Sudan University of Science and Technology
College of Graduate Studies

Molecular Detection of *Helicobacter pylori UreC* and *16sRNA* genes in Saliva from Stool Antigen Positive Patients in Saad Rashwan Medical Center in Khartoum State (2019)

الكشف الجزيئي عن جينات البكتريا الحلزونية البوابية (*UreC, 16sRNA*) في اللعاب من مرضى ايجابيون للبكتريا عن طريق فحص المستضد في البراز من مركز سعد رشوان الطبي في ولاية الخرطوم (2019)

A dissertation submitted in partial fulfillment for the requirements of M.Sc in Medical Laboratory Science (Microbiology)

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بِسْمِ اللَّهِ الرَّحْمَنِ الرَّحِيمِ

قال تعالى " وَمَا أُوتِيتُمْ مِنَ الْعِلْمِ إِلَّا قَلِيلًا "

صدق الله العظيم

سورة الإسراء , الآية(85)

DIDICATION

Every challenging work need self-efforts as well as guidance of older especially those who were very close to our heart .

My humble effort I dedicate ;

To my father.

To my mother.

To my teachers.

To my friends.

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First of all, thanks to **ALMIGHTY ALLAH** for giving me strength to complete this research.

I would like to thank my supervisor, **Dr.Hisham Noraldayem** for his greatest help, starting from topic selection throughout the practical work till completion. I am also grateful to staff of the Research Laboratory, Sudan University of Science and Technology for their help and support during laboratory work. Thanks are extended to my friends and colleagues for their support and encouragement throughout this research. Especial thanks to **Miss. Moram Mustafa Mohammed** for her unlimited help and support to conduct this research.

ABSTRACT

Helicobacter pylori infection is responsible for several gastric disease especially major ulcers in the stomach and small intestine, transmission mechanism of *H. pylori* are still unclear but the fecal oral route might be the most important, and the oral cavity can be an extra gastric reservoir for the organism.

This is a cross sectional study conducted to detect *H. pylori(UreC, 16sRNA)* genes from saliva of stool antigen positive patients in Saad Rashwan medical center from March 2018 to March 2019. Total of fifty (N=50) saliva specimens were collected from patients (males and females of different age).

The DNA was extracted from saliva samples using quinidine chloride method and *16sRNA, UreC* (*glmM*) genes were used in multiplex polymerase chain reaction to detect *H. pylori*.

Out of 50 saliva samples collected in this study one sample (2%) was found positive for *Helicobacter pylori* (*UreC* gene), and all other samples were found negative. This study conclude that *H. pylori* can be found in saliva specimens, which can be a source of transmission.

المستخلص

عدوى البكتريا الحلزونية البوابية هي المسؤولة عن العديد من أمراض المعدة , وخاصة القرحات الرئيسية في المعدة والأمعاء الصغيرة, طرق انتقال البكتريا الحلزونية لا تزال غير واضحة ولكن الإنتقال عن طريق البراز والفم قد يكون الأكثر أهمية , ويمكن أن يكون التجويف الفموي خزانا معويا اضافيا للبكتريا الحلزونية.

هذه الدراسة المقطعية التي اجريت للكشف عن جينات البكتريا الحلزونية (UreC, 16sRNA) من لعاب المرضى الإيجابيين للبكتريا عن طريق فحص البراز في مركز سعد رشوان الطبي. تم جمع ما مجموعه خمسون (N=50) عينة من اللعاب من المرضى (الذكور و الإناث من مختلف الأعمار).

تم استخراج الحمض النووي من عينات اللعاب باستخدام طريقة القوانيدين وتم الكشف عن الجينات 16sRNA,UreC (glmM)باستخدام تفاعل البلمرة المتعدد للكشف عن البكتريا الحلزونية البوابية .

من 50 عينة لعاب تم جمعها لهذه الدراسة عينة واحدة (2%) فقط وجدت إيجابية للحمض النووي للبكتريا البوابية , وقد كانت جميع العينات الأخرى سلبية . وخلصت الدراسة الى أنه يمكن العثور على البكتريا الحلزونية في اللعاب الذي يمكنه ان يكون مصدر لنقل العدوى.

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List of Abbreviations

µm	micro meter
Bab A	Blood Group Antigen-Binding Adhesion
BMI	Basal Metabolic Index
CagA	Cytotoxin Associated Gene
CLO	Campylobacter-Like Organism
D.W	Distill Water
EDTA	Ethyline Diamine Tetra Acetic acid
ELISA	Enzyme Linked Immune Sorbent Assay
H. pylori	Helicobacter pylori
HspB	Heat shock protein B
IFA	Immuno Flouresent Assay
IgA	Immunoglobulin
IgG	Immunoglobulin G
IgM	Immunoglobulin M
Kb	Kilo base
KDa	Kilo Dalton
Mb	Mega base
nm	nano meter
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
PPI	Proton Pump Inhibitors
PUD	Peptic Ulcer Disease

RDT	Rapid Diagnostic Test
RUT	Rapid Urease Test
SPSS	Statistical Package of Social Science
TBE	Tris Borate EDTA
UBT	Urea Breath Test
Ure C	Urease C
US	United State
Vac A	Vacuolating Cytotoxin Gene
VBnC	Viable But non-Culturable

Chapter One

Introduction

Chapter one

1.Introduction

1.1Background

Helicobacter pylori is Gram negative, microaerophillic, spiral rod shaped, that live just beneath the antral gastric mucous layer, on the surface of epithelial cells (Bashir *et al*., 2011).

Helicobacter pylori responsible for one of the most common infection found in the human worldwide, isolated and cultured in early eighteen's by Warren and Marshal, a discovery that allowed them to be awarded Nobel Prize in 2005 (Diab and Abdelgadir, 2017).

It is a major cause of peptic ulcer (PU) disease and a recognized risk factor for gastric adenocarcinoma and mucosa associated lymphoid tissue lymphoma The lifetime risk of an *H. pylori* infected individual developing peptic ulcer disease (PUD) is estimated to be one in six (Amjad *et al*., 2010).

To date, how *H. pylori* transmitted remains unclear and the main suggested route is person to person transfer by oral oral and fecal oral mode (Cellini *et al.*, 2010). Recent studies in Sudan showed that the overall prevalence of *H. pylori* infection in patients with gastritis, assessed by different diagnostic methods, was around 69% (Osman *et al*., 2016).

Several methods may be used to diagnose *H. pylori* infection, histological examination of gastric tissue, bacterial culture, rapid urease testing, use of DNA probe, and PCR analysis (Dunn and Cohen, 2000).

The detection of *H. pylori* in the mouth can occur independently of stomach colonization (Burgers *et al*., 2008) suggesting that the human oral cavity could represent an important extra gastric reservoir of *H. pylori* (Suzukei *et al*., 2008; Kabir 2004), and The low number of *H. pylori* cells isolated from districts different

from the gastric environment might be due both to the difficulty in culturing *H. pylori* from sites colonized by fast growing bacteria and the presence of bacteria entering the viable but not culturable (VBNC) state in which the microorganism modifies its morphology from spiral to coccoid (spherical) form with a loss of cultivability (Cellini *et al*., 1998; Andersen and Rasmussen, 2009).

Consequently, biological samples such as Saliva or feces may not allow the selective growth of a fastidious bacterium such as *H. pylori* requiring more appropriate techniques (Kabir 2003; Rassmussen *et al*., 2010 ; Wnuk *et al* ., 2010). PCR assays, provide a sensitivity in *H. pylori* detection (Bamford *et al* ., 1998; Kabir 2004; Cellini *et al* .,2008) .

1.2 Rationale

Helicobacter pylori is one of the most bacterial pathogen in human. The organism is associated with development of peptic ulcer disease, lymphoproliferative disorders and gastric cancer. Residence in developing country and socio economic condition are regarded as risk factor (Das and Paul, 2007).

Because of the important role of the oral cavity that play in the infection of *H. pylori*, the value of this research to see if the saliva can be sample for diagnosis, to detect the presence of *H. pylori* DNA in saliva, and the role of saliva in transmission and infection of *H. pylori*.

1.3 Objectives

1.3.1 General Objectives

1-To detect *H. pylori 16sRNA, UreC* genes in saliva from stool antigen positive patients in saad rashwan medical center.

1.3.2 Specific objective

1-To detect *H .pylori 16sRNA, UreC* genes in saliva from *H. pylori* positive patients using multiplex polymerase chain reaction.

2- To detect *H. pylori 16s RNA* gene in saliva .

3- To detect *H. pylori UreC* (*glmm*) gene in saliva.

Chapter Two

Literature review

Chapter two

2. Literature review

2. *Helicobacter pylori*

2.1 History

The presence of gastric spirochaetal organisms was first documented over a century ago. Though repeatedly reported in the medical literature, it was felt that these spiral bacteria were merely contaminants and the reports were generally ignored by the medical community. On 22 October 1982, at a meeting of the Royal Australian College of Physicians, successful culture of these Campylobacter like organism (CLO) from gastric biopsy specimens was reported for the first time. Moreover, it was shown that their presence was associated with gastritis and possibly with peptic ulceration (Buckley and Morain, 1998).

The first description of gastric ulceration was reported in 1586 by Italian physician, following by an autopsy report of duodenal ulceration by Johannes Von Murat in Switzerland 1688 and In 1761erythema and erosion of the stomach and duodenum were described in patients with heartburn and upper abdominal pain (Buckley and Morain, 1998).

During the eighteenth and nineteenth centuries, understanding of the function of the upper gastrointestinal tract gradually increased with the description of the digestive properties of gastric secretions and hydrochloric acid in the stomach. In 1825, William Beaumont, an American army physician, reported his famous series of in vivo gastric function experiments in a patient who developed a gastro-cutaneous fistula following a gunshot wound. The symptomatology of peptic ulceration was first described in detail an 1857(Buckley and Morain, 1998).

At this time, gastric ulceration was a common autopsy finding, especially in females, while duodenal ulceration was rarely reported. With the ability to clinically diagnose upper gastrointestinal conditions (Buckley and Morain, 1998). Gastric surgery developed during the second half of the nineteenth century. The odor Billroth described gastro duodenostomy and gastro jejunostomy in 1881. In the same year, the first potentially usable endoscope was reported. In 1897, using bismuth sub nitrate as a contrast material, the ability to diagnose peptic ulceration radio graphically was reported (Buckley and Morain, 1998).

2.2Morphology

Helicobacter pylori organisms are spiral, microaerophilic, Gram negative bacteria that demonstrate bluntly rounded ends in gastric biopsy specimens. However, when cultured on solid medium, the bacteria assume a rod like shape; spiral shapes are infrequent or absent. After prolonged culture on solid or in liquid medium, coccoid forms typically predominate. By electron microscopy, coccoid forms appear as U- shaped bacilli with the ends of the two arms joined by a membranous structure. Coccoid forms are metabolically active; however, they cannot be cultured *in vitro* (Dunn and Cohen, 2000).

In gastric biopsy specimens, *H. pylori* organisms are 2.5 to 5.0 μm long and 0.5 to 1.0 μm wide; there are four to six unipolar sheathed flagella, which are essential for bacterial motility. Each flagellum is approximately 30 μm long and approximately 2.5 nm thick Flagella exhibit a characteristic terminal bulb, which is an extension of the flagellar sheath. The flagellar sheath exhibits the typical bilayer structure of a membrane (Dunn and Cohen, 2000).

Ultra structurally, when tannic acid is used as a mordant, it can be seen that the outer membrane of *H. pylori* is coated with a glycocalyx like structure in gastric

biopsy specimens, the surface of individual bacteria may be linked to gastric epithelial microvilli by thread like extensions of the glycocalyx. The surface of viable *H. pylori* cells grown on agar plates is coated with 12 to 15 nm ring shape aggregates of urease and HspB, a homolog of the GroEL heat shock protein. Urease and HspB are also associated with the surface of viable *H. pylori in vivo* (Dunn and Cohen, 2000).

2.3 Genome and plasmid

The genome size of *H. pylori* range from 1.6 to 1.73 Mb with average 1.6 Mb. The GC composition aver ages 35.2 mol%, with a range of 34.1 to 37.5 mol% . Approximately 40% of isolates contain plasmids ranging in size from 1.5 to 23.3 kb, but the plasmids do not contain recognized virulence factors . The genome possesses at least two copies each of the 16S and 23S rRNA genes. The variable location of multiple genes in genomic maps suggests that extensive rearrangement of the *H. pylori* genome occurs. *H. pylori* exhibits significant sequence diversity in multiple genes including those that encode urease structural and accessory proteins, flagellin, vacuolating cytotoxin, and *CagA* (Dunn and Cohen, 2000).

Examination of allelic variation in six genes by multilocus enzyme electrophoresis has confirmed the genetic diversity of *H. pylori* strains. That are naturally competent for DNA uptake, which through recombination provides a mechanism for the diversity observed. The biological significance of such diversity is not known (Dunn and Cohen, 2000).

2.4 Epidemiology

H. pylori infection is common and the precise mechanism underlying its transmission is not definitively established. Suggested methods of transmission include ingestion of contaminated water, fecal oral means, and even periodontal

pocket serving as a reservoir. Other possibilities include transmission by flies iatrogenic spread with unsterile endoscope and use of PH probe (Wong and Mclean, 2016).

Most children in developing countries are infected before age 10, but in the US and other developed countries infection more commonly occurs in adulthood. *H. pylori* infection is less common in developed countries with a prevalence of approximately 20-30% and in developing countries disease burden is higher with prevalence rates approaching 90% (Wong and Mclean, 2016).

2.5 Transmission

The way in which *H. pylori* infection is acquired remains unclear. *H pylori* has been identified in drinking water using polymerase chain reaction (PCR) amplification. Infection passed through contaminated water has also been suggested, and transmission via food such as uncooked vegetables treated with sewage has been proposed (Stone, 1999).

Although *H. pylori* has been isolated in non-human mammals, in general the bacterium is confined to the gastric mucosa of man. The bacterium has also been found in monkeys. Although a common external source of infection cannot be ruled out, it appears likely that transmission of *H. pylori* is most commonly from person to person, as suggested by a high prevalence of infection amongst those living in institutions, and by intra familial clustering (Stone, 1999).

Transfer of *H. pylori* from stomach to stomach has been shown to occur via inadequately disinfected endoscopy equipment, direct gastric gastric transfer is not the normal route. Three methods by which the normal route. Three methods by which the organism is passed from the human stomach of an infected subject to the stomach of a previously uninfected person without the involvement of an external

source have been suggested: by feces, saliva and vomitus (Stone, 1999).

2.5.1 The fecal oral route

Evidence that transmission occurs mainly in early childhood concurs with the suggested fecal oral pathway. It has been suggested that transmission of *H. pylori* via feces may be restricted to young children with acute infection and adults with reduced acid secretion.

2.5.2 The oral oral route

The oral cavity as a possible reservoir of *H. pylori* has been suggested, with gastric juice transporting viable organisms to the mouth during regurgitation (Stone, 1999).

2. 5.3. The gastric oral route

It has been suggested that *H. pylori* may be passed more directly from stomach to mouth, without the need for an oral reservoir. Transmission via vomitus has been suggested as a possibility and has more recently been proposed as the main route of transfer, specifically during epidemic acute *H. pylori* infection in childhood (Stone, 1999).

2.6 Pathogenicity

After entering the host stomach, *H. pylori* utilizes its urease activity to neutralize the hostile acidic condition at the beginning of infection. Flagella mediated motility is then required for *H. pylori* to move toward host gastric epithelium cells, followed by specific interactions between bacterial adhesions with host cell receptors, which thus leads to successful colonization and persistent infection. Finally, *H. pylori* releases several effector proteins/toxins, including cytotoxin-associated gene A (*CagA*), and vacuolating cytotoxin A (*VacA*) causing host tissue damage (Kao *et al*., 2016).

In addition, the gastric epithelium layer, which forms the major interface between *H. pylori* and the host, secretes chemokines to initiate innate immunity and activate neutrophils, and further lead to the formation of clinical diseases such as gastritis and ulcer (Kao *et al*., 2016).

H. pylori weakens the protective mucus coating of the stomach and duodenum which allows acid to go through to the sensitive lining beneath. Both acid and bacteria irritate the lining and cause a sore or ulcer (Rana *et al.*, 2017).

2.7.Virulencefactors

Among microbial virulence factors identified so far, the *H. pylori* cytoxin associated gene A (*cagA*), its related pathogenicity island (*cag PAI*), vacuolating toxin A *(vacA*) and factors involved in adherence of *H. pylori* to gastric epithelial cells , have been linked to enhanced pathogenicity of the bacterium (Das and Paul, 2007).

The immune reactive 120-145 kDa protein *cagA* is encoded by *cagA* of *H. pylori* ,some of the genes in the *cagPAI* region encode a type IV bacterial secretion apparatus, which can translocate *cagA* into host target cells. Phosphorylation of *cagA* may activate host signaling pathways and subsequently influence host cellular functions, including proliferation apoptosis cytokine release, and cell motility. Individuals infected with *cagA* strains are more likely to have gastro duodenal ulceration than those who are cagA negative (Das and Paul, 2007).

About half of the *H. pylori* strains produce *vacA*, which induces epithelial cell vacuolation and cell death. *VacA* expression is determined by variations in the signal sequence (s1a, s1b, s1c, s2) and mid region (m1, m2) of the *vacA* gene, Infection with s1a/m1 strains is associated with intense inflammation and duodenal ulceration (Das and Paul, 2007).

The outer membrane bound protein, *BabA*, is an adhesin of *H. pylori*, interacting with the blood group antigen Lewis on gastric epithelial cells. The product of *babA1* is identical to *babA2* but cannot interact with Lewis, *H. pylori babA2* strains are associated with an increased risk of peptic ulcers and distal gastric adenocarcinoma, whereas *babA2* strains are more often associated with uncomplicated forms of gastritis (Das and Paul, 2007).

2.8 Risk factors

As the volume of drug distribution is higher in obese patient with high BMI, the risk of drug failure is higher, likely due to reduced concentration of drug at the gastric mucosal level, by contrast, lower basal metabolic index (BMI) patients will have better outcomes (Rana *et al.*, 2017).

Smoking is another risk factor for failure of therapy. The reason for this may be due to reduced gastric blood flow or intra gastric pH in cases of smoking or nicotine which could potentiate vacuolating toxin activity of *H. pylori* in gastric cells (Rana *et al.*, 2017).

2.9 Symptoms

Patients with *H. pylori* infection may clinically present with:

Dyspepsia, heartburn, abdominal pain, diarrhea, halitosis (Rana *et al*., 2017).

2.10. Diseases associated with *H. pylori*

All *H. pylori* infected patients develop chronic gastritis inflammation but this condition usually is asymptomatic This gastritis is not a disease or illness peptic ulcer disease had been considered to be idiopathic or to be due to agents such as aspirin or non-steroidal anti-inflammatory drugs or, rarely, to Zollinger Ellison

syndrome, Crohn's disease, and several other inflammatory disorders (Dunn and Cohen, 2000).

The idiopathic form of peptic ulcer disease represents 60 to 95% of all cases depending on the extent of non-steroidal anti-inflammatory drug use in the population. *H .pylori* is the cause of all the cases in the adults and that treatment that eradicates *H. pylori* leads to cure of the ulcers. Thus in any population *H. pylori* causes the majority of cases of both gastric and duodenal ulcer, Carriage of *H. pylori* also is strongly associated with the risk of development of atrophic gastritis, which is a precursor of lesion to gastric cancer (Dunn and Cohen, 2000).

Thus, not surprisingly *H. pylori* carriage also is associated with adenocarcinoma of the distal but not the proximal (cardia) stomach Infection in associated with both the intestinal and diffuse histologic types of tumors. This association is extremely important since in total, gastric cancer is second leading cause of cancer death in the world (Dunn and Cohen, 2000).

2.11.Diagnosis

Two types of method used to diagnose *H. pylori* infection:

2.1.11.Invasive Methods

2.1.1.11.Rapid Urease Test

The Rapid Urease Test (RUT) is a popular invasive diagnostic *H. pylori* test that is relatively quick cheap and simple to perform. It detects the presence of urease in or on the gastric mucosa. positive results are rarely observed. Best results for RUT are obtained if biopsies are taken from both the antrum and corpus. The biopsy used for RUT can also be used for other tests such as for molecular based tests of

microbial susceptibility or for host factors, false positive results are rarely observed (Kalali *et al*., 2015).

2.11.1.2 Histology

The presence of typical spiral motile bacteria accompanied by inflammatory reaction in the histopathological sections of stomach was the first described method used for the diagnosis of the *H. pylori* (Kalali *et al*., 2015).

Along with routinely applied staining's like Giemsa, hematoxylin, and eosin, there are some more specific staining procedures which facilitate the diagnosis of *H. pylori* infection. However, the accuracy of the histopathological diagnosis of *H. pylori* always depends on the number and the location of collected biopsy materials. While *H. pylori* can be detected in even a single biopsy taken from the correct site, and to achieve a higher sensitivity, multiple biopsies are recommended. Moreover, the possible presence of other bacterial species with a similar morphology to *H. pylori* in the stomach can be another source of error which negatively affects the accuracy of the test (Kalali *et al*., 2015).

2.11.1.3.Culture

Although it should be stated that *H. pylori* culture is not a routine procedure in initial diagnosis, in many bacteriology laboratories *H. pylori* isolation via the culture of biopsy samples is a routine second line approach, Because of the demanding character of this bacterium, this method remains challenging. This technique, although highly specific, is not as sensitive as other tests like histology and the rapid urease test (Kalali *et al*., 2015).

As well as for purposes of scientific research, cultured live *H. pylori* is used for diagnostic approaches and for the detection of antibiotic resistance if treatment

failure is suspected. *H. pylori* requires a microaerophilic atmosphere (5% to 10% oxygen, 5% to 12% carbon dioxide and 80%– 90% nitrogen with humidity) and a complex culture media. The most commonly used media contains Brucella, Columbia Wilkins Chalgren, brain heart infusion or trypticase agar bases, supplemented with sheep or horse blood (Kalali *et al*., 2015).

Because isolation of this microaerophilic organism from gastric biopsy specimens takes a long time, up to 5–7 days, to overcome the problem of growth of other competitors that exist in the sample, the culture media is supplemented with specific antibiotics (Kalali *et al*., 2015).

2. 11.1.4 Polymerase chain reaction

PCR based detection of *H. pylori* could be categorized under invasive as well as noninvasive methods. Molecular diagnostics have dramatically changed the clinical management of many infectious diseases in the past decades. PCR currently remains the best developed molecular technique as it provides a wide range of clinical applications, including specific or broad spectrum pathogen detection, evaluation of emerging novel infections, surveillance, early detection of bio threat agents, and antimicrobial resistance profiling (Kalali *et al*., 2015).

While PCR could be applied for the detection of *H. pylori* in biopsies this technique is more qualified for its use in samples taken from the oral cavity or from stool. In addition to the improved specifications of this technology like high sensitivity and automated specificity, simplicity, and procedures, there are several other advantages to be considered (Kalali *et al*., 2015).

Practically, regardless of genome size, any genomic material could be used as a template sample for PCR, which allows sampling from multiple origins (Kalali *et al*., 2015).

2.11.2Non-Invasive Methods

2.11.2.1.Urea Breath Test

The urea breath test (UBT) is one of the most common noninvasive tests used. This noninvasive test, available in different versions, and has been evaluated in different studies, showing high sensitivity, specificity and accuracy. The test is able to detect the infection indirectly by measuring the existence of bacterial urea produced by *H. pylori* in the stomach. There are different types of this test comprising ^{13}C or ^{14}C isotope labeled urea (Kalali *et al*., 2015).

If *H. pylori* is present, the urease hydrolyses the labeled urea and the exhaled isotope containing ammonia can be detected applying the samples to a measuring device. This test is recommended by the Maastricht IV/Florence Consensus Report as a valuable diagnostic tool for the detection of infection and for therapy control . It has been shown that UBT can distinguish an ongoing from a past infection; hence, it is able to detect the eradication progress after treatment (Kalali *et al*., 2015).

2.11.2.2Fecal Antigen Test

Fecal antigen tests detect antigens in stool samples. ELISA formats comprising monoclonal antibodies against *H. pylori* proteins showed improved results compared to polyclonal approaches.

The current guideline evaluates the use of the stool antigen test as equivalent to the UBT if a validated laboratory-based monoclonal antibody is used (Kalali *et al*., 2015).

2.11.2.3. Serological Test

Immune responses against *H. pylori* are utilized to detect infection by analyzing patients' blood or serum for IgG and IgA antibodies. Serology is the only test which is not affected by those local changes in the stomach that could lead to a low bacterial load and to false negative results. According to guidelines proposed by the Maastricht conference, only IgG detection is considered and the favored method is ELISA (Kalali *et al*., 2015).

Currently, different formats of serological tests are available, including simple ELISAs that use whole lysates or recombinant produced *H. pylori* proteins as antigens. More recently, immune blots, luminex based bead assays and line assays were developed, these allow a more specific evaluation of the infecting *H. pylori* strain in terms of bacterial virulence factors and host immune responses towards the human pathogen (Kalali *et al*., 2015).

2.11.2.4Rapid Diagnostic Test

Results of RDT testing become available within a couple of minutes to a few hours As generally in microbiology laboratories, *H. pylori* RDT kits could also be performed based on the following four approaches:

Testing of *H. pylori* specific antigens, molecular detection of the specific *H. pylori* nucleic acid sequence, rapid biochemical reaction test ,and serologic detection of *H. pylori* specific antibodies (Kalali *et al*., 2015).

2.12 Prevention

H. pylori has high risk for reinfection. There can be two reason:The hygiene habits and the difficulties to reach clean water resources have not been improved, and

The transmission of infection among the people living in the same home has repeated (Yucel, 2014).

In this situation, the eradication of infection cannot be possible without dried sources. Like the eradication of parasitosis, parasitosis is also a disease of bad sanitation, and the screening of whole family and the proper therapy of infected cases can be necessary for accurate eradication of *H. pylori* infection and probably, the chain of infection can be broken. However, it cannot be cost effective (Yucel, 2014).

Also, therapy in children is not recommended except limited indications. Nowadays the most suitable approaches in order to decrease the risk of gastric cancer in the future can be scanning the older people having index cases and the children having gastrointestinal symptoms, and treating infected cases in home. The prevalence of *H. pylori* infection in childhood is decreasing, especially in developed countries. Among the risk factors explored, low socio economic status, limited education, crowded homes and difficult access to sanitized water are the most significant factors affecting the prevalence of *H. pylori*. In future, vaccines may be a chance to prevent infection. According to the initial studies, the decrease gastric *H. pylori* colonization by vaccination with *H. pylori* antigen and adjuvant was possible (Yucel, 2014).

2.13 Treatment

Patients should be asked about any previous antibiotic exposure and this information should be taken into consideration when choosing an *H. pylori* treatment regimen (conditional recommendation, moderate quality of evidence)(Chey *et al.*, 2017).

Clarithromycin triple therapy consisting of a PPI, clarithromycin, and amoxicillin or metronidazole for 14 days remains a recommended treatment option in regions where *H. pylori* clarithromycin resistance is known to be <15% and in patients with no previous history of macrolide exposure for any reason conditional recommendation, low quality of evidence (for duration: moderate quality of evidence). Bismuth quadruple therapy consisting of a PPI, bismuth tetracycline, and a nitroimidazole for 10–14 days is a recommended first line treatment option. Bismuth quadruple therapy is particularly attractive in patients with any previous macrolide exposure or who are allergic to penicillin (strong recommendation, low quality of evidence). Concomitant therapy, consisting of a PPI, clarithromycin amoxicillin and a nitroimidazole for 10–14 days is a recommended first line treatment option (Strong recommendation, low quality of evidence (for duration: very low quality of evidence) (Chey *et al.*, 2017).

2.14 Previous study

In 2018 study was done in Thailand by Wongphutorn *et al* about prevalence and explored risk factor for *H. pylori* infection directly from paired saliva (n = 110) and stool (n = 110) samples from asymptomatic persons in Northeast Thailand. Samples were subjected to indirect immunofluorescence assay (IFA), 16srRNA-based real-time PCR and vacA-based semi-nested PCR. Partial vacA gene sequences of H. pylori were compared between saliva and stool samples.

The overall prevalence of H. pylori infection in this asymptomatic study population was 64%. Age, gender, occupation and frequency of brushing teeth were not found to be associated with H. pylori colonization. The vacA gene was successfully sequenced from both saliva and stool samples of 12 individual, from

110 saliva samples , 65(59.1%), 72(65.5%) and 57(51.8%)were positive according to semi-nested PCR, real time PCR and IFA, respectively.

Another study was conducted in 2017 by Aksit *et al.* in turkey about the investigation of Helicobacter pylori in the dental biofilm and saliva samples of children with dyspeptic complaints, they found *H. pylori* was histo pathologically detected amongst 83% of the children with the dyspeptic condition, and they used real time PCR for detection of 16srRNA and 23srRNA genes of *H. pylori* in saliva and they found (79.3%) from gastric positive patients, and (83%) from gastric negative patients.

Also in 2011Egyptian study done by Sayed *et al.* about Salivary PCR detection of *Helicobacter pylori* DNA in Egyptian patients with dyspepsia, they found that from the 40 gastric *H. pylori* positive patients, 34 patients (85%) had positive salivary PCR. While among the 20 gastric *H. pylori* negative ones, only 6 patients (30%) showed positive salivary PCR.

Another study was conducted in 2011 by Cellini *et al. in* Italy about detection of *Helicobacter pylori* in saliva and esophagus, gastric biopsies were used for *H. pylori* culture and antimicrobial susceptibility tests whereas saliva samples were collected to detect *H. pylori* with a Nested-PCR targeting 16srRNA gene as well as esophagus biopsies which were also investigated with immune histochemical staining. *Helicobacter pylori* was isolated in 18 patients both in gastric antrum and fundus. The molecular analysis, confirmed by comparative sequences evaluation, gave positive results in all saliva and esophageal samples (100%).

Chapter Three

Materials and Methods

Chapter Three

3.Materials and Methods

3.1 Methodology

3.1.1 Study design

Cross sectional (Hospital based) study.

3.1.2 Study area and duration

Study was carried out in Saad Rashwan medical center from March 2018 to March 2019**.**

3.1.3 Study population

H. pylori stool antigen positive patients in Saad Rashwan medical center.

3.1.4 Inclusion criteria

All *H. pylori* stool antigen positive patients.

3.1.5 Exclusion criteria

H. pylori stool antigen negative patients.

3.1.6 Sample size :

A total of 50 saliva samples were collected included in this study.

3.1.7 Sample technique:

Non probability convenience.

3.1.8 Data collection

Data were collected by direct interview (self-administration questionnaire).

3.1.9 Ethical consideration

Permission to carry out the study was obtained from Scientific ResearchCommitte, college of medical laboratory science, Sudan University of science and technology and verbal concent from patients to collect the samples after informed about the value of study.

3.1.10 Data analysis

Collected data was analyzed by using Statistical Package of Social Science (SPSS) version 16.

3.1.11 Selection of patients

From record of Saad Rashwaan laboratory (stool antigen test) **,** positive patients were selected for the research.

3.1.12 Collection of Specimens

Using sterile container gave to patients and asked for collection of the saliva.

3.1.13 Preservation of specimen

The samples were frozen under -20ºC until used.

3.1.14DNA extraction:

Guanidine chloride method for extraction was used to extract *H. pylori* DNA from saliva.

3.1.14.1Procedure

A total of 2 ml of lysis buffer, 10µl of proteinase K, 1 ml of guanidine chloride and 300 µl of ammonium (NH4) acetate were added to the saliva, vortexed, and The mixture was cooled to room زincubated at 37^{o}C overnight or at 65^{o}C for 2 hr temperature, and then 2 ml of pre chilled chloroform were added, vortexed, and centrifuged at 3000 rpm for 5 min. The upper layer of the mixture was transferred

to new tube and 10 ml of cold absolute ethanol were added, shaked, and kept at -20^{o}C for 2hr or overnight. Then the tube was centrifuged at 3000 rpm for 15-20 min., the supernatant was drained carefully, and the tube was inverted on a tissue paper for 5 min. The pellet was washed with 4 ml of 70% ethanol, centrifuged at 3000 rpm for 5 min The supernatant was poured off and the pellet was allowed to dry for 10 min. Then it was re-suspended in 50 µl of distilled water, briefly vortexed, and kept at -20^{o}C overnight (Tang *et al* ., 2006).

3.1.15 Polymerase Chain Reaction

3.1.15.1 Aim:

The polymerase chain reaction is a laboratory technique for DNA replication that allowed a target DNA sequence to be selectively amplified . PCR can use the smallest sample of the DNA to be cloned and amplify it to millions of copies in just few hours.

3.1.15.2Principle:

The PCR involves the primer mediated enzymatic amplification of DNA. PCR is based on using the ability of DNA polymerase to synthesize new strand of DNA complementary to the offered template strand. Primer is needed because DNA polymerase can add a nucleotide only onto a preexisting 3-OH group to add the first nucleotide. DNA polymerase then elongate its 3 end by adding more nucleotides to generate an extended region of double stranded DNA.

3.1.15.3 Procedure

This was performed using multiplex polymerase chain reaction and the following *Helicobacter pylori 16sRNA* and *UreC(glmM*) genes carried out by thermal thycler Heat force,960). Multiplex PCR was carried out in a 25µl volume of reaction mixture using the maxim PCR premix kit (iNtRON Biotechnology, Seongnam,

Korea), it is a premixed reagent ready to use, contain all the reagents required for PCR (except water, template and primers).

Amplification was performed for each gene in a final volume of 25 µl of PCR mixture containing 4µl of pre mixture, .5 µl of each primer (forward and reverse) (Table 3.1), 17 µl of double distill water, 2 µl of DNA .

DNA amplification was carried as follows: Initial denaturation 94 ^{o}C for 3 minutes in the first cycle, followed by 35 cycle of the second denaturation for 30 seconds at 94 ^{o}C. Annealing for 30 second at 53 ^{o}C. Following by the extension at 72 ^{o}C for 45 seconds, for the last cycle time was increased to 5 minutes to ensure complete extension to the amplified fragment (Essawei and Hammoudeh, 2013).

3.1.15.4 PCR product separation

A total of 4µL amount of each PCR product were separated in agarose gel. After preparation of agarose gel(appendix5), mixture allowed to be cooled, and stained with 2µl ethidium bromide. Mixed well and poured in a casting tray that had been taped up appropriately and equipped with spacer and suitable comb to form wells, any bubbles were removed and gel allowed to setting at room temperature, after solidification the comb and spacers were gently removed) and then viewed under UV Trans illuminators. A result was considered positive when a band of size 284 bp for *UreC* gene and 387bp for *16sRNA H. pylori* specific gene were visible in the gel. Standard procedures for reducing contamination were strictly followed.

Table 3.1: Primer sequences used for detection of *Helicobacter pylori* genes.

Target gene	Primer Name	Sequence (5-3)	Size (bp)
ureC (*glmM*)	Hp-F	GGATAAGCTTTAGGGGTGTTAGGGG	
	Hp-R	GCTTACTTTCTAACACTAACGCGC	284
16Srna	16sRNA-F	AGAGTTTGATCCTGGCTCAG	387
	16sRNA-R	TACGGTTACCGGTTACCGACTT	

(Essawei and Hammoudeh, 2013)

Chapter Four

Results

Chapter Four

4.Results

This is cross sectional study was done in Saad Rashwan medical center to detect *H. pylori* DNA in saliva of stool antigen positive patient using multiplex polymerase chain reaction.

Out of 50 saliva samples investigated (2%) was found positive for *H. pylori* DNA showing 284bp target band for *UreC* gene. This indicated that all saliva sample negative for *16SRNA* gene and only 1 sample showed positive for *UreC* gene.

Table.4.2 Frequency and percentage of study result

Variable	Freguency	Percentage
Male	25	50%
Female	25	50%
Positive	1	2%
Negative	49	89%

Table 4.3 Show the distribution of study result according to gender and age group

Age group	Male	%	Female	%	Positive	%	Negative	%
10-20	3	%6	2	%4	0	%0	5	%10
20-30	7	%14	10	%20	0	%0	17	%34
30-40	10	%20	9	%18	1	%2	18	%36
40-50	5	%10	4	%8	0	%0	9	%18
Total	25	%50	25	%50	1	%2	49	%98

Table 4.4 Detection of *H. pylori* genes according to gender

Gender	UreC (Positive)	UreC (Negative)	16s RNA (Positive)	16s RNA (Negative)	
Male	1	24	0	25	Frequency
%50	%2	%48	%0	%50	Percentage
Female	0	25	0	25	Frequency
%50	%0	%50	%0	%50	Percentage

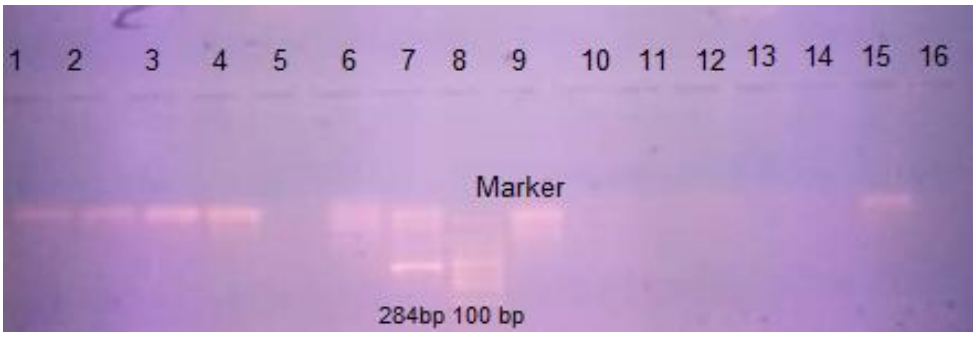

Figure 4.1 Multiplex PCR for *H. pylori* using species specific primers.

lane 7: positive control show 284 band for *UreC* gene ; lane 8: marker 100bp , the other lanes are negative samples.

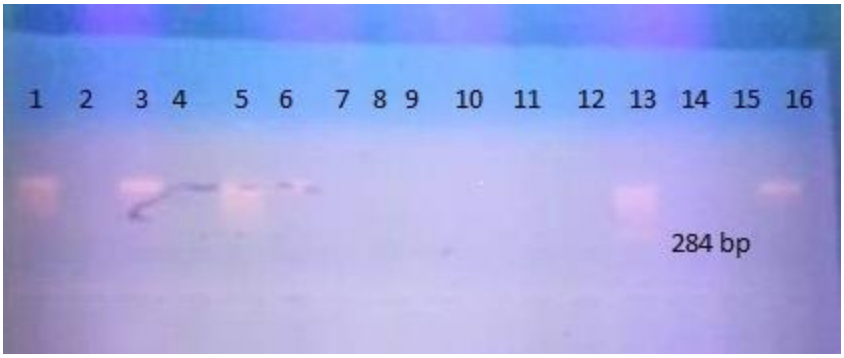

Figure 4.2 Multiplex PCR for *H. pylori* .

Lane 13 : positive sample show 284 band for *UreC* gene. The other lanes are negative samples.

Chapter Five

Discussion

Chapter Five

5.1Discussion

Some studies indicate the oral cavity as a transient or permanent location of *H. pylori* (Souto and Colombo, 2008). In this study multiplex polymerase chain reaction was used to detect presence of *H. pylori* DNA in fifty saliva specimens from patients that diagnosed as *H. pylori* positive by stool antigen test, and study on *16sRNA* and *UreC* genes showed that only (2%) was found positive only for *UreC* gene, and this finding is not with agreement with any studies.

This result is in disagreement with many study that show high percentage of *H. pylori* in saliva , Wongphutorn *et al*(2018) in Thailand whom used semi nested and real time PCR report that From 110 saliva samples, 65 (59.1%), 72 (65.5%) were positive according to semi nested PCR, real time PCR respectively.

Sayed *et al* (2011), In Egypt whom found that from 40 gastric *H. pylori* positive patients , 34 patients (85%) had positive salivary PCR .

Aksit *et al* (2017)in turkey whom study that used real time PCR whom found that the percentage of *H. pylori* from gastric positive patients was 79.3% and from gastric negative patients 83%.

In Cellini *et al* (2010), in Italy whom found that the percentage of *H. pylori* DNA was 100% from saliva and esophagus. All the previous studies had high result of *H. pylori* DNA in saliva in contrast to this study, the reason of the differences may be explained by the different type of PCR or the method of diagnosis that used, the differences in sample collection technique and the inclusion, exclusion criteria that used for select the patients. .

5.2. Conclusion

The study conclude that *H. pylori* DNA can be found in saliva sample of patient diagnosed with stool antigen test , and the oral oral route play important role in transmission of *H. pylori* from person to person.

5.3. Recommendations

1.Multiplex PCR technique can be used with different gene (*cagA*) of *H. pylori* to screen saliva

2.Increase sample size is critical for best result .

3**.**Other type of PCR (real time PCR) can be used to confirm presence of *H. pylori* DNA in saliva .

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Appendix 1

Sudan University of Science and Technology

College of Graduates Studies

Questionnaire

Molecular Detection of Helicobacter pylori 16SRNA, UreC genes from stool antigen positive patients of Saad Rashwan medical center

Patient number:…… date :……

Age:…… sex: ……

Previous infection by *H. pylori*……yes……no……..……

Result of stool antigen test ……positive …….negative……

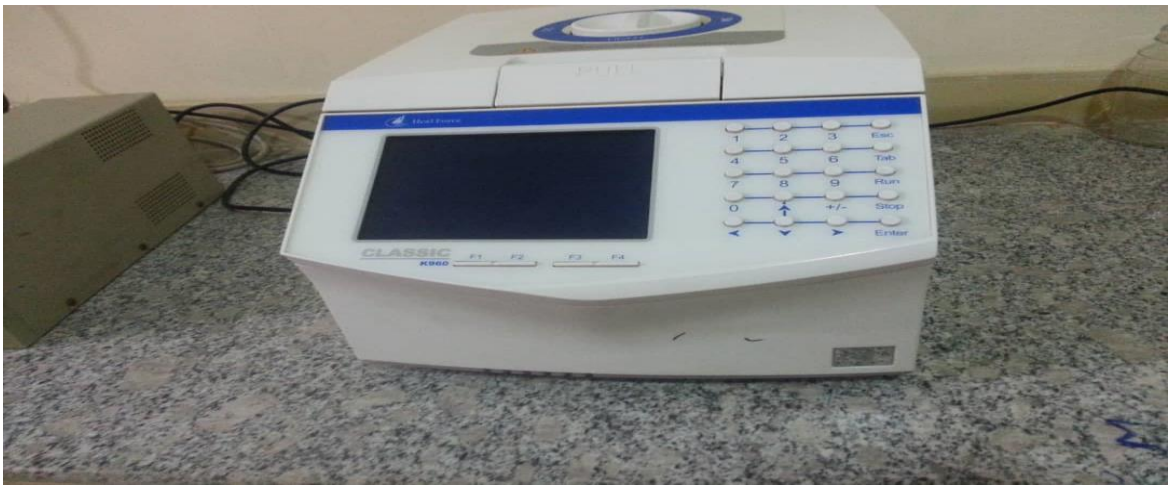
Education…….. Nationality……

Treatment……. Smoking…….

Gastritis……..Symptoms…….

Appendics2

Devices



1.Thermocycler(Heat force 960)

3.Transilluminater

3.PCR product

4.Agarose gel electrophoresis

Appendix 3

Chemicals and reagent:

1/ Lysis buffer: cell lysis.

2/ Proteinase K: breakdown of protein.

3/ Chloroform: phase separation in DNA extraction.

4/Absolute ethanol: DNA precipitation

5/70% ethanol: washing the precipitated DNA.

6/ Distilled water: reagent preparation and re-suspension of DNA.

7/ Agarose gel: gel electrophoresis.

8/ 1X TBE buffer: gel preparation and running.

9/ Loading dye: Tracking DNA in gel.

10/ Ethidium Bromide: Visualizing DNA in gel.

11/ Master Mix: to perform PCR process.

Appendics4

Instrument

1/Disteller : for preparation of distill water for reagent preparation.

2/ Centrifuge : for separation of sample in DNA extraction.

3/ Refrigerator : for preservation of samples, DNA extract and PCR product.

4/ Microwave: for agarose gel preparation.

5/ Gel electrophoresis system : for separation of PCR product.

6/ vortex mixer: for mixing the samples.

7/ Thermal cycler : to amplify the target sequence.

Appendix5

Preparation of agarose gel:

0.75g of agarose powder were dissolved into 100ml of 10X TBE [TBE was prepared by taken amount of 108g of Tris base and added to 55g of boric acid and 40ml of 0.5% EDTA and then dissolved into 1L of D.W].

Preparation of ethidium bromide :

Ethedium bromide was prepared by taken 10mg of ethedium bromide and dissolved into 0.5ml D.W then kept into brown bottle.