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Molecular Detection of *Helicobacter pylori* Virulence Genes in Patients with Gastritis, Gastrodudenities and Peptic Ulcer in Khartoum State

الكشف الجزيئي لجينات الضراوة في البكتريا البوابية المعِدية لدى المرضى المصابين بالتهاب المعدة والاثنى عشر والقرحة في ولاية الخرطوم

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الآيـــة

قال تعالى:

﴿اللَّهُ نُورُ السَّمَاوَاتِ وَالْأَرْضِ مَثَلُ نُورِهِ كَمِشْكَاةٍ فِيهَا مِصْبَاحٌ الْمِصْبَاحُ فِي زُجَاجَةٍ الزُّجَاجَةُ كَأَنَّهَا كَوْكَبٌ دُرِّيٌّ يُوقَدُ مِن شَجَرَةٍ مُّبَارَكَةٍ زَيْتُونَةٍ لَّا شَرْقِيَّةٍ وَلَا غَرْبِيَّةٍ يَكَادُ زَيْتُهَا يُضِيءُ وَلَوْ لَمْ تَمْسَسْهُ نَارٌ نُّورٌ عَلَىٰ نُورٍ يَهْدِي اللَّهُ لِنُورِهِ مَن يَشَاءُ وَيَضْرِبُ اللَّهُ الْأَمْثَالَ لِلنَّاسِ وَاللَّهُ بِكُلِّ شَيْءٍ عَلِيمٌ﴾

صدق الله العظيم

سورة النور، الآية (35)

Dedication

This is dedicated to….

My Mother (Nawal Salih Edrees)

All that I am or hope to be, I owe to my angel mother, she is my pillar of strength and her immense believe in me had lifted me up.

My Father (Alsafi Abduelrhman Murgan)

A man of few words in many ways, words that impact me all the way, his words leave a deep impression my father has always been a Hero of mine.

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One looks back with appreciations to the brilliant teachers with indebted gratitude to those who touched me not only sciences but also manners and morality.

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ABSTRACT

Helicobacter pylori is a Gram negative, microareophilic bacteria usually found in the stomach, produce urease, oxidase,hydrogenase and catalase enzymes ; cause very harmful damage to the stomach epithelial cells by different mechanisms and this harmful expressed by human body as gastritis (acute or chronic), duodenal ulcer ,peptic ulcer and *Helicobacter pylori* may extend to cause mucosa associated lymphoid tissue lymphoma and cancer. The aim of this study was to detect *Helicobacter pylori* in Gastric and Duodenal biopsies from symptomatic patients by using *urea A* gene specific primer and the frequency of *Helicobacter pylori* virulence genes $IceA_1$, $IceA_2$, *glmM* and *HPU* genes among Sudanese patients attending to Fedil special hospital in Khartoum by using polymerase chain reaction assay. Participants of this study were of different age groups, different signs and symptoms and different life habits whose visited Fedail special hospital in Khartoum state at gastric endoscopic unit. Total number of 61 gastric biopsy samples were collected from antrum region of the stomach by gastro endoscopist .There were 50(82%) of all isolated biopsies positive for *Helicobacter pylori urea A* gene, those positive *urea A* gene isolates were analyzed for detection the frequency of the virulence genes, Most of study population were females26(52%) compare to males 24(48%)and the frequency of each virulence genes were $IceA_1$ 8(16%),$IceA_2$ 31(62%),*gLmM* 7(14%) and4 (8%)for *HPU* and the main virulence factors in all isolated biopsies are $IceA_2$ gene.This study clarified that $IceA_2$ gene is the common isolated gene by 31(62%) compared to other virulent genes.

مـــلخص الأطروحة

البكتريا البوابية المعِدية هي بكتريا سلبية لصبغة الجرام ,تحتاج الى أقل كمية من الهواء للنمو توجد عادةً في المعدة وتنتج انزيمات الurease ,oxidase ,hydrogenase and catalase

تتسبب هذه البكتريا في ضرر كبير لخلايا المعدة بعدة طرق , هذا الضرر يكون على هيئة التهاب معدة (سواء حاد أو مزمن)، قرحة الإثنى عشر وقرحة المعدة وقد تمتد هذه البكتريا وتسبب الليمفوما والسرطان.

الهدف من هذه الدراسة هو الكشف عن البكتريا البوابية المعِدية في خزعات المعدة والاثنى عشر من المرضى الذين يعانون من اعراض التهاب المعدة عن طريق إستخدام بادئات *Urea A* gene وايضاً تهدف الدراسة الى كشف معدل تردد جينات الضراوة $HPU, IceA_1, IceA_2, glmM$ لهذه البكتريا في المرضى السودانيين في ولاية الخرطوم في مستشفى فضيل عن طريق تفاعل تسلسل البلمرة.المشاركون في هذه الدراسة من المرضى ذوي أعمار مختلفه وأعراض مختلفة وعادات مختلفة في مستشفى فضيل التخصصي في وحدة مناظير الجهاز الهضمي.عدد المرضى الاجمالي كان 61 مريض أُخذت منهم عينة خزعة من المعدة من الantrum عن طريق أخصائي مناظير الجهاز الهضمى . عدد 50(82%) من الخزعات المعزولة اعطت نتائج ايجابية في تفاعل تسلسل البلمرة مؤكدة وجود البكتريا الحلزونية المعدية فيها عن طريق بادئات ال *Urea A* gene تم اخذ هذه العينات وتحليلها للكشف عن معدل تردد جينات الضراوة فيها وكانت النتائج كالآتي:

8 (16%) for $IceA_1$, 31 (62%) $IceA_2$, 7 (14%) $glmM$ and 4 (8%) HPU

الغالبية المشاركة في هذه الدراسة من الاناث (52%)26مقارنة بالذكور24(48%).هذه الدراسة تخلص الى أن $IceA_2$ هو اكثر الجينات ضراوة بنسبة 62%مقارنة ببقية الجينات الضارية.

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MALT	Mucosa-associated lymphoid tissue
Hop protien	Hsp70-Hsp90 organizing protien
VacA	*Vaculating cytotoxic A*
Cag/PAI	*Cytotoxin-associated gene*/Pathogenicity island
IceA	*Induced by contact epithelial cell*
ureA	*Ureas A*
PPI	Pepton Pump Inhibitor
PUD	Peptic ulcer disease
IL-1	Interleukin -1
UreC	*Urea C*
bp	Base pair

CHAPTER ONE
INTRODUCTION

1.1. Introduction:

Helicobacter pylori previously *Campylobacter pylori* is an important human pathogen involved in the pathogenesis of atrophic gastritis, gastro duodenal ulcer, gastric cancer, Mucosa Associated Lymphoid Tissue (MALT) lymphoma, idiopathic thrombocytopenic purpura, iron deficiency anemia and vitamin B12 deficiency, *H. pylori* is a spiral shaped gram negative, microaerophilic,flagellate bacterium that has been implicated as a major human gastric pathogen (Blaser,2006).

Over 80% of individuals infected with the bacteria are asymptomatic, and it may play an important role in the natural stomach ecology (Blaser, 2006).

H. pylori found in the stomach (Blaser, 2006), measured 0.5-0.9mM wide by 2-4mM long (Ketley,2007).Like *Campylobacters* it requires carbon dioxide for growth but it has a tuft of sheathed unipolar flagella ,Unlike the unsheathed flagella of *compylobacters* (Ketley,2007).

H.pylori requires oxygen but at lower concentration than in the atmosphere .It contain hydrogenase that can produce energy by oxidizing molecular hydrogen (H_2) made by intestinal bacteria, it produces oxidase, catalase and urease. *H. pylori* possess five major outer membrane protein (OMP) families (Kusters *et al.*, 2006). *H. pylori* have four to six lophotrichous flagella, all gastric and enterohepatic are highly motile due to flagella (Josenhans *et al*., 2000).

H. pylori consists of a large diversity of strains, and hundreds of genomes have been completely sequenced (Tomb *et al.*, 1997).

In particular, many virulence genes of *H. pylori* play an important role; Different genotypes of *H. pylori* produce different virulence factors (Shiota *et al*., 2013).

Among the most remarkable finding of two *H.pylori* genome-sequencing projects were the discovery of a large family of 32 related outer membrane proteins (Hop proteins) that includes most known *H.pylori* adhesins and the discovery of many genes that can be switched on and off by slipped strand mispairing-mediated mutagenesis . Proteins encoded by such phase-variable genes include enzymes that modify the antigenic structure of surface molecules, control the entry of foreign DNA into the bacteria, and influence bacterial motility. The genome of *H.pylori* changes continuously during chronic colonization of an individual host by importing small pieces of foreign DNA from other *H.pylori* during persistent or transient mixed infections (Suerbaum *et al*., 1998).

The vacuolating cytotoxin *(vacA)* gene encodes for the vacuolating cytotoxin, the pore forming toxin which causes progressive vacuolation and injury to gastric epithelium (Gangwer *et al*., 2010).

In the other way most strains of *H.pylori* possesses the *Cag* pathogenicity island (*cag-PAI*), (Censini *et al*., 1996).

The Induced by contact to epithelial cells *IceA* gene was identified in the *H.pylori* isolated from peptic ulcer disease and gastritis patients ,there are two allels of *IceA* ,$IceA_1$,and $IceA_2$ (Yakoob *et al* , 2015).

H. pylori eradication therapy is recommended whenever *H. pylori* infection is detected. The standard first line therapy is a one-week Triple therapy consisting of proton-pump inhibitors such as omeprazole and the antibiotics clarithromycin and amoxicillin (Malfertheiner *et al* ,2012). In areas with higher rates of clarithromycin resistance, other options are recommended (Malfertheiner *et al* ,2017)

H. pylori infections like other major chronic infectious diseases (i.e., syphilis and tuberculosis) are associated with a long latent period before presenting clinically. As such, many infections will be discovered during this latent period. A number of methods to *H. pylori* infection have been developed and they are generally grouped as being “invasive” meaning that they require gastric tissue or mucus, or “non-invasive” requiring only blood, breath or stool analysis (Stenström *et al* ,2008).

1.2. Rationale:

At least half the world's population is infected by *H.pylori*, making it the most widespread infection in the world (cover and Blaser, 1992). *H. pylori* produce different virulence genes associated with different clinical conditions of chronic active gastritis, gastric and duodenal ulcer disease. *H. pylori* are one of the important causes of gastric carcinoma (Uemura *et al* , 2001).

In Sudan *H. pylori* infection is one of the major health problems. Most of the previous studies and the routines tests for detection of *H. pylori* infection focusing depends on serology which have a cross reaction with other infections. In this study we focusing in detection of *H .pylori* in symptomatic patients and to determine Virulence genes *ure/ A* , $IceA_1$,$IceA_2$,*HPU* and *glmM* genes of *H. pylori* by Polymerase Chain Reaction.

1.3. Objective:

1.3.1. General objective:

To Detect *Helicobacter pylori* virulence genes from biopsy samples in patients with gastritis, Gastrodudenities and peptic ulcer by multiplex PCR in Khartoum state.

1.3.2. Specific objective:

1. To Detect of *Helicobacter pylori* in gastric and duodenal biopsies from symptomatic patients by using *urea A* gene specific primer.
2. To Determine the frequency of $IceA_1$,$IceA_2$, *HPU* and *glmM* virulence genes by polymerase chain reaction (PCR).

CHAPTER TWO
LITERATURE REVIEW

2. Literature review

2.1. History

H.pylori was identified in 1982 by barry marshal and robin warren, who found that it was present in patients with chronic gastritis and gastric ulcers, conditions that where not previously believed to have a microbial cause. It is also linked to the development of duodenal ulcers and stomach cancer (Blaste, 2006). However, over 80% of individuals infected with the bacteria are asymptomatic and it has been postulated that it may play an important role in the natural stomach ecology (Blaster, 2006).

Professor Walery Jaworski of Jagiellonian University in Krakow investigated sediments of gastric washings obtained from humans in 1899.Among some rod like bacteria, he also found bacteria with a gastric helical shape, which is called *Vibrio rugula*. He was the first to suggest a possible role of this organism in the pathogenicity of gastric disease. This work was included in the 'Handbook of Gastric Diseases" but it did not have much impact as it was written in polish(Konturek, 2003) .Warren and Marshall contended that most stomach ulcers and gastritis were caused by infection with this bacterium and not by stress or spicy food as has been assumed before (Marshall and Warren ,1984).

The community began to come around after further studies that were done, including one in which Marshall drank a petri dish of *H .pylori*, developed gastritis and bacteria were recovered from his stomach lining, Thereby satisfying three out of the four of Koch's postulates. The fourth was satisfied after a second endoscopy ten days after inoculation revealed signs of gastritis and the presence of the *H.pylori* .Marshall was then able to treat him using a fourteen day therapy with bismuth salts and metronidazole. Marshall and warren went into show that antibiotics are effective in the treatment of many cases of gastritis.The International Agency for Research on Cancer confirmed that *H. pylori* infection was the most significant risk factor for gastric cancer and that the eradication of *H. pylori* can reduce the risk of gastric cancer (International Agency for Research on Cancer , 2013).

2.2. Taxonomy and Classification

The presence of spiral shaped bacteria on human gastric mucosa was first recognized nearly one hundred years ago, These bacteria were originally named *campylobacter pylori* (Warren, 1983).In 1989, a new genus *Helicobacter,* was proposed, and *C. pylori* was renamed *Helicobacter pylori* (Goodwin *et al*., 1989).

Recently the genus *helicobacter* has been include with the genus *Wolinella* in the family *helicobacteraceae* ,which the family *Compylobacteraceae,*constitutes the *Epsilonproteobacteria*.According to the usual site of colonization ,*Helicobacter* species can be divided into gastric and enteric or entero hepatic *Helicobacter* types .

Table (2.1): classification of *Helicobacter pylori* (Marshall *et al*., 1985):

Kingdom	Bacteria
Phylum	*Protebacteria*
Class	*Epsilon Proteobacteria*
Order	*Campylobacterales*
Family	*Helicobacteraceae*
Genus	*Helicobacter*
Species	*H.pylori*
Binomial Name	*Helicobacter pylori*

There are many species of *Helicobacter* bacteria and have many primary mammalian host (Kusters *et al* .,2006) as in table 2.2. below:

Table (2.2): Characteristics of selected *Helicobacter* species (kusters *et al*., 2006):

Species	**Primary mammalian host**	**Pathology**	**Animal model**
Gastric *Helicobacter* spp.			
H.pylori	Human,primate	Gastritis, peptic ulcer disease, gastric adenocarcinoma, MALT lymphoma	Mouse, Mangolian gerbil, guinea pig, gnotobiotic piglet
H.felis	Cat, dog , mouse	Gastritis in natural host; may cause peptic ulcer or gastric adenocarcinoma in mouse.	Mouse
H.mustelae	Ferret	Gastritis, peptic ulcer disease, gastric adenocarcinoma, MALT lymphoma.	None
H.acinonychis	Cheetah, tiger, other big cats.	Gastritis, peptic ulcer disease	Mouse
H.heilmannii	Human , dog , cat, monkey, cheetah, rat	Gastritis, dyspeptic symptoms, MALT lymphoma.	Mouse
H.hepaticus	Mouse ,other rodent	Proliferative typhlocolitis, hepatitis, hepatocellular carcinoma.	none

2.3. Structure:

2.3. 1. Morphology

H. pylori is a helix-shaped ,Gram-negative bacterium , can be demonstrated in tissue by Gram stain, Giemsa's stain, haematoxylin–eosin stain, Warthin–Starry silver stain, acridine orange stain, and phase-contrast microscopy. It is capable of forming biofilms (Stark *et al.,* 1999).

2.3.2. Physiology

H. pylori is microareophilic that is it requires oxygen, but at lower concentration than in the atmosphere. It contains a hydrogenase that can produce energy by oxidizing molecular hydrogen (H_2) made by intestinal bacteria and It produces oxidase, catalase, and urease. (Olson and Maier ,2002).

H. pylori possess five major outer membrane protein (OMP) families (Kusters *et al*., 2006), The largest family includes known and putative adhesins. The other four families are porins, iron transporters, flagellum-associated proteins, and proteins of unknown function (Kuster *et al*., 2006).

2.3.3. Genome

H. pylori consists of a large diversity of strains, and hundreds of genomes have been completely sequenced (Testerman *et al.,* 2014).*H.pylori* exhibits unusual genetics flexibility and it is hypothesized the variability within the genome could potentially account for the organisms ability to adapt to the dynamic environment within the host gastric niche, facilitating chronic colonization. These adaptations include reversible and irreversible changes to the genome as well as regulatory mechanisms that modulate gene expression.

2.4. Transmission and Epidemiology

Helicobacter pylori are one of the most common bacterial infections worldwide. However, the majority of those infected do not develop clinical manifestations of disease (Blaser, 2006).

2.4.1. Transmission

2.4.1.1. Person to Person Route

Person to person route is believe to be the primary route of transmission in developed countries, and is also important in developing countries.Close personal contact, particularly with in the family including parent to child, sibling to sibling and husband to wife, has been constant demonstrated as a risk factor for Transmission of infection (Escobar and Kawakami, 2004). The prevalence of infection was significantly greater in wife with infected partners, compared to wife whose husband was not infected (34.9% VS 14.5%) (Brenner *et al*, 2006).Person to person transmission can occur in several ways (Parsonnet *et al.*, 1999).

2.4.1.2. Oral-Oral Route

H.pylori DNA has been detected in the saliva of *H.pylori* positive subjects by PCR (Madiniar *et al* ., 1997). *H.pylori* organisms have also been successfully detected from the dental plaque of infected persons (Nguyen *et al., 1993*).

2.4.1.3. Faecal-Oral Route

H.pylori has been detected in faeces by culture and it's DNA by PCR (Namavar *et al*., 1995).

2.4.1.4. Waterborne Transmission

Studies in the peoples in republic china and in Latin America found that the source of water used for consumption, bathing or swimming could possibly be associated with *H.pylori* infection (Goodman *et al.*, 1996).

2.4.1.5. Iatrogenic Transmission

Endoscopy used routinely in upper gastrointestinal procedures may be the source of iatrogenic infection as a result of improper sterilization between procedures (Tytgat, 1995).

2.4.2. Epidemiology

The prevalence of *H. pylori* shows large geographical variations. In various developing countries, more than 80% of the population is *H. pylori* positive, even at young ages. The prevalence of *H. pylori* in industrialized countries generally remains under 40% and is considerably lower in children and adolescents than in adults and elderly people (Pounder and Ng , 1995) .In Western countries, the prevalence of this bacterium is often considerably higher among first- and second-generation immigrants from the developing world (Perez-Perez *et al*., 2005).

2.5. Pathology and clinical manifestation

Until the discovery of *Helicobacter* in 1982, ulcers were thought to be caused by stress. Now it is known that ulcers, in addition to gastritis, are caused by a bacterial infection of *H. pylori*. Though relatively easy to treat with antibiotics, *H. pylori* can be a risk factor for gastric cancer if it becomes a long-term infection.

Gastric-biopsy specimen showing *Helicobacter pylori* adhering to gastric epithelium and underlying inflammation.

H.pylori is the etiological agent of acute and chronic gastritis, peptic ulcer disease (75% of gastric ulcers and 90% of duodenal ulcers), and two forms of gastric cancer mucosa associated lymphoid tissue lymphoma (MALT) and Gastric adenocarcinoma (Ernest and Gold 2000).

The association with the development of two forms of cancer led to classification of *H.pylori* by world health organization as the only bacterial class1 carcinogen (IARC, 1994)

2.5.1. Acute and chronic gastritis

Colonization with *H.pylori* virtually always leads to infiltration of the gastric mucosa in both antrum and corpus with neutrophil and mononuclear cell. This chronic active gastritis is the primary condition related to *H.pylori* colonization and other *H.pylori* associated disorders in particular result from this chronic inflammatory process,Chronic gastritis is likely to underlie H. pylori-related diseases (Shiotani and Graham , 2002).

2.5.1.1. Acute gastritis

The acute phase of colonization with *H. pylori* may be associated with transient nonspecific dyspeptic symptoms, such as fullness, nausea, and vomiting, and with considerable inflammation of both the proximal and distal stomach mucosa, and pan gastritis. This phase is often associated with hypo chlorohydrin(Granstrom *et al* .,1997).

2.5.1.2. Chronic gastritis

When colonization does become persistent, a close correlation exists between the level of acid secretion and the distribution of gastritis. This correlation results from the counteractive effects of acid on bacterial growth versus those of bacterial growth and associated mucosal inflammation on acid secretion and regulation. This interaction is crucial in the determination of outcomes of *H. pylori* infection. In subjects with intact acid secretion, *H. pylori* in particular colonize the gastric antrum, where few acid-secretory parietal cells are present. This colonization pattern is associated with an antrum-predominant gastritis. Histological evaluation of gastric corpus specimens in these cases reveals limited chronic inactive inflammation and low numbers of superficially colonizing *H. pylori* bacteria. Subjects in whom acid secretion is impaired, due to whatever mechanism, have a more even distribution of bacteria in antrum and corpus, and bacteria in the corpus are in closer contact with the mucosa, leading to corpus-predominant pan gastritis. The reduction in acid secretion can be due to a loss of parietal cells as a result of atrophic gastritis, but it can also occur when acid-secretory capacity is intact but parietal cell function is inhibited by vagotomy or acid-suppressive drugs, in particular, proton pump inhibitors (PPIs) (Kuipers *et al* ., 1995a).

2.5.2. Peptic ulcer disease (PUD)

People infected with *H. pylori* are at increased risk of developing peptic ulcers (Marshall *et al.,* 1988).

2.5.3. Ulcer complication

Complication of ulcer disease include: Bleeding, perforation and stricture formation .Bleeding is the most common complication of ulcer disease and is estimated to occur in 15 to 20% of ulcers. Approximately ,40% of patients presenting with upper gastrointestinal bleeding have a bleeding ulcer .The treatment of bleeding ulcer by endoscopic therapy can be performed by several methods ,including injection of adrenalin ,coagulation with the heater probe or clipping of the bleeding (Gisbert *et al* ,2004).

2.5.4. Patients at risk of gastric cancer

The IARC have classified *H. pylori* as a Type 1 carcinogen (IARC, 1994) However, *H. pylori* is not the absolute or only cause of gastric cancer, Cure of histological gastritis, the immunological- inflammatory response to *H. pylori*, only occurs following successful eradication. The risk of development of atrophy and cancer is the presence of *H.pylori* is gain related to host and bacterial factors, which influence the severity of the chronic inflammatory response .As such the risk is increased in subject colonized with *cagA* positive strains

(Parsonnet *et al.*, 1997), but also in those with a genetic predisposition to higher *IL-1* production in response to colonization (El-Omar *et al.,* 2000).

2.6. Laboratory Diagnosis of *H.pylori*

2.6.1. Invasive diagnosis

2.6.1.1. Stool Antigen

The stool antigen test is considered as valuable noninvasive alternative to diagnose *H.pylori* when Urea Breath Test is not available (Gisbert ,*et al.,* 2006).

2.6.1.2. Histology

Nodular Gastritis increases with gastritis score (Koh *et al*., 2007), advantage of this method is the possibility to send specimens via regular mail at room temperature, especially for epidemiological studies lacking freezing equipment. Fixation with 10% formaldehyde provided very stable specimens, in which the morphology of the bacteria was maintained (Mégraud *et al.,* 2007).

2.6.1.3. Culture

Culture remains a reference method as it allows the direct detection of *H. pylori* organisms even though it presents a limited sensitivity and is a time-consuming procedure (Patel *et al.,* 2014).

2.6.1.4. Rapid Urease Test

In the presence of *H. pylori* urease, urea is hydrolyzed to produce ammonia and bicarbonate, leading to a pH increase in the gastric mucosa, and this is indicated by a change in the color of phenol red from yellow to pink or red. After developing a medium to detect *H. pylori* with a pH indicator (McNulty *et al.,* 1989).

2.6.2. Noninvasive diagnosis

2.6.2.1. Urea breathing test

This method showed higher sensitivity and specificity than serology, this test cannot provide information about genotypes and antibiotic resistance and it requires specialized equipment, which may not be available in routine clinical laboratories.

Recently, a new portable ^{14}C-based urea breath test was produced (Heliprobe, Noster AB, Stockholm, Sweden). Which is accurate, reliable, easy to use, fast (20 minutes), inexpensive, and uses low radioactivity of ^{14}C-based urea capsule comparable to natural radiation (Jonaitis *et al.,* 2007).

2.6.2.2. Antibody-based tests

Serological tests that detect anti- *H. pylori* IgG antibodies, serology is the most efficient diagnostic method (Burucoa *et al.*, 2013).

2.7. Virulence factors of *H.pylori*

In particular, many virulence genes of *H. pylori* play an important role in pathogenicity; Different genotypes of *H. pylori* produce different virulence factors (Shiota *et al,* 2013).

2.7.1. *Vaculating cytotoxic A (VacA)*

Approximately 50% of all *H.pylori* strains secrete *VacA* , a highly immunogenic 95-kDa protein that induces massive vacuolization in epithelial cell *in vitro* and gastric tissue damage *in vivo* leading to gastric ulcer (Cover and Blasser , 1992).

2.7.2. *Cytotoxin-associated gene A (CagA)*

The *CagA* protein is a highly immunogenic protein (120-145kDa) encoded by the *CagA* gene (Covacci *et al*., 1993).This gene is presented in approximately 50 to 70% of *H.pylori* strains (Ching *et al.,* 1996).

H.pylori infection is associated with MALT lymphoma and gastric adenocarcinoma .

CagA is also a highly antigenic protein that is associated with a prominent inflammatory response by eliciting interlukin-8 production (Yamaoka, 2010).

2.7.3. *Urea A gene (UreA)*

The urease is predominantly located in the bacterial cytoplasm, but is also found on the cell surface. It has long been believed that the external urease was responsible for creating a cloud of ammonia around the bacterial cell, thus lowering the pH of the surroundings and allowing the bacteria to grow in the generally acidic environment. Urease is composed of two types of subunit, *UreA* and *UreB,* encoded by the *ureAB* genes that are organized in an operon, which is transcribed as a bicystronic RNA. A total of 12 of each type of subunit are associated to form the active holoenzyme (Kuster, 2006).

Enzymatic reaction:

Urease catalyzes the hydrolysis of urea to yield ammonia and carbonate .The letter compound spontaneously decomposed to yield another molecule of ammonia and carbonic acid:

$H_2N\text{-}CO\text{-}NH_2 + H_2O \xrightarrow{Urease} NH_3 + H_2N\text{-}C(O)OH$

$H_2N\text{-}C(O)OH + 2H_2O \rightarrow NH_3 + H_2CO_3$

In aqueous solutions, the released carbonic acid and two molecules of ammonia are in equilibrium with their deprotonated and protonated forms; respectively .The net effect of these reactions is an increase in PH.

$H_2CO_3 \leftrightarrow H^+ + HCO_3^-$

$2NH_3 + 2H_2O \leftrightarrow 2NH_4^+ + 2OH^-$

(Kuster,2006).

2.7.4. *Induced by contact with epithelium gene A (Ice A)*

The *IceA* gene was identified in the *H.pylori* isolated from peptic ulcer disease and gastritis patients. There are two alleles of *IceA* ,$IceA_1$,and $IceA_2$ (Yakoob *et al*., 2015).

Some study have suggested that the *IceA* may significantly associated with disease of digestive system (van Doorn *et al*., 1998).

2.7.5*. glmM gene (UreC)*

The *UreC* gene encodes for a phosphor-glucos-amine mutase ;this gene is unrelated to the urease production so it was renamed *glmM* .This gene is considered as "housekeeping" gene ,and it participate directly in cell wall synthesis (DeReuse ,1997).

2.7.6 .*HPU*

H.pyloriś Urease is an important virulence factor produced in large amount by these bacteria. This protein is able to activate several cell types like neutrophils, monocytes, platelets endothelial cells and gastric epithelial cells .Immunization of urease has had much success in mice (Ermak *et al*.,1997).

Table (2.3): *Helicobacter Pylori* Virulence Factors & Their Association with Gastric Colonization and Disease:

Virulence gene	**Subtypes**	**Predicted role**	**Associated disease**
ureA	none	Acid resistance (conversion of urea into CO2 and 2NH3)	**None**
cagPAI (cagA, cagE, cagT)	none	Expression of the type IV secretion system and translocation of bacterial products to the host cell (induction of cell shape changes, apoptosis and proinflammatory cytokines)	Strains positive for *cagA, cagE* and *cagT* are associated with peptic ulcer, atrophic gastritis and gastric cancer
vacA	*s1/m1,s1/m2,s2/m1 s2/m2,i1 ,i2*	Cytotoxinassociated with membrane channel formation (vacuolation and apoptosis), disruption of lysosomal activity, interference with cytoskeleton, and cell signaling and immune modulation	*s1/m1* and *s1/m2* strains are closely associated to peptic ulcer, athrophic gastritis and gastric cancer
iceA	$iceA_1$, $iceA_2$	*iceA1*,encodes,arestriction endonuclease	*iceA1*-positive strains are associated with PUD

2.8. Treatment

Multiple antibiotic regimens have been evaluated for *H. pylori* therapy(,Graham *et al* , 2008 and Qasim, *et al,* 2005).The treatment regimen that is selected must consider local antibiotic resistance patterns (if possibly known), previous exposure and allergies to specific antibiotics, cost, side effects, and ease of administration. All patients with evidence of active infection with *H. pylori* should be offered treatment. The standard first-line therapy is a one-week "triple therapy" consisting of proton pump inhibitors such as omeprazole and the antibiotics clarithromycin and amoxicillin, Variations of the triple therapy have been developed over the years, such as using a different proton pump inhibitor. In areas with higher rates of clarithromycin resistance, other options are recommended (Malfertheiner *et al.*, 2007).

Most patients will be better served by first line treatment with bismuth quadruple therapy or concomitant therapy consisting of a PPI, clarithromycin, amoxicillin and metronidazole. When first line therapy fails, a salvage regimen should avoid antibiotics that were previously used (Chey *et al.*,2007) .

2.9. Prevention

H. pylori are a major cause of certain diseases of the upper gastrointestinal tract. Rising antibiotic resistance increases the need to search for new therapeutic strategies; this might include prevention in the form of vaccination. Much work has been done on developing viable vaccines aimed at providing an alternative strategy to control *H. pylori* infection and related diseases, including stomach cancer(Blanchard, 2010) Researchers are studying different adjuvants, antigens, and routes of immunization to ascertain the most appropriate system of immune protection; however, most of the research only recently moved from animal to human trials. An economic evaluation of the use of a potential *H. pylori* vaccine in babies found its introduction could, at least in the Netherlands, prove cost-effective for the prevention of peptic ulcer and stomach cancer(de Vries *et al* ., 2009).A similar approach has also been studied for the United States.

The presence of bacteria in the stomach may be beneficial, reducing the prevalence of dermatitis ,inflammatory bowel disease, gastro esophageal reflux disease, and esophageal cancer by influencing systemic immune responses ,(Salama *et al*., 2013).

CHAPTER THREE

MATERIALS AND METHOD

3. Materials and methods

3.1. Study Design

This is a descriptive, cross sectional hospital based study.

3.2. Study area and duration

This study was conducted in Khartoum state at Fedial hospital during May to September 2018.

3.3. Sample size

Sixty one (n=61) biopsies samples were collected from antrum region of the stomach.

3.4. Inclusion criteria

Patients who are suffering from Dyspepsia and gastritis and indicated for endoscopy were included in this study.

3.5. Exclusion criteria

Patients who have gastrointestinal bleeding were excluded in this study and

Patient who are not indicated for gastric endoscopy were excluded.

3.6. Sample Technique

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

3.7. Study populations:

Samples were collected from patients suffers from gastrodoudenitis, Gastritis and peptic ulcer disease.

3.8. Data Collection:

Data were collected through a self-administered questionnaire. Questionnaire was designed to record demographical clinical data.

3.9. Ethical consideration

Permission to conduct this study was obtained from College of Graduate Studies, Sudan University of science and technology and verbal consent were obtained from patients and head of endoscopies unit at Fedail hospital.

3.10. DAN extraction

DNA was extracted using Vivantis GF-1 Nucleic acid extraction kit (Vivantis, MALAYSIA). Thiskit applies the principle of spin mini-column technology and the use of optimized buffers ensure that only DNA and \or RNA is isolated while cellular protiens, metabolites ,salts and other impurities are removed during subsequent washing step. First endoscopy biopsy samples were collected and extracted in plain containers followed by a lysis buffer and

proteinase K. The DNA was extracted according to manufacturer's instructions. Finally the DNA was eluted in 200µl elution buffer provided with the kit.

3.11. Target amplification by polymerase chain reaction (PCR)

Amplification was conducted in a total volume of 25 µL. The reaction mixture contained 12.5 uL, 2X ready PCR mix (Thermo Scientific) and consisted of 1.25 U Taq-Pol, 75 mM Tris-HCL (pH 8.8), 1.5 mM MgCl2, and 0.2 mM of each dNTP. The reaction mixture contained 12.5 uL master mix, 1.0 uM of each forward and reverse primers (Table 1), 2 ug DNA template, and 8.5 uL RNase free water to a total volume of 25 uL. The amplification was carried out in a C-1000 thermal cycler (Bio-Rad, USA) according to the following program: an initial denaturation step at 95°C for 10 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing, primer specific shown in Table 1 for 1 min, and a final extension step at 72°C for 5 min. Amplified PCR products were resolved by agarose gel electrophoresis (5 V/60 min) using 2% agarose in Tris Acetate-EDTA (TAE) buffer containing 0.8 ug/mL of ethidium bromide. Molecular size ladder of 100 bp (Fermentans, Germany) and 50 bp were used to determine the size of the bands. The gel was viewed and photographed on a Camera phone . The primers used for the amplifications were obtained from Invitrogen (Rhenium, Jerusalem), shown in Table below.

Table (3.1): Primers, sequence, product size and annealing temperature:

Primer	5´ -3´ Sequence	Product size (bp)	Annealing (C°)
UreA-F UreA-R	AACCGGATGATGTGATGGAT GGTCTGTCGCCAACATTTTT	217	58
IceA1-F IceA1-R	CGTTGGGTAAGCGTTACAGAATTT TCATTGTATATCCTATCATTACAAG	558	56
IceA2-F IceA2-R	GTTGTCGTTGTTTTAATGAA GTCTTAAACCCCACGATTAAA	120	50
HPU1 HPU2	GCCAATGGTAAATTAGTT CTCCTTAATTGTTTTTAC	411	45
GlmM-F GlmM-R	AAGCTTTTAGGGGTGTTAGGGGTTT AAGCTTACTTTCTAACACTAACGC	294	55

3.12. Data analysis

Data was analyzed by using Statistical Package for Social Science Program (SPSS) version (11.5).

CHAPTER FOUR

RESULTS

4. RESULTS

4.1. Age group

Most of study population were females 26 (52%) 18 suffering from gastritis, 4 suffering from severe gastritis, 3 suffering from gastric ulcer and 1 suffering from dysphagia compared with males 24 (44%) 21 suffering from gastritis 1 from sever gastritis , 1 suffering from ulcer and 1 suffering from chronic gastritis.

The age ranges from 10 – 90 with mean 37 years.

4.2. Gender

The overall results revealed that total of 5 females and 3 males positive for *IceA1*,14males , 17 females were positive for *IceA2* ; 3 males and 4 females were positive for gLmM and 4 males positive for HPU compared to 0 positive for females .

Table (4.1): The Frequency of positive virulence genes of *H.pylori* in isolated biopsies:

Urea A gene of *H.pylori*	Positive result		Total
	Male N(%)	Female N(%)	
IceA$_1$	3(6%)	5(10%)	8(16%)
IceA$_2$	14(28%)	17(34%)	31(62%)
gLmM	3(6%)	4(8%)	7(14%)
HPU	4(8%)	0(0%)	4(8%)
Total	22(44%)	26(52%)	50(100%)

4.3. Analysis result

Antral biopsies were collected from 61 patients, Molecular identification of *Helicobacter pylori* were performed in all biopsies by PCR using primers (*UreA*) to amplify a 217 bp product for the *urea* gene; the rate of positive *H.pylori* in the biopsies tested was 82% (50/61) .

The positive *UreA* gene biopsies were tested for the frequency of *H.pylori* virulence genes *IceA1*,*IceA2*,*gLmM* and *HPU* and the frequency of positive virulence genes summarized in **table (4.2)** bellow:

Table (4.2) :The frequency of *H.pylori* virulence gene among positive sample :

Gene	$IceA_1$	$IceA_2$	gLmM	HPU	UreA
Positive	8(16%)	31(62%)	7(14%)	4(8%)	50(82%)
Negative	42(84%)	19(38%)	43(86%)	46(93%)	11(8%)
Total	50(100%)	50(100%)	50(100%)	50(100%)	61(82%)

The overall results revealed that the *IceA2* gene is the most frequent gene31(62%) positive samples and the same gene is responsible for Gastritis 24(78%) of positive sample shows Gastritis as main gastroduedenal symptoms see **Table Below:**

Table (4.3): Association between gastroduedenal symptoms and *IceA2* gene

Gene	Gastritis		Total
	yes	No	
IceA2 positive	24	7	31(78%)
IceA2 negative	15	4	19(22%)
Total	39	11	50(100%)

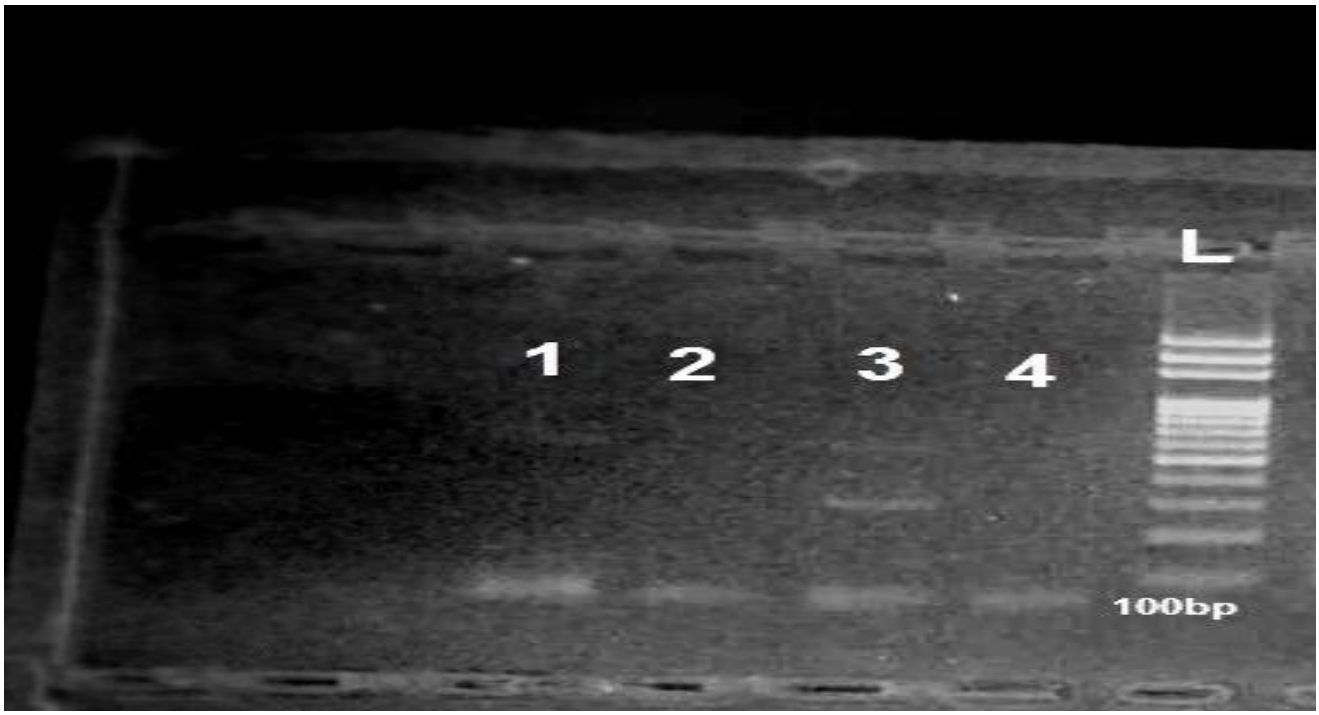

Figure (4.1) agarose gel electrophoresis of multiplex PCR product 1,2 Lane shows primer dimer ; Lane3 gLmM AND IceA2 positive ; Lane L 100 bp ladder

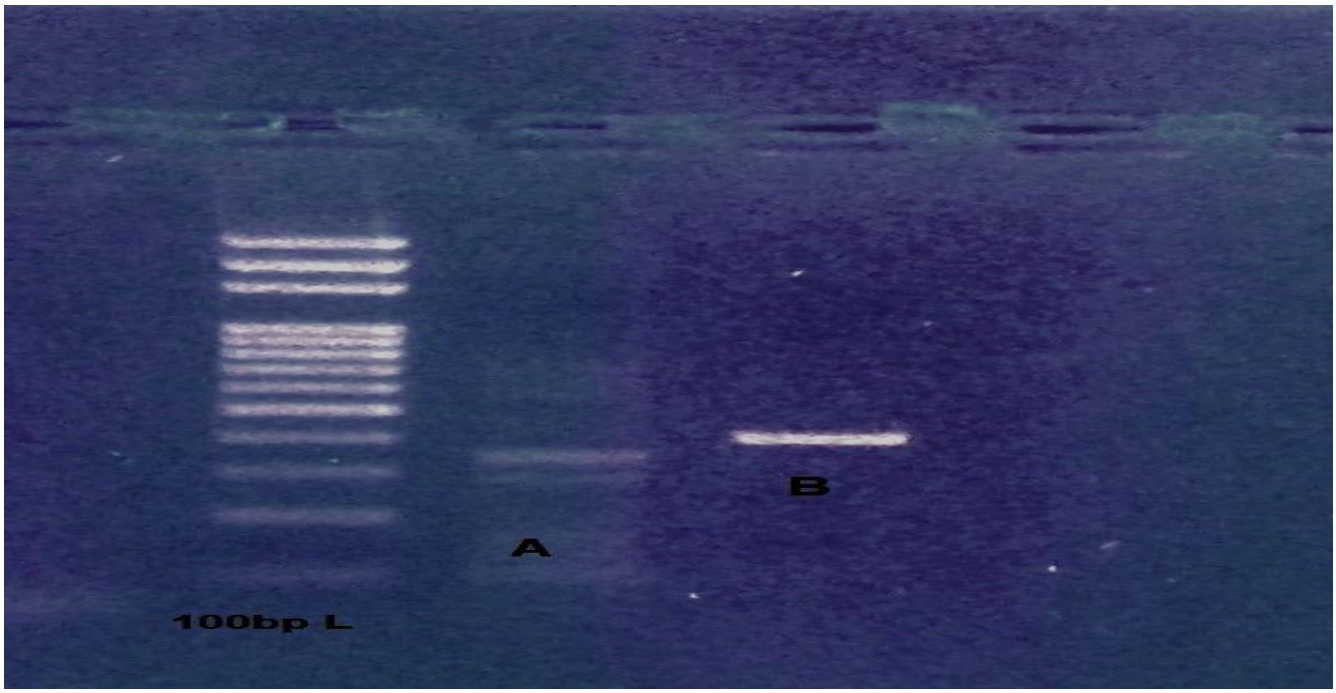

Figure (4.2) Agarose gel electrophoresis of PCR product ;Lane A and B positive for HPU gene

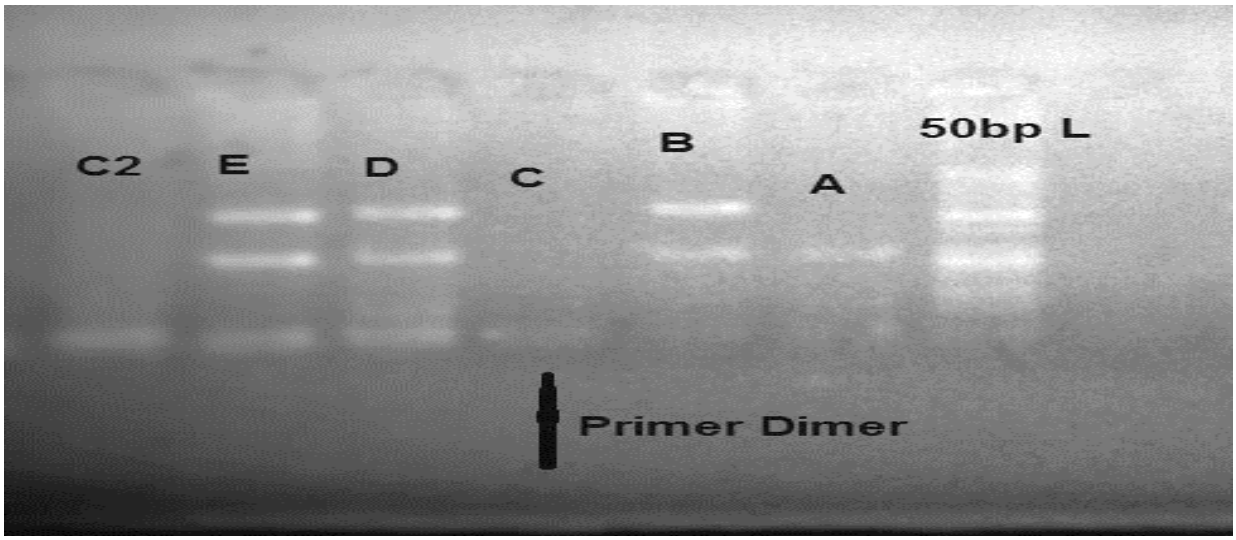

Figure (4.3) agarose gel electrophoresis of multiplex PCR product;C2 Lane shows primer dimer ,Lane B ,D and E gLmM and IceA1 positive ,Lane A positive for gLmM only and lane L is a 50 bp ladde

CHAPTER FIVE
DISCSION

5.1. Discussion

The clinical development of *Helicobacter pylori* infection is determined by the interaction of several factors of the host and bacteria. Among the factors of the micro-organism, there are evidence of the involvement of certain *H.pylori* genotypes in more sever pathologies (Van Doorn *et al* .,1998).

In this study we determined the frequency of *IceA$_1$*,*IceA$_2$*, *gLmM* and *HPU* from *H.pylori* isolates from strictly selected patients with gastric pathology.

UreA gene has been used to identify *H.pylori* in gastric biopsies, from 61 samples, 50 (82%) samples were positive and this agrees with the result obtained from Iraqi 73.9% and palastin study shows more than 90% (sugimoto *et al*., 2009).In this study *IceA$_2$* gene was the most frequent gene (62%) contrary to *IceA*$_1$ gene (16%). A Brazilian study reported a rate of *IceA$_2$* is 90.1% (Ashour *et al.,* 2001) another study reported that the prevalence of *IceA1* (64.58%)compared to *IceA2* (and 39.89%)(Wei *et al* ., 2012).In East Asia, the *IceA$_1$* genotype has been reported to be the predominant (76%) while in Portugal and Colombia *IcaA$_2$* is predominant(Wu *et al* ., 2005).

Our finding revealed that the *HPU* gene and *glmM* gene together were not common in Sudanese population and this result need to confirm.

This result agrees with another result that report a low prevalence of *gLmM* gene(20%) and disagree with another result that report *gLmM* gene as Housekeeping gene(De Reuse *et al* ., 1997).

The induced by contact of epithelium has two subtypes *IceA$_1$* and *IceA$_2$* and it has been reported that the sensitivity and specificity of the frequency genes is 16% and 62% respectively. In this study there is no association between *IceA$_1$* gene with gastritis and peptic ulcer unlike to other reports (Proenc$_s$a-Modena *et al.,* 2009).

There are significant variations reported regarding the prevalence of *IceA$_1$* and *IceA$_2$* genes. The *IceA$_2$* has been reported to be the prevalent gene while *IceA$_2$* the prevelant gene in other (Yamoka *et al.,* 1999).

The result of the present study along with the previous reports suggests that PCR has a great value in *H.pylori* diagnosis.

There is a high prevalence of gastritis among investigated patients 78% and our results were disagreements by previous study showed that 20% of patients have chronic gastritis .it

seems that the distribution of gastric pathological among patients was varied and depend on the selection of patient and/or the host and environmental factors(Wroblewisky *et al*.,2010).

In this study *UreA* gene and *IceA*$_2$ has a value in the diagnosis of *H .pylori* in symptomatic patients.

IceA$_2$ gene was found to be the most prevalent gene in gastritis and this result did not agree with another report that revealed no significant association between the *IceA*$_2$ and clinical outcomes(Wang *et al*., 2007), and the other genes, *HPU, gLmM and IceA*$_1$ showed low rate.

5.2. Conclusion

The study concluded that:

- *Helicobacter pylori* bacteria were directly detected from biopsy samples using *ureaA* specific gene in 82% of symptomatic patients.
- The most frequent virulence factor of *H. pylori* is $IceA_2$ gene.
- The analysis showed that infection with the $IceA_2$-positive *H. pylori* significantly increased the overall risk for gastritis by 78% , The $IceA_1$, *gLmM* , *HPU* genes status and clinical outcome of *H. pylori* infection have no significant correlation.

5.3. Recommendations

- Further studies with large sample size from different Sudanese locations are needed to determine the epidemiology of virulence gene of *H.pylori*.
- Further studies are needed to identify the most frequent virulence factor associated with the gastritis and gastric cancer among Sudanese population and the physiology and clinical pathology of these infections to consider possible prevention measures.
- We recommended to introducing PCR in routine diagnosis of *H.pylori* due to its high sensitivity, specificity and rapidity.
- Further studies are needed to study routes of transmission of *H. pylori* and possible prevention measures.
- More studies should be established in an antimicrobial resistance surveillance system to monitor the changes in resistance among *H. pylori* strains in Sudan.

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Appendix I

Preparation of 10 X TBE buffer

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

Preparation of 1X TBE buffer

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

Preparation of ethidium bromide solution

Ten milligrams of ethidium bromide powder were dissolved into 500 µl deionized water, and kept into brown bottle.

Preparation of agarose gel

Amount of 2 gram of agarose powder dissolved by boiling in 100 ml 1X TBE buffer, then was cooled to 55°C in water bath, 5 µl of (10mg\ml) Ethidium bromides 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.

Visualization of the DNA

The gel casting tray was put into the electrophoresis, tank flooded with 1x TBE buffer just to cover the gel surface (Appendix),10 µl of PCR products; from each samples was added to wells of electrophoreses, 5 µl of DNA ladder (100-bp DNA ladder, iNtRON, Korea),was added to the well in each run. The gel electrophoresis apparatus was connected to power supply (Primer, 100 V, 500 mA, UK). The electrophoresis was carried out at 75Volts for 30 minutes and the gel tray was removed from the electrophoresis apparatus and the buffer was discarded. Then the gel was visualized for DNA bands by U.V trans illuminator and photographed (Uvitec – UK).

Appendix1I-Questionnaire

Sudan University of Science and Technology

Collage of graduated studies

Molecular Determination of *Helicobacter pylori* Virulence Genes in patients with Gastrodudenities, Gastritis and peptic ulcer in Khartoum stat

By: Nosaiba Alsafi Abduelrhman

Supervisor: Dr. Hisham noureldaym Altayeb

ID: _____

Hospital:_____

Unit: _____

Age: _____ **Sex: Male** ☐

Female ☐

Diagnosis: 1- Duodenal Ulcer ☐ **Gastric Ulcer** ☐

3-Int.metaplasia ☐ **4-Non atrophic gastritis** ☐

5-Gastric Cancer: Intestinal ☐ **Diffuse** ☐

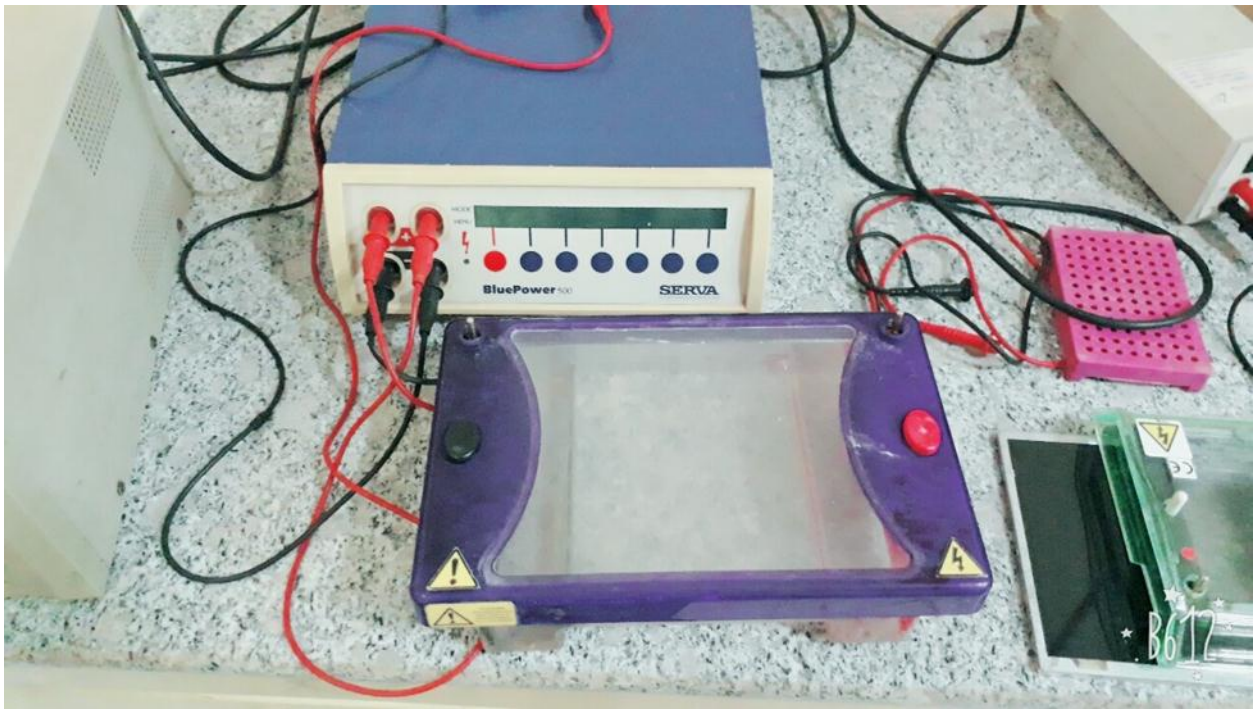
Comment:_____

Patient sign:_____

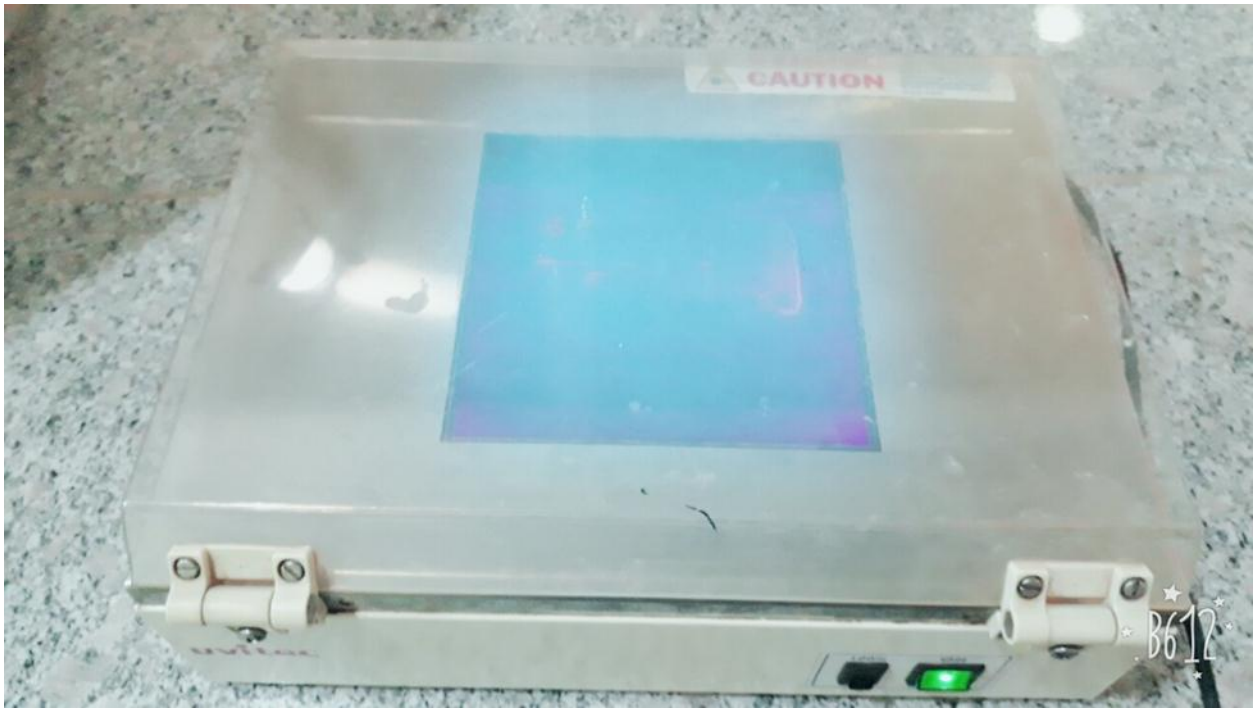
Appendix III

Color plate (1):Thermo cycler device (A)

Thermo cycler Device (B)



Color plate (2):Gel electrophoresis and power supply device



Color plate (3):UV Light trans illuminators device