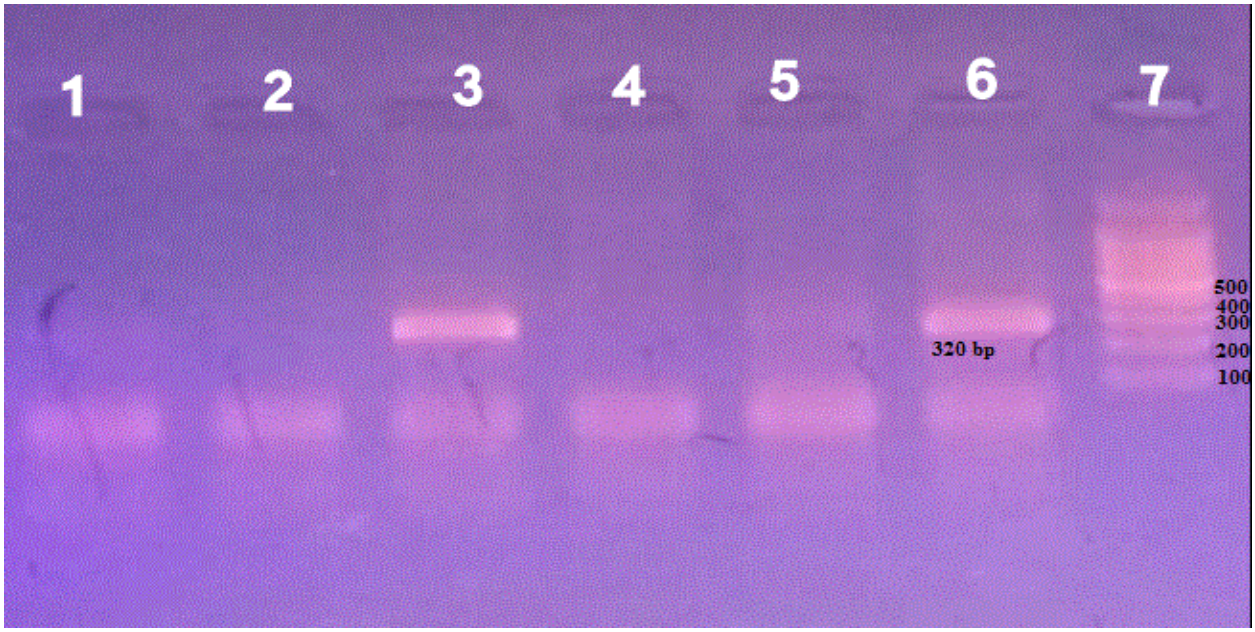

Figure 4. 8 PCR amplification of *H. pylori 23S rRNA* gene.

Lane 7 marker (100-1500 bp). Lanes 3 and 6 include amplicons of wt *23 s RNA* (320 bp)

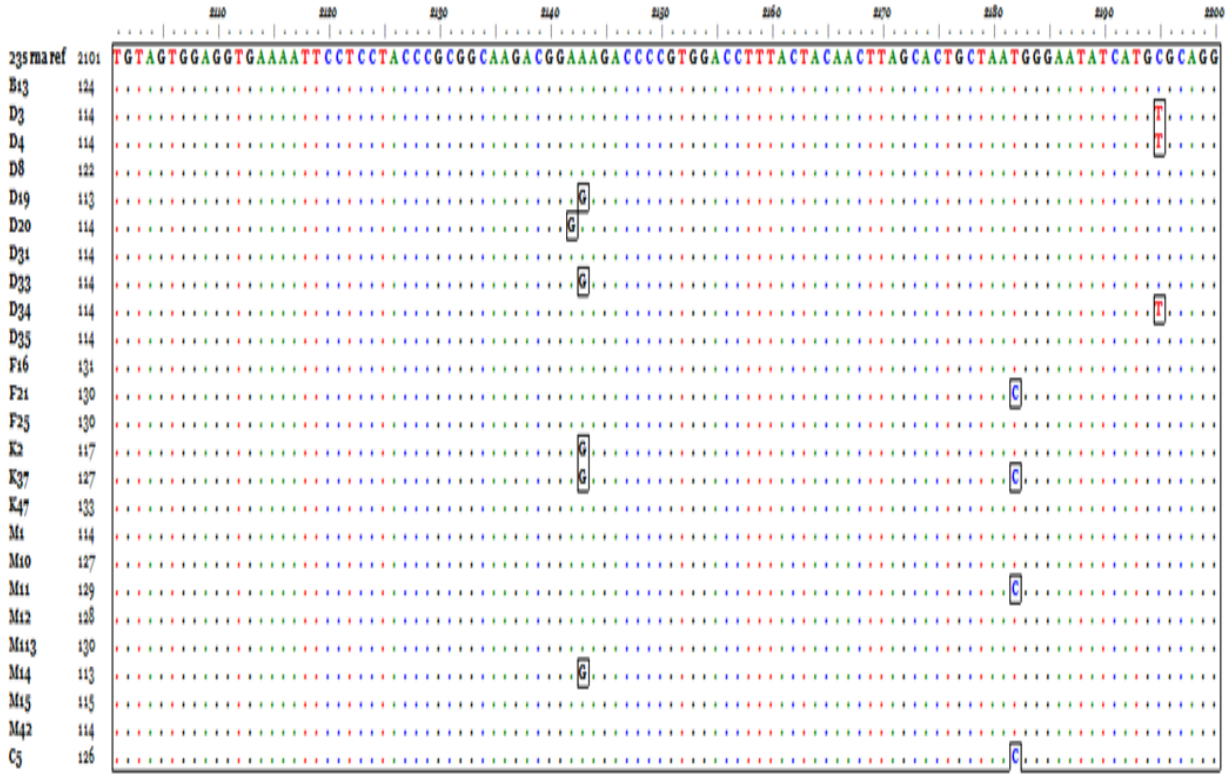

Figure 4. 9 Multiple sequence alignment of *23S RNA* gene. *23S RNA* gene sequences compared to reference gene, the mutant nucleotides appear in boxes.

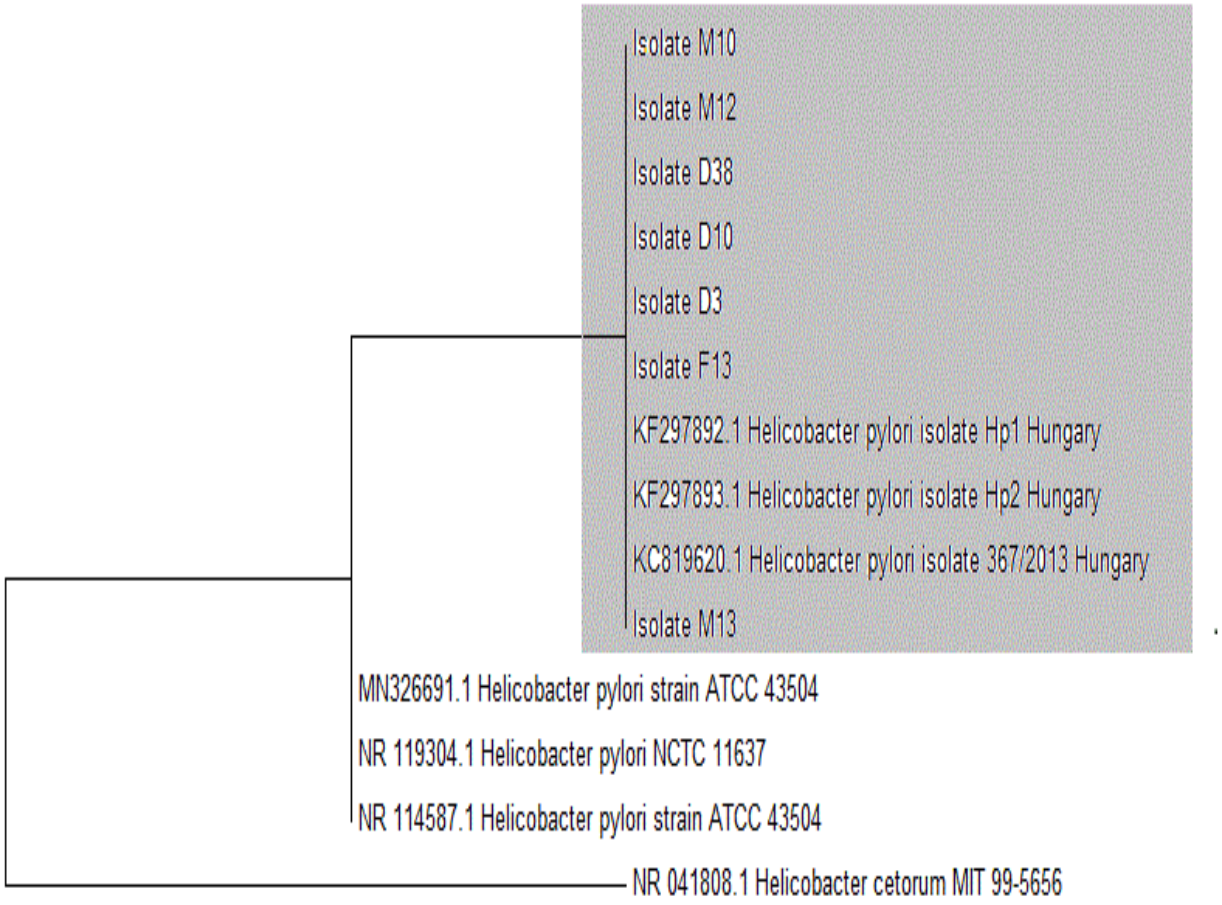

Figure 4. 10 Phylogenetic tree based on interspaced gene sequences of 7 *H. pylori* isolates. The phylogenetic tree analysis was constructed using the neighbor-joining method in MEGA 7.

CHAPTER FIVE

5 DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.1 Discussion

Helicobacter pylori infection has been related to severe gastroduodenal diseases. There is a lot of genomic and allelic variation in the *Helicobacter* bacterium. This unique property allows the bacterium to actively participate in a variety of gastrointestinal problems in infected people all over the world (Ramis *et al.*, 2013). Several diagnostic approaches for *Helicobacter* infections have been proposed. The availability of resources, the sample population, the status of the patients, and the investigator's competence or experience are usually used to select an appropriate diagnostic approach (Suerbaum and Josenhans, 2007).

In this study, out of 384 patients with gastric pain, 70% were diagnosed as gastritis, 9.9% as gastric ulcer, 7.3% as duodenal ulcer, 4.2% as esophagitis, and 8.6% were normal patients. This finding agrees with other studies, which found that gastritis is the most prevalent gastric disease (Ghaith *et al.*, 2016; Shah *et al.*, 2016).

The overall prevalence of *H. pylori* among the studied patients was 28.4% (109/384), based on PCR result. This is in agreement with what was reported in past literature. Similar study conducted in Khartoum State reported that; the prevalence rates of *H. pylori* among gastric biopsies from Sudanese patients were 21.1% using PCR targeting *16 S rRNA* gene (Rahama *et al.*, 2014) and 22.2% using culture (Mamoun *et al.*, 2015). Mohamed *et al.* reported a prevalence of 23.2% among Sudanese patients with colon polyps and colon cancer lesions using immunohistochemistry technique (Mohamed *et al.*, 2020). Another study conducted in Kassala State, east of Sudan to determine the prevalence of *H. pylori* infections among schoolchildren showed that *H. pylori* seropositivity was found to be 21.8% (Abbas *et al.*, 2018). This variation could be attributed to that in our study, PCR was directly done from specimens without culturing step, which may minimise detection chance due to difficulties of cultivation. Study conducted to determine the prevalence of *H. pylori* infection in Saudi patients presenting with dyspepsia, the overall prevalence of *H. pylori* in Jazan Province was 46.5% (Akeel *et al.*, 2018). It is consistent with the prevalence rates 47.3% obtained from some studies conducted in Nigeria with the use of biopsy based method (Bojuwoye *et al.*, 2016). Furthermore, several studies from the Middle East have demonstrated that prevalence ranges between 44 %, and 49% (Chong *et al.*, 2008; Tanih *et al.*, 2010). In many studies worldwide (United States, Taiwan, Kazakhstan, and China), the prevalence of *H. pylori*

among subjects with dyspepsia was 28.9%, 22.5%, 62.7%, and 84% respectively (Shokrzadeh *et al.*, 2012; Ramis *et al.*, 2013; Fang *et al.*, 2020; Mezmale *et al.*, 2021). A recent review, including global studies published between 2000 and 2014, examined the prevalence of *H. pylori* infection in various African, South American, Asian, and European general adult populations. This review showed that the prevalence of infection varied by country (18.2–60.5% in Uganda, 68.6% among pregnant women in Chile, 80% in Bolivia, 83.4% in China, 29.4%-54.5% in Japan, 76.5% and 47.9% among the Yami and Han ethnic groups in Taiwan, respectively, 72.1% in Italy, and 84.2% in Poland) (Alsulaimany *et al.*, 2020). Another systematic review published in 2017 compiled 184 global studies conducted between 1970 and 2016 in a total of 62 countries. The selected samples were representative of the general population, and the prevalence of *H. pylori* infection among all participants was 48.5%. While the infection was widely spread in developing countries (70.1% in Africa, 69.4% in South America, and 66.6% in Western Asia), the prevalence was significantly lower in developed countries (24.4% in Oceania, 34.3% in Western Europe, and 37.1% in North America) (Hooi *et al.*, 2017). These variations in prevalence rates of *H. pylori* in different studies across the world might be attributed to different contributing factors including geographical location, low socioeconomic status, vegetarian diet (in the Chinese study), environmental factors (e.g., contaminated or untreated water), poor health, crowded living conditions, and day care center attendance (leading to person-to-person transmission among children) (Aziz *et al.*, 2014; Alsulaimany *et al.*, 2020). In addition to the variability in the methods of *H. pylori* detection, size of the study and exclusion of prior antibiotic use, all these can play roles in these variations (Ashtari *et al.*, 2015).

In this study, there was no significant association between the presence of *H. pylori* among different age groups. The highest rate of *H. pylori* infection in this study (22.4%) was seen in the age group between 25-64 years, 3.1% in participants equal to or more than 65 years of age, and 2.9% among the age group between 14-24 years. In an agreement to our finding, study conducted by Adlekha *et al.*, reported that, there was no statistically significant difference in age related distribution of *H. pylori* infection (Adlekha *et al.*, 2013). Many studies have demonstrated positive correlation between *H. pylori* prevalence rates and increase in age (Pajavand *et al.*, 2015; Aitila *et al.*, 2019; Mezmale *et al.*, 2021). The finding would suggest that colonization with *H. pylori* organism starts early in life. During neonatal life, sources of infections would be limited to person to person from caretakers, family

members, or nursery attendants. As age increase, exposure to various infection sources increases hence the ultimate rate of infection (Aitila *et al.*, 2019). In contrast, other studies have shown the decreased rate of *H. pylori* infection with an increase in age (Aziz *et al.*, 2014; Ashtari *et al.*, 2015).

In terms of gender, our findings indicated that, there were no statistically significant differences in the prevalence of *H. pylori* (14.8% in males and 13.5% in females). This finding in an agreement with many studies that show no statistical association between sex and *H. pylori* infection (Adlekha *et al.*, 2013; Aitila *et al.*, 2019; Mezmale *et al.*, 2021). This suggests that maintaining all exposures constant, both males and females would be infected equally (Tadesse *et al.*, 2014). Some authors reported a high prevalence of *H. pylori* among females (Pajavand *et al.*, 2015). While other authors reported a high rate of *H. pylori* infection among males, a large number of studies showed no gender differences (Kadi *et al.*, 2014; Nguyen *et al.*, 2015). A recent meta-analysis study conducted by Zamani *et al.* in 2018 that involved 183 studies from 73 countries in six continents revealed that although males were predominant of *H. pylori* infection across all continents, none of the differences reached statistical significance. The issue of gender disparity in *H. pylori* infection is an intriguing topic and further research is needed to understand the mechanisms by which sex may influence the acquisition and/or persistence of infection (Zamani *et al.*, 2018).

In the current study, the most common endoscopic findings among the *H. pylori* positive subjects were gastritis (19.5%), duodenal ulcer (DU) (3.4%), gastric ulcer (GU) (3.1%), esophagitis (0.8%), and normal gastric mucosa (1.6%), whereas, in *H. pylori* negative subjects, the rates of gastritis, DU, GU, esophagitis and normal gastric mucosa were 50.5%, 3.9%, 6.8%, 3.4% and 7.0% respectively. So we did not find association between these endoscopic findings and *H. pylori*. This result in agreement with Akeel *et al.* which reported that no significant association between *H. pylori* and endoscopic findings (Akeel *et al.*, 2018). In another studies conducted by Ayana *et al.* and Mohammed *et al.* who found significant correlation between endoscopic findings and *H. pylori* (Ayana *et al.*, 2014; Mohammed, 2014).

The prevalence of *H. pylori* virulence genes from different parts of the world are different, and there is a direct association between specific genotypes and certain clinical manifestations (Pajavand *et al.*, 2015). Previous studies on the prevalence of *H. pylori* virulence genes and its associations with the clinical outcomes in Sudan are so scarce. Our

data revealed that among 109 positive *H. pylori* strains, 38.5% were positive for *cagA* gene, 44.0% for *dupA*, 37.6% for *cagE* gene and 18.3% for *iceA1* gene. Previously, similar study conducted in Egypt reported that *H. pylori cagA* gene-positive was detected in 45.9 % strains (El-Khlousy *et al.*, 2016). The *cagA*, *vacA*, *iceA1*, and *iceA2* genes were detected in 49.2%, 100%, 42.2 %, and 32.8% respectively in Saudi Arabia (Akeel *et al.*, 2019). According to a recent South African study, *cagA* gene was detected in 62% (145/234) and *dupA* gene in 53.4% (125/234) *H. pylori* strains (Idowu *et al.*, 2019). In another study, the *cagA, cagE*, and *iceA1* genes of *H. pylori* were positive in 69%, 51%, and 26% of 160 *H. pylori* strains, respectively (Dabiri *et al.*, 2017).

The *vacA* genotype was found in all of the isolates in our study (100%). Dabiri *et al.* (2017) and Akeel *et al.* (2019) revealed a 100% *vacA* detection rate in Iran and SouthWestern Saudi Arabia, respectively, which was similar to our findings. In a study conducted in Morocco, the rate of *vacA* identification was determined to be 99% (El Khadir *et al.*, 2017), while low rates of *vacA* gene detection were observed in Ethiopia, Netherlands, and Palestine (90, 93%, and 43.2% respectively) (Essawi *et al.*, 2013; El Khadir *et al.*, 2017).

The most common *vacA* subtypes in the current investigation were *s1/m1* (70.0%), followed by *s2/m1* (11.0%) and *s2/m2* (11.0%), then the lowest genotype is *s1/m2* subtype (7.3%). Similar to our finding, many authors reported that *vacA* gene subtype *s1/m1* is the most predominant *vacA* genotype (Basso *et al.*, 2008; Román-Román *et al.*, 2017; Sallas *et al.*, 2017; Idowu *et al.*, 2019). In addition, most investigations found that in isolated *H. pylori* strains, *vacA s1/m2* was the most prevalent genotype (Marie, 2012; Essawi *et al.*, 2013; Keikha *et al.*, 2020). Many researchers have discovered a link between *H. pylori* pathogenicity and vacuolating toxin activity. The toxin activity of *vacA s1/m1* genotype is high, the *s1/m2* genotype is moderate, and the *s2/m2* genotype is low (El-Khlousy *et al.*, 2016). In general, *vacAs1/m1*strains have been linked with higher degrees of inflammation and cell infiltration when compared to *vacAs2/m2*strains. Furthermore, *vacAs1/m1* strains produce large amounts of vacuolating toxin and induce a higher vacuolating activity in gastric epithelial cells than *vacAs2/m2* strains (Ramis *et al.*, 2013).

Numerous studies have found that *vacA s1/m1* is present in 24–84 % of people worldwide (Kim *et al.*, 2001; Akeel *et al.*, 2019). The *cagA* gene was found in 30.7% of the isolates with the *vacA s1/m1* genotype in this study. This relationship has previously been observed in other investigations (Marie, 2012). This variation in genotype prevalence rates could be

related to differences in the study population, ages, and sample collection site (Akeel *et al.*, 2019).

Only the *H. pylori dupA* gene showed statistical significance (*p*. value=0.016) with the distributions of *H. pylori* virulence genes with endoscopic results (clinical outcome). There was no association between *H. pylori cagA*, *cagE*, *vacA*, and *iceA1* with clinical outcomes, according to our findings. Several researchers from all around the world have found a link between the existence of the *dupA* gene in *H. pylori* strains and clinical outcomes (Lu *et al.*, 2005; Argent *et al.*, 2007; Hussein, 2010; Jung *et al.*, 2012). Furthermore, Akeel *et al*. (2019) reported that; no statistical significance was identified between the *cagA* gene and its association with clinical outcomes, which agrees with our findings, but also discovered that *iceA* genes and *vacA* subtypes were linked to clinical outcomes. Our findings are in contrast with those of a study conducted in Riyadh, Saudi Arabia, which reported a statistically significant link between *cagA* and *vacA* and clinical outcomes (Marie, 2012). The severity of gastritis and clinical consequences may be influenced by many factors like host and environmental factors (Akeel *et al.*, 2019).

Worldwide, the prevalence of clarithromycin-resistant strains of *H. pylori* is 19.4% (Jaka *et al.*, 2019). Generally, countries with an antibiotic resistance rate of more than 20% alter their treatment strategies (Park *et al.*, 2016). Our study revealed a higher frequency (36%) of mutations associated with clarithromycin resistance using DNA sequencing of V domain of *23S rRNA gene*. While using the allele-specific PCR, the frequency of mutations associated with clarithromycin resistance in our specimens was 17% (9/53). These variations could be due to the low sensitivity of allele-specific PCR compared to DNA sequencing (Imyanitov *et al.*, 2002). Also, in this study, allele-specific PCR targeted only two common mutations (A2142G and A2143G), while sequencing revealed all SNPs in the amplified region.

The point mutation A2142G was detected in 17% (9/53) of specimens using allele-specific PCR. This percentage is a noticeable amount compared with Tran (Ha *et al.*, 2018) study in Vietnam, where found this mutation in about 3.6%, variation in the population may represent a critical factor. Like Ghaith's (Ghaith *et al.*, 2016) study, point mutation A2143G was fallen to be detected by PCR although different PCR protocols were tried; this could be justified according to Cheng (Cheng *et al.*, 2013), which is that there is only one nucleotide difference between wild-type DNA and point mutation in DNA sequence. Therefore, the unusual mutations between large excess wild-type alleles are difficult to detect by traditional gene

variation assays. In contrast, both mutations A2142G and A2143G appeared by DNA sequencing technique, and they are already known to cause reduced affinity of the ribosome for Clarithromycin (Binh *et al.*, 2014). As it appeared in our results, differences in detection methods has a larger impact. Fallen in the detection of A2143G mutation by PCR and its appearance by DNA sequencing techniques may suggest that the percentage of clarithromycin resistance gene mutation may be more than the above results. DNA sequencing also showed the presence of T2182C mutation in some specimens. According to Jung suggestion, this mutation is nonspecific (Jung *et al.*, 2012). In contrast, Khan confirmed that this mutation is associated with clarithromycin (Khan *et al.*, 2004). Besides, point mutation C2195T was detected by sequencing, and according to Fasciana, it has no relation with clarithromycin resistance (Fasciana *et al.*, 2015).

5.2 Conclusion

In this study, the prevalence of *H. pylori* infection and diversity of virulence genes were found to be high among Sudanese gastritis patients especially in age groups 25-64 years. The *vacA* gene was the most prevalent *H. pylori* genotype.

No association was found between endoscopic findings and *H. pylori* positivity. Furthermore, there was an association between the *H. pylori dupA* gene and clinical outcome. In addition, there were some variations in the distribution of virulence genes by age and gender.

A high frequency (36%) of mutations associated with clarithromycin resistance using DNA sequencing of the *23S rRNA* gene's V domain.

The results of this study provide important implications for public health strategies for the prevention of *H. pylori* infection in our community.

5.3 Recommendations

Based on this study we recommended that:

1. Treatment failure of gastritis and peptic ulcer should be followed by culture and or urea breath test to ensure complete eradication of *H. pylori*.
2. Laboratory personnel should be trained on microbiological (isolation and identification) and molecular procedures of *H. pylori* pathogen.
3. Further studies should be done using PCR (two conserved target genes) to compare and make a logical conclusion in the diagnosis of the Helicobacter infection in patients at risk and to avoid false-positive results.
4. Information about clarithromycin resistance should be taken into consideration to avoid eradication therapy failing.
5. In depth studies are needed to better understand the genetic diversity of this bacterium and the factors associated with *H. pylori* infection in our region.
6. Health education of the public especially clinicians to avoid empiric treatment, as much as possible, it may increase resistance and eventually leads to treatment failure.

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APPENDICES

Appendix I

Molecular reagents

10 X TBE buffer

Formula in grams per liter

Tris base……. 108 gm

Boric acid…….……..…..55gm

EDTA……..….....40 ml of 0.5M

Deionized water……..……..…..…1 liter

Preparation

Amount of 108 gm. Tris base were weighed and added to 55gm of boric acid and 40 ml of 0.5M EDTA then dissolved into 1 liter deionized water pH 8.0.

1X TBE buffer

Formula in ml per liter

10 X TBE…….10 ml

Deionized water…….90 ml

Ten ml of 10 X TBE buffer was added to 90 ml deionized water and heated until completely dissolved.

Ethidium bromide solution

Formula in grams per 1ml

Ethidium bromide……10 mg

Deionized water……I ml

Preparation

Twenty milligrams of ethidium bromide powder were dissolved into 1000 µl deionized water, and kept into brown bottle.

Agarose gel

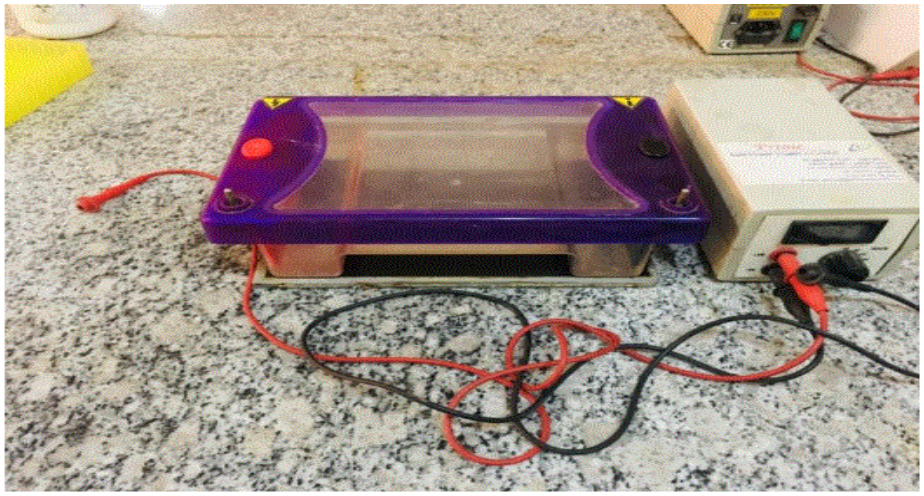
Preparation

Amount of 2 gm of agarose powder dissolved by boiling in 100 ml 1X TBE buffer, then was cooled to 55°C in water bath, then, 1.5 µl of Ethidium bromides stock (10 mg/ml) per 100 ml gel solution for a final concentration of 0.5 ug/ml were added, mixed well and poured on to the casting tray that has been taped up appropriately and was equipped with suitable comb to form well in place. Any bubbles were removed and the gel was allowed to set at room temperature. After solidification, the comb was gently removed and the spacer from the opened sides was removed.

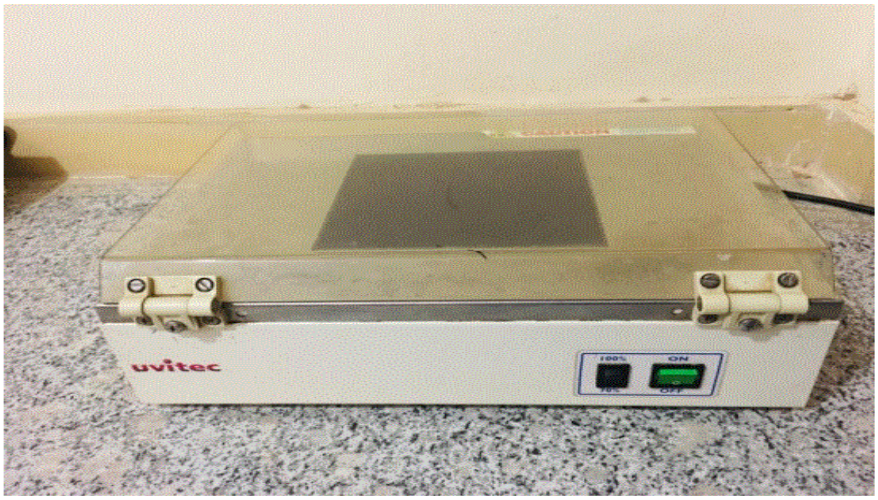
Appendix II

Thermocycle Device

Microwave



Gel Electrophoresis and Power Supply Device



UV Light Transilluminater Device

Appendix III

Sudan University of Science and Technology

College of Graduate Studies

Questionnaire for requirement of PhD degree

Molecular Characterization of *Helicobacter pylori* Virulence Genes and Antimicrobial Resistance Genes in Gastric Biopsy among Symptomatic Patients, Khartoum State

التوصيف الجزيئي لجينات عوامل الضراوة والجينات المقاومة للمضادات الحيوية للبكتريا الحلزونية في خزعات المعدة بين المرضى الذين تظهر عليهم الأعراض في ولاية الخرطوم

- Date:..... - Hospital:.....
- Participant name:.....
- ID number..... - Age:.....years
- Sex: Male ( ) Female ( )
- Past history of *H. pylori* infection Yes ( ) No ( )
- Clinical diagnosis (endoscopy):

a. Gastritis ( ) b. Gastric Ulcers ( ) c. Duodenal Ulcer ( ) d. Esophagitis ( )

e. Normal gastric mucosa

- Lab investigation and results:

PCR result for presence of *16S rRNA* gene:……

PCR result for presence of virulence genes:

- *cagA*……
- *cagE*……
- *vacA*……
- *iceA*……
- *dupA*……

PCR result for presence of resistance genes:……

NGS result:……

بسم الله الرحمن الرحيم

وزارة الصحة ولاية الخرطوم

الادارة العامة للاستراتيجية

ادارة الابتكار والتطوير والبحث العلمى

Date: 1/3 /2018

ETHICAL CLEARANCE CERTIFICATE

This is to certify that the Khartoum State Ministry of Health research department was approved the request **of Miss/ Maram Mustafa Mohammed Ali**

conduct the research entitled: "**Molecular Characterization of Virulence And Antimicrobial Resistance Genes Mutations of *Helicobacter pylori* in Public Hospitals in Khartoum State 2018**

The researcher may therefore start with research as from the date of this certificate.

Dr. Mona Fath Alrahman Omer

Directorate of research department

وزارة الصحة - ولاية الخرطوم
ادارة البحوث
RESEARCH DEPT
الادارة العامة للتخطيط والتنمية

تلفون ٧٦٠١٤٠ فاكس ٠١٨٣٧٦٠١٤٠
٠١٨٣٧٧٠٩٦٢

جامعة السودان للعلوم والتكنولوجيا

كلية علوم المختبرات الطبية

استمارة الموافقة على المشاركة فى بحث عن التعرف على الجينات المسببة لمقاومة المضادات الحيوية وجينات الضراوة في البكتريا الحلزونية المعزولة من خزعات المعدة وعلاقتها بالمضاعفات السريرية

أنا مرام مصطفي محمد علي من كلية المختبرات الطبية بجامعة السودان اقوم بالتعرف على انواع الجينات المسببة لمقاومة المضادات الحيوية وجينات الضراوة في البكتريا الحلزونية ونسبة انتشارها في ولايه الخرطوم لتحديد العلاج المناسب ووضع الاستراتيجيات المستقبليه لمواجهة لهذه المشكله.

أصبحت مقاومة الميكروبات للمضادات الحيويه مشكلة مزمنة في التدخل العلاجي للعدوى. ومن اهم اسباب مقاومة البكتريا للمضادات الحيويه هو انتاجها لبعض الجينات التي تودي لمقاومة المضاد الحيوي.

وحتي نستطيع أن نقوم بهذا الدور نود ان نطلب منك ان تشارك فى هذا البحث. واذا وافقت سنقوم بأخذ بقايا خزعة المعده التي تم أخذها مسبقا بواسطة الطبيب او الكادر الطبى المدرب لاجراء الفحوصات والتأكد من احتمال اصابتك بالبكتريا المقاومه للمضاد الحيوي.

ونعلمك بان موافقتك على المشاركة فى البحث اوعدم موافقتك لن يحرمك من حقك فى العلاج والرعاية الطبية المطلوبة. ونفيدك بانك لن تتلقى اى عائد مادى لمشاركتك فى البحث وستكون مشاركتك طوعية لكنها ستساهم وتساعد في تطوير سبل العلاج.

نود اخطارك بان المعلومات الخاصة بك ستكون سرية ولن يطلع عليها الاخرون ماعدا الطبيب الذى يتولى علاجك ورؤساء الفريق البحثى. وسيشارك معك حوالي 300 شخص.

نود موافقتك على المشاركة فى هذا البحث ويمكنك أن تسال وتستفسر عن البحث ومشاركتك فيه من الاطباء والفريق الطبى المشرف على البحث قبل موافقتك النهائية. ونرجو اثبات موافقتك بامضاء الاستمارة المرفقة بعد قرائتها

للبالغين (اكبر من 18 عام)

أنا .....اؤكد فهمى لمحتوى الاستمارة وافيد بموافقتى على المشاركة فى البحث

واعلم أنه من حقى رفض المشاركة و الانسحاب من المشاركة فى اى وقت ولن يؤثر ذلك فى حقوقى .

واوافق على اخذ العينه المطلوبة للبحث.

الامضاء.....

الشاهد:.....