Molecular Detection of Metronidazole Resistant Genes ($RdxA$ and $FrxA$) of Helicobacter pylori Isolated from Stool Specimen at Alinjaz Diagnostic Center in Khartoum State

الكشف الجزيئي للجينات المقاومة للميترونيديازول للبكتريا الحلزونية المعزولة من عينات البراز بمركز الإنجاز التشخيصي بولاية الخرطوم

A dissertation Submitted in Partial Fulfillment of the Requirement for the award of the Degree of Master in Medical Laboratory Sciences (Microbiology)

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Jan 2019
قال تعالى:

(قالوا سبحانك لآ علمنا إلا ما علمتنا إنيك أنت العليم الحكيم)

صدق الله العظيم

سورة البقرة الآية (32)
DEDICATION

Behind any successful work there are self-efforts as well as guidance from those who are close to our hearts.

I dedicate this humble effort to my parent for their love, encouragement, prayers of day and night and supporting me morally and financially.

For all those who encouraged me to fly towards my dream.

Along with all hard working and respected teachers, and for all gentle readers.
AKNOWLEDGMENT

First of all, thanks to ALMIGHTY ALLAH who helped me throughout this research.

Then completion of this research could not have been possible without participation and assistance of so many people whose names may not be all enumerated.

Whatever I have done is only due to such guidance and assistance and we wouldn’t forget to thank them.

My deepest thanks to my supervisor Prof. YousifFadlallaHamedelnil for his support, guidance, advices, valuable comments and suggestions that benefited me much in the completion of this research.

Also extend my heartfelt thanks to my family and well-wishers.
Abstract

The bacterium *H. pylori* is a fastidious, microaerophilic spiral Gram negative microorganism. This study aimed for molecular detection of metronidazole resistance genes (RdxA and FrxA) in *H. pylori* isolated from stool specimens in Elinjaz Diagnostic Center, in Khartoum State. A total of 65 samples were collected in this study, and bacteria were identified by the immune chromatography test (ICT), chemical method (Guanidine) was used for DNA extraction, and multiplex PCR was conducted to detect metronidazole resistance genes (RdxA and FrxA) in *H. pylori* isolates. RdxA and FrxA genes were not detected in the isolates.

As conclusion there were no detected resistance genes to metronidazole in *H. pylori* in this study.
المستخلص

هدفت هذه الدراسة إلى الكشف الجزيئي للجينات المقاومة للميترونيديازول (RdxA and FrxA) في البكتيريا الملولية البوابية المعزولة من عينات البراز في مركز الإنجاز التشخيصي بولاية الخرطوم. جمعت 65 عينة من البراز وتم التعرف عليها البكتيريا بطريقة فحص الاستضراب المناعي. استخدمت الطريقة الكيميائية (الجواندين) لاستخراج الحمض النووي. وأخيراً اجري اختبار تفاعل البلمرة المتعددة للكشف عن الجينات المقاومة للميترونيديازول في الملوية البوابية. جين لم يتم الكشف عن بين المعزولات FrxA وRdxA.

خلاصة البحث، أنه لم يتم الكشف عن جينات مقاومة للميترونيديازول في الملوية البوابية في هذه الدراسة.
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<tr>
<td>ICT</td>
<td>Immune Chromatography Test</td>
</tr>
<tr>
<td>HPTI</td>
<td>Helicobacter pylori Type I</td>
</tr>
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<td>HP</td>
<td>Helicobacter pylori</td>
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<tr>
<td>TNF</td>
<td>Tumor Necrosis Factor</td>
</tr>
<tr>
<td>IgG</td>
<td>Immune globulin G</td>
</tr>
<tr>
<td>MIC</td>
<td>Minimum Inhibitory Concentration</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribo Nucleic Acid</td>
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<tr>
<td>pH</td>
<td>Potential Hydrogen</td>
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<td>IARC</td>
<td>International Agency for Research of Cancer</td>
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<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene Diamine Tetra Acetic acid</td>
</tr>
<tr>
<td>MALT</td>
<td>Mucosa-Associated Lymphoid Tissue</td>
</tr>
<tr>
<td>Cº</td>
<td>Celsius</td>
</tr>
<tr>
<td>C13</td>
<td>Carbon-13</td>
</tr>
<tr>
<td>CO2</td>
<td>Carbon dioxide</td>
</tr>
<tr>
<td>D.W</td>
<td>Distilled Water</td>
</tr>
<tr>
<td>Min</td>
<td>Minute</td>
</tr>
<tr>
<td>µl</td>
<td>Microliter</td>
</tr>
<tr>
<td>Ml</td>
<td>Milliliter</td>
</tr>
<tr>
<td>R.T</td>
<td>Room Temperature</td>
</tr>
<tr>
<td>Rpm</td>
<td>Round per minute</td>
</tr>
<tr>
<td>Kda</td>
<td>Kilo Dalton</td>
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<tr>
<td>Ag</td>
<td>Antigen</td>
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Chapter One

Introduction
1. Introduction:

*Helicobacter pylori* (*H. pylori*) is a motile, curved and Gram negative bacillus (Rafeey et al., 2007). *H. pylori* certainly is the most prevalent human infection, the frequency of infection due to *H. pylori* is nearly 50% in the world and in developing country is as high as 80-90% (Ghotaslou et al., 2013). This bacterium colonizes the stomach of human and its infection is correlated with gastritis, peptic ulcer disease and extra digestive disease (Gasbarrini et al., 2010). *H. pylori* is also considered as a human carcinogen (Malfertheiner et al., 2007). Since, *H. pylori* eradication therapy represents a key clinical essential.

Unfortunately therapy against *H. pylori* has turned out to be more difficult over the years, principally due to the great decrease of standard eradication therapies efficacy. Although *H. pylori* is sensitive to many antibiotics in vitro, just a few antibiotics can be used in vivo to treat infected patients. Management of *H. pylori* infections are recommended in all suggestive individuals (Smith et al., 2014).

Failure of treatment in *H. pylori* infection has become an actual subject for physicians.

The causes of treatment failure are many that can be grouped into microorganism – related factors, host – related factors and treatment related factors. *H. pylori* resistance to antibiotic is widely recognized as the chief reason for treatment failure (Di Mario et al., 2006).

Furthermore, antibiotic resistance should be considered as a lively idea, since it is prevalence can change not only among diverse countries, but also between two different periods in the same area (Seo et al., 2013).
The rate of antibiotic resistance in *H. pylori* has been evaluated worldwide (Kupcinskas et al., 2013).

Antibiotic use for infections other than *H. pylori* is accounting for the extensive raise antibiotic resistance rate in *H. pylori* (Papastergiou et al., 2014).

1.2 Rationale:

*H. pylori* has been linked to chronic active gastritis, peptic ulcers disease, gastric cancer, and mucosa-associated lymphoid tissue lymphoma. *H. pylori* has been classified as definite class I carcinogen by the world health organization may exceed 70% in some developing countries (Kabir, 2001). *H. pylori* is most important pathogen associated with significant disease fatality. Due to the rising frequency of antimicrobial resistance, management of *H. pylori* remains as challenge for physician in most parts of world. Metronidazole is used against *H. pylori* infections and is one of the few anti-bacterial agents as drug of choice that is effective in eradicated the microorganism. Some researcher reported the rate of treatment failure is more than 20% with triple therapy in which metronidazole is the drug of choice; also *H. pylori* resistance to metronidazole is the chief solitary reason responsible for management failure (Dammann et al., 2000). Metronidazole resistance is the most common antibiotic resistance in *H. pylori* in developing countries (Frenk and Clemens, 2003). In Sudan the rate of *H. pylori* resistance to metronidazole is high (71.5) (Hekma, 2000). For all this reasons this research was conducted.
1.3 Objectives:

1.3.1 General Objective:

To study the molecular characterization of metronidazole resistant genes (RdxA and FrxA) in \textit{H. pylori} isolated from stool specimens in Elinjaz Diagnostic Center, in Khartoum State.

1.3.2 Specific Objectives:

1. To detect the \textit{H. pylori} from stool specimens using immune chromatography test.
2. To detect the metronidazole resistant genes (RdxA and FrxA) in \textit{H. pylori} using multiplex PCR.
Chapter Two
Literature review
Chapter Two

Literature review

2. Literature review:

2.1 History:

The bacterium *H. pylori* was initially named *Campylobacter pyloridis*, then *C. pylori* (after a correction to the Latin grammar) and in the 1989, after DNA sequencing and other data showed that the bacterium did not belong in the *Campylobacter* genus, it was placed in its own genus, *Helicobacter*. The name *pylori* means "of the pylorus" or pyloric valve (the circular opening leading from the stomach into duodenum), and it is Greek word means gatekeeper. In 1875, German scientists found helical shaped bacteria in the lining of the human stomach. The bacteria could not be grown in culture and the results were eventually forgotten (Surbaum and Josehans, 2007). In 1893 the Italian researcher Giulio Bizzozero described helical shaped bacteria living in the acidic environment of the stomach of dogs (DeGroot et al., 2005). Professor Walery Joworski of the Jagiellonian University in Krakow investigated sediments of gastric washing obtained from humans in 1899. Among some rod-like bacteria, they also found bacteria with a characteristic helical shape, which called *vibrio rugula*. The first to suggest a possible role of this organism in the pathogenicity of gastric disease (Anderson, 2007). Then the bacterium discovered in 1979 by Australian pathologist Robin Warren, who did further research on it with Barry Marshall beginning in 1981, they isolated the organisms and were the first successfully culture them. In their original paper, Warren and Marshall contended that most stomach ulcers and gastritis were
caused by stress or spicy food as had been assumed before (Good win et al., 1989).

2.2 Classification:

The most important stage in the development of the taxonomy of gastric microorganisms was the proposal in 1989 to establish a new genus called *Helicobacter* to mean a spiral rod – and that *C. pylori* should be transferred to that genus as *H. pylori* (Table 2.1).

Table (2.1): Classification of *H. pylori*.

<table>
<thead>
<tr>
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<th>Bacteria</th>
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<tr>
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<td><em>Proteobacteria</em></td>
</tr>
<tr>
<td>Class</td>
<td><em>EpsilonProteobacteria</em></td>
</tr>
<tr>
<td>Order</td>
<td><em>Campylobacterales</em></td>
</tr>
<tr>
<td>Family</td>
<td><em>Helicobacteraceae</em></td>
</tr>
<tr>
<td>Genus</td>
<td><em>Helicobacter</em></td>
</tr>
<tr>
<td>Species</td>
<td><em>H. pylori</em></td>
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<tr>
<td>Binomially name</td>
<td><em>Helicobacter pylori</em></td>
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(Goodwin et al; 1989)

2.3 Cellular morphology:

*H. pylori* is a Gram – negative, s-shaped or curved rod 0.5-0.9 mm wide by 2-4 mm long with 1 to 3 turns when observed in vivo. No spores are formed in blood agar culture (in vitro), and spiral forms are less obvious with cells appearing more frequently as singly curved rods (Axon, 1996).

Cells of *H. pylori* typically have up to six polar flagella filaments.
The cells are mostly actively motile although more some culture may appear to be non-motile in hanging drop preparations. Other forms of *H. pylori* reported in culture and occasionally in vivo include spherical, V- shaped, U shape (ox – bow) and straightened forms. Infrastructure features flagella of *H. pylori* are sheathed with covering that is continuous with the outer membrane components of the body wall. Freeze – fracture ultra structure studies suggest that the normal configuration of flagella is seven. Flagella are each about 30 nm in diameter with filament of 12-15 nm. Some flagella have distinctive terminal bulbs but no function has been assigned to such structures (Allen *et al.*, 1997).

Electron microscopy also reveals the presence 40 nm thick glycocalyx or capsule – like polysaccharide rich layer external to the cell wall unit membrane, which is thicker in vivo than in cultured bacteria. General physiological properties of *H. pylori* is a microaerophilic , growing best in an atmosphere of 5% oxygen with 5-10 % CO$_2$ on blood containing media such as Oxide brain heart infusion agar (BHI) and 5 % horse blood agar enriched with 1% IsoVitale X , which is a chemically defined supplement containing B,2,L-glutamine , L- cysteine , and various other growth promoting compounds . It has a respiratory type of metabolism. The cultures grow optimally at 37°C after 3-5 days.

All strains grow over a relatively narrow temperature range of 33-40 °C, whereas some grow poorly at 30°Cand42°C, none grow at 25 °C. *H. pylori* will grow on a suitable culture medium over a wide pH range (5.5-8.5) with good growth between pH 6.9 and 8.0. *H. pylori*does not tolerate low pH in vitro (Blaser and Atherton, 2004).
2.4 Virulence Factors:

*Helicobacter pylori*’s pathogenic properties are provided by its special ability to survive in a gastric acid milieu, it is able to move and multiply for decades in the mucus immediately adjacent to the apical pole of epithelial cells in spite of the local and cellular reaction that it causes with its host. The bacteria’s virulence is on the one side an effect of the direct action of its products, and on the other side of the induction and modulation of the associated inflammatory reaction.

Many studies on persons infected with *H. pylori* have evidenced number mechanism through which the bacterium perturbs the local equilibrium of the gastric mucosa (Nedrudet et al., 2002).

1. *Helicobacter pylori*’s motility is also involved in the infection persistence colonization is possible even in the of *H. pylori* variants that have mutations of the flagellins, but preservation of the bacterial reserve in the mucus layer (chronic infection) necessitates the normal expression of both A and B flagellins. *H. pylori* bacteria adhere to the surface of gastric epithelial cells at the level of several membrane segment that contain cadherins, integrines and antigens of blood type H1 and Lewis (Zhong et al., 2008).

2. The ammonia production (caused by the action of bacterial urease) determines the increase of intracellular pH for the mucosal gastric cells in the superficial epithelium; the ammonia rapidly spreads through the membrane and reacts with H+ ions, forming ammonia ions that produce consecutive alkalinization of the intra environment. Modifications of the pH lead to an increase of intracellular Ca²⁺ concentration that activates calcium – dependent intracellular cascades and result in the final release
of chemostactic factors (especially interleukin 8 - IL8) for inflammatory cells (Zhong et al., 2008).

*H. pylori* produces proteases that break down glycoprotein of the mucus layer and phospholipases that damage the epithelial cell by direct interfering with the protective factors of the mucosa. Moreover, the phospholipases are direct involved in leucotrienes release, thus multiplying the noxious effect upon the mucosa.

Once the inflammatory alteration of the gastric mucosa appears, other mechanisms intervene and accelerate local lesions. The afflux of PMN (characteristic event for active gastritis) leads to mieloperoxidase release into the interstitial tissue, which react with hydrochloric acid thus determining the formation of hypochloric acid; the latter, combined with local ammonia transforms into ammonia hypochlorite. Both the hypochloric acid and the ammonia hypochlorite are extremely aggressive towards human cell and tissue, determining tissue necrosis (D’elio et al., 2004).

Colonization of the corporeal gastritis mucosa determines modifications of the local histology. Chronic antral gastritis leads to a decrease in number of D cell (somatostatine producing cell); consecutive hypergastrinemia stimulates the proliferation of parietal cells in fundic and corporeal areas and implicitly gastric acid hypersecretion. The increase of the production of gastric acid implies an increased chlorhydro – peptic aggression of the duodenal mucosa and the increased risk of duodenal ulcer. Chronic corporeal gastritis eventually associates atrophy with subsequent disappearance of parietal cells and hyposecretion of acid (Antony, 1999).
3. The vacuolization cytotoxine gene (VacA) is present in all HP types; the VacA gene codifies a protoxin of a molecular mass of approximately 140 KD with a signal sequence in the antino–terminal position. In vitro VacA is associated to different phenotypes: cytotoxicity causing vacuolization when VacA is accumulated in the endosome membranes of epithelial cells by apoptosis. In vivo the role of VacA cytotoxin is controversial. Studies done on animals did not confirm the role of VacA in the occurrence of epithelial lesions.

4. The flagellin’s genes – flaA, flaB. The Helicobacter mobility is an indispensable factor for bacterial colonization of the gastric mucosa. The researchers estimate that 80% of bacterial populations multiply in the mucus, the remaining 20% colonizing the entire surface of gastric epithelial cells. Among the proteins involved in the biogenesis of the machine that ensures the mobility of the HP FlagE (the hook protein) and FlbA and FlgR were studied.

5. The ureasis genes – ure A and ure B, ure C and ure D, ure E, ure F, ure G, ure H and ure I. The ureasis is an essential determinant of the bacterial virulence. The nonureolitic mutants are incapable to colonize the gastric mucosa. The ureasis is codified by an operon that contains structural genes of the enzyme (ureA and ureB) and five other genes (ure IEFGH) whose products determine the enzyme’s activation by incorporation of the nickel ions; an extremely compact ferric complex is created, thus offering the special acid resistivity to the enzyme. One of the unique characteristics of the ureasic operon in H. pylori is related to the presence of the gene ure I; it codifies a membrane protein involved in a membrane pore formation; this pore opens at low levels of pH and therefore allows
the efficient transport of ure I when the bacterium is found in an acid milieu. UreI is crucial for the bacteria and constitutes a sort of acidity sensor.

6. Adhesion factors. Several types of adhezines have been identified to date. Third adhezines permit HP to stick to the surface of epithelial cells.

They are coded by the babA gene, the babP gene, the alp A and alp B and later by the gene sabA. The genome’s analysis has revealed all these genes belong to a family of 32 genes that codify the external membrane’s proteins.

7. Pic B has the capacity to induce the production of interleukin IL8 (Labigne and Reuse) by the gastric epithelium.

8. Superoxide dismutase (SOD) catalyses the transformation of superoxide and peroxide into hydrogen and oxygen ions; these are the enzyme that allow H. pylori to resist to oxidative stress generated by phagocytes.

9. Cag A, the gene of an associated cytotoxin codifies a protein with a high molecular weight (120 – 140 kda), the pathogenicity’s island – the cag region, formed by zones cag I and cagII, is similar to those discovered in Salmonella or Escherichia coli. The H. pylori strains were classified in two categories – some containing a complete and functional pathogenicity island (cag PAI) and others without any pathogenicity island or with a less active one. The island codifies a secretion system able to translocate a protein into cells, the immune – dominant protein CagA with a variable molecular mass (120 – 140 kda) through which the bacterium is put connection with the epithelial or macrophage cells.
2.5 Transmission:

It is not known how *H. pylori* is transmitted or why some patients become symptomatic while other do not. The bacteria are most likely spread from person to person through fecal – oral or oral – oral routes. Possible environmental reservoirs include contaminated water sources. Iatrogenic spread through contaminated endoscopes has been documented but can be prevented by proper cleaning of equipment. (Nedrudetal., 2002).

2.6 Pathophysiology:

Adaption to the stomach's acidic environment to avoid the acidic environment of the interior of the stomach (lumen), *H. pylori* usesits flagella to burrow into the mucus lining the stomach to reach the epithelial cells underneath, where there is a more neutral pH.*H. pylori* is able to sense the pH gradient in the mucus and move towards the less acidic region (a process called chemotaxis).

This also keeps the bacteria from being swept away into the lumen with the bacteria's mucus environment, which is constantly moving from its site of creation at the epithelium to its dissolution at the lumen interface. *H. pylori* is found in the mucus, on the inner surface of the epithelium, and occasionally inside the epithelial cells themselves. It adheres to the epithelial cells by producing adhesions', which bind to lipids and carbohydrates in the epithelial cell membrane. One such adhesion is BabA, which bind to the Lewis b antigen displayed on surface of stomach epithelial cells. In addition to using chemotaxis to avoid areas of low pH, *H.pylori* also neutralizes the acid in its environment. It does this by producing large amounts of urease, which breaks down the urea present in the stomach to carbon dioxide and ammonia. The ammonia, which is
basic, then neutralizes stomach acid, by inducing inflammation, gastritis, and ulcer *H. pylori* harms the stomach and duodenallinum (Guarner et al., 2003).

2.7 **pathology**:

More than 50% of world’s population harbor *H. pylori* in their upper gastrointestinal tract. Infection is more prevalent in developing countries, and incidence is decreasing in Western countries.

*H. pylori* helix shape (from which the generic name is derived) is thought to have evolved to penetrate the mucoid lining of the stomach (Yamaoka, 2008).

Colonization and long – term persistence of *H. pylori* can induce a complex immune responses that can potentially severe mucosal damage, including atrophy, intestinal metaplasia and dysplasia. This makes *H. pylori* the etiologic 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 and gastric adenocarcinoma) (Ernst and Gold; 2000).

The association with the development of two forms of cancer led to the classification of *H.pylori* by the World Health Organization as the only bacterial class 1 carcinogen (Yamaoka, 2008).

2.7.1 **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 cells. 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 (Perez – Perez et al., 2005).
2.7.2 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, or pangastritis (Kajkawahetal., 2007). This phase is often associated with hypochlorhydria, which can last for months. It is unclear whether this initial colonization can be followed by spontaneous clearance and resolution of gastritis and, if so, how often this occurs. Follow up studies of young children with serology or breath test suggested that infection may spontaneously disappear in some patients in this age group, this has not been observed in adults other than under specific circumstances, such as development of atrophic gastritis (Perez – Perez et al., 2005).

2.7.3 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 a corpus predominant pangastritis. The reduction in acid secretion can be due to 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 acid suppressive drugs, in particular, proton pump inhibitors (PPIs) (Kuipers et al., 1995 b)

2.7.4 Peptic Ulcer:

Gastric or duodenal ulcers (commonly referred to as peptic ulcers) are defined as mucosal defects with a diameter of at least 0.5 cm penetrating through the mucus mucosa (Engel et al., 1995). Both gastric and duodenal ulcer disease are strongly related to H. pylori. In initial reports from all over the world in the first decade of gastric ulcers occurred in the presence of H. pylori infection (Fox et al., 2002).

2.7.5 Helicobacter and cancer:

Two related mechanisms by which H. pylori could promote cancer are under investigation (Axon, 2007). One mechanism involves the enhanced production of free radicals near H. pylori and an increased rate of host cell mutation. The other proposed mechanism has been called a “perigenetic pathway” and involves enhancement of the transformed host cell phenotype by means of alterations in cell proteins such as adhesion protein. It has been proposed that H. pylori induce inflammation and locally high levels of TNF- alpha and/or interleukin6.

According to the proposed perigenetic mechanism, inflammation – associated signaling molecules such as TNF- alpha can alter gastric epithelial cell adhesion and lead to the dispersion and migration of mutated epithelial cells without the need for additional mutations in tumor suppressor genes such as genes that code for cell adhesion proteins (Kuipers et al., 1995 a).
2.8 Diagnosis of *H. pylori* Infection:

Several diagnostic tests are used to detect *H. pylori* infection. These tests, including invasive and noninvasive techniques, have high sensitivity and specificity. The advantages of the various techniques are described below (Benjamin *et al.*, 2000).

2.8.1 Invasive Techniques:

2.8.1.1 Culture:

Because of fastidious nature of *H. pylori*, culturing the bacterium is often tedious and is no more sensitive or specific than simple histologic analyses. Culturing *H. pylori* also involves the cost of endoscopy, making the method even less practical.

2.8.1.2 Histologic analysis of biopsy:

Routine histologic analysis of biopsy samples is common and practical. This technique is helpful, because one can visualize the mucosa, permitting detection of histologic gastritis and lesions such as MALT-Type lymphomas, which are tumor of lymphoid tissues. There are, however, clear drawbacks that should be considered. First, the organism may have a patchy distribution, especially at the base of the stomach, so more than two biopsy specimens are necessary for accurate results. Also, standard staining techniques (i.e., eosinstaining) are usually unreliable for detection of *H. pylori* by microscopy. Adding to the impracticality of this method is that it requires endoscopy and diagnosis cannot be obtained until several days after the procedure (Chev and Lai, 2009).
2.8.1.3 Camplyobacter – like Organism (CLO) test:

This test is based on the fact that mucosal biopsy specimen can be inoculated into a medium containing urea and phenol red, a dye that turns pink in a pH of 6.0 or greater (Axon, 1996). The pH will rise above 6.0 when H. pylori, the campylobacter – like organism, metabolizes urea to ammonia by way of its urease activity. This test is commercially available and therefore quite inexpensive. Only one – half hours is required for diagnosis of infection, and the test has shown 98% sensitivity and 100% specificity. These qualities have made the CLO test the invasive technique of choice for diagnosis H. pylori infection (Benjamin et al., 2000).

2.8.2 Noninvasive Technique

2.8.2.1 Breath test:

Although H. pylori itself can be detected noninvasively, its urease activity can be detected by way of a breath test. In this test, urea that is radioactively labeled with carbon 13 and carbon 14 is ingested (Benjamin et al.; 2000). Bacterial urease splits off labeled carbon dioxide, which can be detected in the breath. Accuracy is not a problem for either of these breath tests, since both elicit 100% sensitivity and specificity. The breath test technique reflects only current infection with H. pylori but can demonstrate very rapidly the existence of infection. A disadvantage of this technique is that it may involve a small amount of exposure to radiation. Although carbon 13 is a stable isotope and does not emit radiation, its detection required a mass spectrometer, which may not readily available. The breath test is not yet commercially available (Benjamin et al., 2000).
2.8.2.2 Detection of IgG antibody:

When a host recognizes *H. pylori* an immune response immediately stimulates IgG and secretory antibody IgA. Therefore serologic testing for antibodies to *H. pylori* using the Enzyme–Linked Immuno Sorbent Assay (ELISA) has become widely accepted diagnostic. The test is simple, inexpensive, and readily available. ELISA detects IgG with a sensitivity of up to 99% and is 100% specific. Since spontaneous clearing of *H. pylori* by IgG or IgA is rare, an elevated antibody titer indicates current infection. This test also detects the decline in antibody titer after removal of the organism; however, the rate of decline of IgG after eradication is still not known. This technique, although useful and accurate, still has certain limitations. In order to determine a clear decline in antibody titer, the patient must be monitored for at least six months, and the cutoff for a significant decline is unclear. In addition, in order to control the inherent variability of the test, the base and follow-up titer must be measured simultaneously. Still, the outstanding accuracy and low cost makes this test an attractive choice for detecting *H. pylori* infection (Benjamin et al., 2000). In current practice, endoscopy is still required for diagnosis of infection by *H. pylori*. The full range of noninvasive techniques is expected to be more readily available soon, with the antibody test ideal for assessing current infection, and the carbon 13-urea breath test the method of choice for determining the response to infection (Benjamin et al., 2000).
2.9 Treatment of \textit{H. pylori}:

The National Institute of Health recommends that all patients’ infected with \textit{H. pylori} be treated with an antibiotic. However, although the bacterium is sensitive to most antimicrobial therapy in vitro, in vivo results have been disappointing. Researchers have attributed this discrepancy to the locale of \textit{H. pylori} infection, under the mucus gel layer in the stomach. Environments which are this acidic often decrease the antimicrobial activity of most antibiotics (Axon, 1996).

2.9.1 Triple Therapy:

Eradication of \textit{H. pylori} is defined as the absence of the organism four or more weeks after eradication therapy. Since the eradication rate for single drug therapy is only 19\% and that for double – drug therapy is still only 48\%, researchers have found that combining three antibiotics offers a better chance for eliminating the bacterium. The highest eradication rate, 82\%, was achieved by combining bismuth, metronidazole, and tetracycline. There are obvious drawbacks to this type of treatment. First of all, it is inconvenient for patient, so it is difficult for doctors to convince their patients to comply with the therapy. Second, such multidrug therapy is almost always associated with many adverse side effects, namely diarrhea, nausea, and vomiting, which occur in approximately 20\% of all patients (Longan and Walker, 2001).

2.10 The problem of Antimicrobial Resistance:

Acquired antibiotic resistance implies that the organism was originally sensitive but became resistant after exposure to an antibiotic. The chance of developing resistance to an antibiotic is depend on the frequency of the natural mutation that
confers resistance as well as whether the mutation that result in a resistant phenotype enhances, impairs or is neutral regards to the ability of the resistant organism to compete with antibiotic – sensitive organisms. Consider an example in which the frequency of a mutation result in an antibiotic resistance occurred in one in every ten million bacteria. If the total number of bacteria in the stomach were $5 \times 10^9$, 500 resistant organisms would be present even if the patient had not received the antibiotic. The proportion of $H.pylori$ exhibiting the resistant phenotype would also be so low [$<0.001\%$] that culture and susceptibility testing of a gastric mucosal biopsy would not identify that any resistant organism were present. Failure of antibiotic therapy would be attributed to the organism having acquired antibiotic resistance. Penicillin and Amoxicillin resistant was more prevalent in the U.S.A than in Europe. All penicillin resistance strains isolated in U.S.A exhibited beta-lactamase activity, whilst 80% of beta lactamase negative strains isolated in Europe were also Penicillin- resistant. Metronidazole resistance was (87%) in Saudi Arabia strain of $H.pylori$ (Alknaryet et al., 1997).

Antibiotic resistance in the $H.pylori$ is a growing problem. In the United State the frequency of resistance to Metronidazole range from about 20%to more than 50%, on average it is about 25% but is higher in women and immigrants from semitropical countries where metronidazole is widely used. Clarithromycin resistance has steadily increased and at present is in the range of 7-15% in U.S trials, pretreatment Amoxicillin MIC $>0.25\mu g/ml$, were found on approximately 3% of the patients (Engstrandet et al., 1997).

2.11 Pharmacological resistance:
Pharmacological resistance describes a situation in which therapy fails because the antibiotic does not achieve the appropriate concentration or duration at the sites where the antibiotic-sensitive bacterium is residing and multiplying (Bager et al., 1997). *H. pylori* also inhabit extra gastric sites such as gastric metaplasia in the duodenal bulb. *H. pylori* organisms within the stomach have a potentially formidable survival advantage because they live in an environment where the diffusion of antibiotic is limited and where the pH is lower than required for the antibiotics to be effective.

*H. pylori* is also relatively slow growers and, because most antibiotics are effective only with actively multiplying organisms, slow growth may provide a survival advantage. The topical effect of antibiotics is also limited by the short time they are in the stomach (Peters and Clissold, 1992).

2.12 Mode of action, resistance mechanisms metronidazole:

2.12.1 Mode of action

Electron reduction processes, leads to the formation of nitro-anion radicals and subsequent DNA damages (Gerrits et al., 2004).

2.12.2 Resistance mechanisms

Poor drug uptake and/or increased drug efflux, enhanced activity of DNA repair enzymes, increased oxygen scavenging abilities and decreased antibiotic activation arising from change in metronidazole – reducing enzymes (Gerrits et al., 2004).

2.12.3 The roles of frxA and rdxA in metronidazole resistance
In one analysis deficiencies in either rdxA, frxA or both explained the resistant phenotype, even though with mutations in frxA alone yielding low-level metronidazole resistance in clinical isolates, moderate –to-high-level metronidazole resistance in laboratory mutants with disrupted gene (Jeong et al., 2000). In contrary to this, it has been observed that Pylori strains become resistant to metronidazole in two ways: by inactivation of rdxA (type 1) or by inactivation of both rdxA and frxA(type11), and rarely , if ever by inactivation of frxA alone, disruption of rdxA alone can produce metronidazole resistance at all level of resistance of type 1 strains (Jeong et al., 2000).

2.13 Immune Response :

The human immune response to *H. pylori* involves the activation of neutrophils, monocytes and macrophages, and the production of serum antibody IgG and secretory antibody IgA. In addition, T cell proliferates as in a cell mediated response. However, as stated earlier, *H. pylori* infection, once acquired, persists indefinitely. (Wyatt and Rathbone, 1988). Therefore, although there is a definite and immediate immune response to *H. pylori*, the host is still unable to eliminate the parasite.

The intensity of the host immune responses can culminate in one of several ways:

The most common result is chronic superficial gastritis, which is an inflammation of the stomach lining due to the infiltration of lymphocytes, plasma cells, eosinophils, and monocytes into the mucosal lining of the stomach,
which causes injury to the gastric glands. The immune response can actually benefit *H. pylori* by releasing nutrients locally for the organism.

The host could be harmed by the immune response due to the direct damage of epithelial cells, which affects their function and vitality.

The host, in order to avoid this type of cell damages will often down-regulate its immune response, making it even more difficult to completely eliminate *H. pylori* from the affected area.

The immune response can also cause inflammation of the duodenum, leading to duodenal ulcers.

Atrophic gastritis, which is nonspecific inflammation of the entire lining of the stomach, may be the result of the infiltration of lymphocytes into the stomach. MALT-type and other lymphomas, which are tumors of the mucosal and lymphoid tissues, can also result from *H. pylori* infection.

The effects of infection by *H. pylori* represent a delicate equilibrium between the host inability to remove the organism and its ability to contain the damage caused by the pathogen. It is the integrity of this equilibrium that allows *H. pylori* to persist in most cases for a lifetime in their hosts (Wyatt and Rathbone, 1988).

### 2.14 Other body site *H. pylori* may found:

Stomach was supposed to be the only reservoir of infection in humans. Nevertheless *H. pylori* infection was detected in other sites recently. It was found in dental plaque and saliva and also in oropharyngeal lymphatic tissue. This finding is of great importance because of known carcinogenic potential of *H. pylori*.
It was declared type I carcinogen by IARC. The question of direct contribution of *H. pylori* to oral and oropharyngeal disease was not resolved yet (Nguyen *et al.*, 1995).

### 2.15 Prevention:

Since the source of *H. pylori* is not yet known, recommendations for avoiding infection have not been made. In general, it is always wise for persons to wash hands thoroughly, to eat food that has been properly prepared, and to drink water from a safe, clean source (Broutet *et al.*, 2001)
Chapter Three

Materials and Methods
Chapter Three

3. Materials and Method

3.1 Study design:

This was descriptive, cross sectional study.

3.2 Study area and duration:

This study was conducted at Alinjaz Diagnostic center, in Khartoum State. The PCR was performed at research laboratory in Sudan University of Science and Technology, during the period from June to December 2018.

3.3 Study population:

Sixty-five stool specimens were collected from patients of different age and both gender having H. pylori infection detected by ICTAg.

3.4 Sample size:

A total of sixty - five (n=65) stool specimens were collected from patients having H. pylori infection.

3.5 Sampling technique:

The study was based on convenience non probability sampling technique.

3.6 Data collection:

Questionnaire including personal and clinical data was used.
3.7 Ethical approval:

Approval to this study was obtained from the committee of Sudan University for Science and Technology College of Medical laboratory Science.

3.8 Laboratory Methods:

3.8.1 ICT Technique:

The stool was collected into clean, dry plastic jars with screw lids, then small amount of stool dissolved in the dilution buffer and mix well, and then squeezed 2 drops of the sample solution in the sample well of the H.pylori Ag device.

3.8.2 Extraction of DNA for the PCR:

DNA was extracted by Guanidine Chloride method by taking 1 gram of stool dissolved in 2ml of WBCs lysisbuffer, 1mlGuanidine Hydrochloride (57.2 grams dissolved in 100 ml D.W), 300 µl of NH4 acetate (57.81 gramsdissolved in 100 ml D.W) and 10µl of proteinase K wereadded, in 15 ml falcon tube, then the samples were incubated over night at 37. After overnight incubation the samples were cooled at room temperature, and then 2ml of pre – chilled chloroform was added, after that samples werecentrifuged for 5min at 6000 r.p.m. upper layer was collected in new falcon tube, 10 ml of cold absolute ethanol was added to collected samples, then the samples were incubated overnight at -20 0C . After overnight incubation the samples were centrifuged for 10 min at 6000 r.p.m then the supernatant was drained, pellet was washed with 4 ml of 70% Ethanol, then was centrifuged for 10 min at 6000 r.p.m. supernatant was poured off and pellet was allow to dry, pellet was dissolved in 100 µl of dd H2O and then kept at -20.

Table (3.1): primers sequences used for detection of H.pylori metronidazole resistant genes
<table>
<thead>
<tr>
<th>Target Genes</th>
<th>Primer Name</th>
<th>Sequences (5'-3')</th>
<th>Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RdxA</td>
<td>RdxA1</td>
<td>GCCACTCCTTGAAACTTTAATTTAGG</td>
<td>749</td>
</tr>
<tr>
<td></td>
<td>RdxA4</td>
<td>CGTTAGGGATTTTTATTGTATGCTAC</td>
<td></td>
</tr>
<tr>
<td>FrxA</td>
<td>FrxA1</td>
<td>CGAATTGGATATGGCAGCCG</td>
<td>913</td>
</tr>
<tr>
<td></td>
<td>FrxA4</td>
<td>TATGTGCATATCCCTGTAGG</td>
<td></td>
</tr>
</tbody>
</table>

**3.8.3 Preparation of 10x Tris Base EDTA (TBE) buffer:**

Amount of 108 g of Tris base was added to 55 g of boric acid and 40 ml of 0.5% EDTA and then dissolved into 1L of D.W.

**3.8.4 Preparation of 10X TBE buffer:**

10 ml of 10X were added to 90 ml D.W and mixed.

**3.8.5 Preparation of ethidiumbromide:**

10 mg of ethidium bromide were dissolved into 0.5 ml D.W and kept into brown bottle to avoid light.

**3.8.6 Preparation of agarosegel:**

Amount of 1.5 g ofagarose powder were dissolved into 100 ml of 10X TBE, then the mixture had been cooled, then 2ul of ethidium bromide was added, mixed well and poured in a casting tray that had been tapped up appropriately and equipped with spacers and suitable comb to from wells, any bubbles were removed and the gel allowed setting at room temperature after solidification the comb and the spacers were gently removed.
3.8.7 Master Mix:

Maxime PCR pre Mix Kit ( INtRonBiothochnology , Seongnam , Korea ) is a premixed ready to used , it contains all the reagent required for PCR ( except water , template and primers ) and additional compound needed for direct leading onto agarose gel tracking blue dye that allow the monitor progress during the electrophoresis .

3.8.8 Preparation of reaction mixture:

Multiplex PCR was carried out in 25µl volume using the Maxime PCR Pre Mix Kit ( INtRonBiothochnology , Seongnam , Korea ) , premix were dissolved by 15 µl of D.W , for each gene 0.75 µl of forward primer , 0.75µl of reverse primer , 3µl of DNA were added .

3.8.9 Detection of metronidazole resistant genes:

Multiplex PCR assay was performed to detect metronidazole – resistance genes (RdxA and FrxA) using specific primers in table 3.1.

PCR amplification involved initial denaturation at 95 ⁰C for 3 min, followed by 30 cycles of denaturation at 95⁰C for 1min, annealing at 55⁰C for 1min, extension at 72 ⁰C for 1 min and a final at 72 ⁰C for 5 min.

3.8.10 Visualization of PCR product:

The amplicons were separated at 120 V for 30 min in a 1.5% (Wt/vol) agarose gel containing ethidium bromide, bands were visualized under U.V trans illuminator ( UVitec – UK ) to detect the specific amplified products by comparing with 100 base pairs standard ladders ( INtRonBiothochnology , Seongnam , Korea ).
3.9 Data analysis:

Data were analyzed using Statistical Package for the Social Sciences (SPSS) version 16.
Chapter Four
Results
4. Result

4.1 Detection of *H. pylori* from faecal specimen by ICT:

Out of 100 patients, 65 (65%) were positive for *H. pylori* by ICT, and 35 (35%) were negative for *H. pylori* by ICT (table 4.1)

**Table (4.1): frequency of *H. pylori* by ICT in faecal specimen**

<table>
<thead>
<tr>
<th>ICT</th>
<th>Frequency</th>
<th>Percent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>65</td>
<td>65%</td>
</tr>
<tr>
<td>Negative</td>
<td>35</td>
<td>35%</td>
</tr>
<tr>
<td>Total</td>
<td>100</td>
<td>100%</td>
</tr>
</tbody>
</table>

**Figure (4.1): Frequency of *H. pylori* by I.C.T in faecal specimen**
4.2 Distribution of \textit{H. pylori} infection according to gender

Out of 65 patients infected by \textit{H. pylori}, 35(54\%) were females and 30(46\%) were males.

\textbf{Table 4.2 Frequency of \textit{H. pylori} infection according to gender}

<table>
<thead>
<tr>
<th>Gender</th>
<th>Frequency</th>
<th>Percent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Females</td>
<td>35</td>
<td>54%</td>
</tr>
<tr>
<td>Males</td>
<td>30</td>
<td>46%</td>
</tr>
<tr>
<td>Total</td>
<td>65</td>
<td>100%</td>
</tr>
</tbody>
</table>

\textbf{Figure 4.2: Frequency of \textit{H. pylori} infection according to gender}
4.3 Detection of *H. pylori* resistance genes to metronidazole from faecal specimens positive by I.C.T using PCR technique

For all 65 *H. pylori* positive in faecal specimen by ICT RdxA and FrxA genes are not detected.

**Table (4.3): frequency of RdxA gene detected by PCR:**

<table>
<thead>
<tr>
<th>RdxA gene</th>
<th>Frequency</th>
<th>Percent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>0</td>
<td>0%</td>
</tr>
<tr>
<td>Negative</td>
<td>65</td>
<td>100%</td>
</tr>
<tr>
<td>Total</td>
<td>65</td>
<td>100%</td>
</tr>
</tbody>
</table>

**Figure (4.3): Frequency of RdxA gene detected by PCR**
Also FrxA gene was not detected as shown in table (4.4) figure (4.4)

Table (4.4): Frequency of FrxA gene detected by PCR

<table>
<thead>
<tr>
<th>FrxA gene</th>
<th>Frequency</th>
<th>Percent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>0</td>
<td>0%</td>
</tr>
<tr>
<td>Negative</td>
<td>65</td>
<td>100%</td>
</tr>
<tr>
<td>Total</td>
<td>65</td>
<td>100%</td>
</tr>
</tbody>
</table>

Figure (4.4): Frequency of FrxA gene detected by PCR
Figure (4.5): PCR Result. M: size marker, 100bp. S: samples.
Chapter Five

Discussion
5. Discussion

5.1 Discussion

One of main causes of therapeutic failure is resistance of microorganism to anti-infective used (Berradettet al., 2017).

Sixty five samples were randomly tested for the present study, 30 of them were males (46%), and 35 were females (54%). Both sexes affected by H.pylori, but in this study it was observed that females predominance in Khartoum State, this agree with study done by Imtithalet al., (2017) in Sudan, also agree with study done by Seyda et al.,(2007) in Turkey. In this study it was observed that no H.pylori resistance to metronidazole in Khartoum State (0.00%). In Sudan published studies related to my study were many few. The result obtained in the study were not agreed to Hekmastudy, (2000) in Sudan where the rate of H.pylori resistance to metronidazole (71.5%), and also not agreed to Berradettet al., (2017) in France where the rate of H.pylori resistance to metronidazole (12.2%) and also not agreed to Dalia et al., (2017) in Egypt where the rate of H.pylori resistance to metronidazole (25%). Also height rate of H.pylori resistance to metronidazole were reported by Ghotaslou et al; (2015) in South America where the rate of H.pylori resistance to metronidazole (52.8%), Tanihet et al., (2011) in South Africa where the rate of H.pylori resistance to metronidazole (95.5%), Suk et al., (2008) in Senegal where the rate of H.pylori resistance to metronidazole (85%). Also low rate of H.pylori resistance to metronidazole were reported by Kimenga et al., (2014) in Kenya where the rate of H.pylori resistance to metronidazole (4.5%).

The difference between my result in this study and results in others studies may be due to difference in the type of specimen and the protocol used in the DNA
extraction. All these studies not taking stool sample, the sample was used in these studies is gastric biopsy, and also these studies used tissue DNA Extraction Kit for DNA extraction.
5.2 Conclusion

The findings in this study show no prevalence of metronidazole resistance genes of *H. pylori* from Elinjaz Diagnostic Center in Khartoum State.

5.3 Recommendations

1. More research should be done in this problem.
2. Remove the inhibitors from stool sample.
3. Larger sample size is needed for better result.
References:


Wyatt J. I and Rathbone B.J (1998), Immune response of the gastric mucosa to *Campylobacter pylori.* *Scand .J.Gastroenterol;* **142**: 44- 49


Appendixes
Appendix (1)

Sudan University of Science and Technology
Questionnaire

Molecular Detection of Metronidazole Resistance Genes (RdxA and FrxA) of Helicobacter pylori

Name: ........................................................................................................ NO (......)

Age: ......................................... Sex: ........................................................

Medical history:

Using of antibiotic □

ICT result:
................................................................................................................
................................................................................................................

PCR result: ................................................................................................
........................................................................
Appendix (2)

H Pylori Rapid Test - Bioplus Ltd.
Colored Plate 2:2
Thermocycler

Colored plate 2:3
Microwave