Sudan University of Science and Technology

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Molecular Detection of *Helicobacter pylori* in Drinking Water in Khartoum State, 2019.

الكشف الجزيئي عن البكتريا الحلزونية البوابية في مياه الشرب في ولاية الخرطوم, 2019

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بسم الله الرحمن الرحيم

قال تعالى " وَمَا أُوتِيتُمْ مِنَ الْعِلْمِ إِلَّا قَلِيلًا "

صدق الله العظيم

سورة الإسراء الآية(85)

Dedication

I dedicate this work to:

My dear brother, My beloved mother

My sisters and teachers who prayed for my prosperityin education, and encouraging me in my life …

My friends and love …. With them I enjoyed my

Life and I am nothing without them.

Acknowledgement

First and foremost, praise to ALLAH, who give me the strength to do this work.

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Abstract

Helicobacter pylori (*H. pylori*) is one of most the causative agent of chronic bacterial infection in humans, and act as predisposing factor for peptic ulcer and gastric cancer. The infection has strongly association with lack of access to clean water and proper sanitation, However *H. pylori* loses its ability to survive in an infectious state in the environment because it rapidly loses its cultivability. The aim of this study was to detect *H. pylori* in water using culture and molecular methods.

One hundred water samples were collected from tap water with and without filters, cooler and Zeer from different area in Khartoum state. Samples were filtered through 0.45µm filter membrane (cellulose membrane filter). Each membrane was takensliced and immersed in 2 ml of Brain Heart infusion broth (BHI) media (Himedia, India) for overnight. After that each 2 ml of BHI was taken and cultured for *H. pylori* on special Columbia media (Himedia, India) contain special selective supplement and incubated in closed jars with special kits to provide environment with the oxygen tension lowered to 5-15% and carbon dioxide raised to 1-10% at temperature 37ºC for three days and incubated for a week before being discarded as negative, the identification was depending on their colonial morphology.

DNA was extracted by quinidine chloride method from 2 ml Brain Heart Infusion (BHI) broth media and PCR technique were applied to these samples to detect *H.pylori* genes (*16sRNA* specific for *Helicobacter pylori* and *Urease C*).

Out of 100 samples in cultural method there was no growth, and in molecular methodthere was no positive sample for both *urease C* and *16sRNA* genes (0%),

This finding indicates that water may not act as source of transmission for *H. pylori* infection, or may be due to the addition chlorine to water system in Khartoum.

ملخص الأطروحة

البكتريا الحلزونية البوابية هي واحدة من أكثر العوامل المسببة للعدوى البكتيرية المزمنة في البشر، وتعمل كعامل مسبب للقرحة المعوية وسرطان المعدة. وقد ارتبطت العدوى ارتباطًا وثيقًا بعدم الوصول إلى المياه النظيفة والصرف الصحي المناسب ، إلا أنها تفقد لقدرتها على البقاء في حالة معدية في البيئة الخارجية لأنها تفقد قدرتها على النمؤ بسرعة. كان الهدف من هذه الدراسة هو الكشف عن البكتريا الحلزونية البوابية في الماء باستخدام طرق التزريع وعن طريق تفاعل البلمرة المتعدد.

تم جمع مائة عينة ماء من الصنبور مع مرشحات وبدون مرشحات والمبردات والازيار من مناطق مختلفة بولاية الخرطوم. تم ترشيح العينات من خلال غشاء المرشح 0.45µm (مرشح غشاء السليلوز) . تم أخذ كل غشاء من شرائح مغموسة في 2 مل من مرق حساء القلب (BHI) ، وتم تحضينها لليوم التالي. بعد ذلك تم أخذ 2 مل من BHI وزرعها على وسائط كولومبيا الخاصة التي تحتوي على ملحق انتقائي خاص وحضنت في الجرار المغلقة مع مجموعات خاصة لتوفير بيئة منخفضة التوتر و الأوكسجين إلى 5–15 ٪ و ثاني اكسيد الكربون الذي تم رفعه إلى 1–10٪ عند درجة الحرارة 37 درجة مئوية لمدة ثلاثة أيام وحضنت لمدة أسبوع قبل أن يتم التخلص منه بشكل سلبي.

تم استخلاص الحمض النووي بواسطة طريقة كلوريد القوانيدين من 2 مل من تزريع مرق حقن القلب (BHI) ثم تم استخدام تقنية تفاعل البلمرة المتعدد على هذه العينات للكشف عن جينات 16s RNA و Urease C الخاصة بالبكتريا .

من بين 100 عينة لم يكن هنالك نمو باستخدام التزريع ، وفي الطريقة الجزيئية لم تكن هناك عينة إيجابية لكل من جيناتurease C 16 sRNA . هذا النتائج تشير إلى أن المياه قد لا تكون كمصدر للإصابة بالعدوى بالبكتيريا وقد يرجع ذلك إلى إضافة الكلور إلى نظام المياه في الخرطوم

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ABBREVIATIONS

Ure C	Urease C
C.pylori	*Campylobacter pylori*
H.pylori	*Helicobacter pylori*
VBnC	Viable bot non-culturablecoccus
LPS	Lipopolysaccharide
TLR	Toll like receptor
VacA	Vacuolatingcytotoxin gene
GC	Gastric carcinoma
Dup A	Duodenal Ulcer (DU) promoting gene
IceA	Induced by contact with Epithelium gene
IL	Inter leukin
hor	Hop-related group
le^{b}	Lewis b
Bab A	Blood group antigen-binding adhesion
Sab A	Sialic acid-binding adhesion
Oip A	Outer inflammatory protien
SOD	Super oxide dismutase
CLO	Campylobacter-like organism
HPSA	*H.pylori* stool antigen
BHI	Brain heart infusion broth
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
CDC	Center for Disease Control and Prevention
TBE	Tris Borate EDTA

CHAPTER ONE

INTRODUCTION

Chapter one

1.Introduction

1.1. Background

H.pylori is a type of bacteria. These germs can enter your body and live in digestive tract. After many years, it can cause sores, called ulcer in the lining of stomach. As more of the world gets access to clean water and sanitation, fewer people than before are getting the bacteria. With good health habits, you can protect yourself and children from *H.pylori*(Blahd.,2016) .

*H. pylori*primary habitat is human gastric mucosa, the organism is found worldwide. Although acquired early in life in underdeveloped countries, it is exact mode of transmission is unknown (Forbes,*et al*., 2007).

An oral-oral, fecal- oral or common environmental source have been proposed routes of transmission, with transmission of *H.pylori* primarily occurring in families. Recent finding suggest that mother-to-child transmission is most probable cause of intra-familial spread. *H.pylori* colonizes the mucous layer of the antrum and fundus of the stomach but does not invade the epithelium(Forbes,*et al*., 2007).

H.pylori grow optimally at pH of 6.0-7.0 and would be killed or not grow at pH within the gastric lumen. Gastric mucus is relatively impermeable to acid and has a strong buffering capacity. The pH is about 7.4 in epithelial side and *H.pylori* is found deep in the mucous layer near the epithelial surface where physiologic pH is present (Brooks,*et al*., 2013).

H.pyori was detected using molecular methods in untreated water. The presence of *H.pylori* in wells correlated with infection in consumers, and consumption of untreated well water should be considered a risk factor for *H.pylori* infection (Baker KH, *et al*., 2001). The infection with *H.pylori*is seemed to be different in developed and developing countries, as it shows to be higher in developing countries than the developed countries (Grahm, *et al*.,1991 ; Cullen,*et al*., 1993).

1.2. Rationale:

The evidence relating to survival of *Helicobacter pylori* outside of the gastric environment is extremely limited. To date the primary transmission routes of *H.pylori* have yet to be confirmed and when this is achieved preventive mechanism can be implemented to reduce and prevent human from this pathogen (Forbes ,*et al* .,2007; Webb , *et al*., 1994).

Because infection is acquired orally from contaminated foods or water especially in developing countries where contaminated water is untreated, crowded condition and poor hygiene contributes to higher *H.pylori* prevalence.

To provide an improved means to investigate the possible role of water as a disease vector, Due to increase frequency of *H.pylori*infections in hospitals, the research was done to detect the role of water in the transmission of disease and to detect the gene that is present in this bacteria.

The seriousness of *H.pylori* infection with high prevalence rate and suggestion of many studies that *H.pylori* can transmitted by contamination with mentioned that there isa high rate of contamination in Khartoum state, this study aimed to investigate the presence of*H.pylori*in drinking water in Khartoum state.

1.3. Objectives:

1.3.1 General Objective

To detect the present of *Helicobacter pylori* in water in Khartoum state by culture and PCR technique.

1.3.2 Specific Objectives

1.3.2.1-To isolate *H. Pylori* in water samples using cultural technique

1.3.2.2-To detect *H. Pylori genes(Urease C & 16sRNA)* in water samples by using *PCR*.

1.3.2.3- To compare the results of drinking water from different sources (Cooler, Zeer and tap water with and without filters).

Chapter two

Literature review

Chapter two

2. Literature review

2.1 Helicobacter pylori:

Helicobacter pylori is Gram-negative spirally-shaped bacterium, it is about 0.5-0.9µm in wide and 2-4µm long. It is strictly micro-aerophilic and required carbon dioxide for growth, but has a tuft of sheathed unipolar flagella unlike unsheathed flagella of *Campylobacter*, it is biochemically inactive but it produces powerful urease (Greenwood,*et al*., 2003).

2.2 Classification:

Helicobacter pylori have been classified as most important stage in development of taxonomy of gastric microorganisms. It was proposed 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*(Forbes,*et al*., 1998).

Table 2.1 Taxonomy of *Helicobacter pylori*.

kingdom	bacteria
Phylum	proteobacteria
Class	Epsilon Proteobacteria
Order	campylobacterales
Family	Helicobacteraceae
Genus	*Helicobacter*
Species	*H. pylori*
Binomial name	*Helicobacter pylori*

2.3 Cellular morphology:

H.pylori has S-shaped morphology with 1 to 3 turns, 0.5 µm in length, with a tuft of 5 to 7 polar sheathed flagella but it lacks fimbrialadhesins. This morphology has correlated with maximum in vitro motility. Some *H.pylori* has granules located in the cytoplasm were the largest, 0.05-0.2 µm in size, amorphous and vacuole-like, and generally regarded as source of energy and phosphorus reservoir (Rourke.,2001) .

H.pylori is gram-negative bacterium, although usually spiral-shaped, the bacterium can appear as a rod, while coccoid shapes appear after prolonged in vitro culture or antibiotic treatment. These coccoids cannot be cultured invitro and are thought to represent dead cells(Kusters,*et al*., 2006). However results obtained from electron microscopy revealed that *H.pylori* long-term cultures not only consist of classic, spiral shaped bacteria, but also contain alternative forms of coccoid cells. Coccoid forms can be divided into two types: viable but non-culturablecoccus(VBnC) and a degenerative form, coccoid stage which is probably the effect of bacterial cell death. There is no evidence for reversion from coccoidstate to the viable and infectious spiral forms (Rudnika*et al*., 2014).

*H.pylori*motileusingits flagella which is driven by proton motive force. The motility and the shape of the bacteria is specifically adapted to the gastric mucus. Flagella have a molecular weight of 50000-62000. The shape helps bacteria to move easily in viscous environments. They have blubs on the ends of flagella which favors the adhesion. Flagella are 4µm in length, and the diameter of each flagella is 30nm (Mobley *et al*., 2001).*H.pylori* has 6-8 flagella at one end and is found in very acidic environments, at a pH of 2.0 or less. The bacterium has been cultured in micro aerobic (low oxygen conditions) but it adapts to high oxygen at high culture densities. It is commonly found inside the lining of stomach and the duodenum. *H. pylori* are aslowgrowing organisms that can cause peptic ulcers and gastritis that can lead to gastric cancer and gastric MALT(mucosa-associated lymphoid tissue) lymphoma(Marshall *et al.,* 1991).

2.4 Virulence factors:

*H.pylori*is not like other bacterial pathogen, it is typically colonizes the host for life unless specific treatment is given. There are several properties contribute to *H.pylori* persistence. All *H.pylori* clinical isolates express urease. Urease converts urea to ammonia plus carbon dioxide changing the pH of surrounding area.*H.pylori* has non-toxic lipopolysaccharide (LPS) which contribute to persistence unlike LPS from other species. *H.pylori* LPS is recognized by TLR-2 rather than TLR-4 (Roesler*et al*., 2014).

a. CagPAI

CagPAI is 40 Kb regions of chromosomal DNA encoding about 31 genes that forms a type IV secretion system and can be divided into two regions, *cag 1* and *cag 2* according to a novel insertion sequence. This secretion system forms a pilus that delivers *Cag A*, an oncoprotein, into the cytosol of gastric epithelial cells through a rigid needle structure covered by *CagY*, a *VirB10*-homologous protein, and *CagT*, a *VirB7*-homologus protein, at the base. The present of *cagA*gene has been associated with higher grades of inflammation which may lead to the development of the most sever gastrointestinal disease, such as peptic ulcer disease and GC. Furthermore, *cagA* is a polymorphic gene that presents different numbers of repeated sequences located in its 3' region. Each repeated region of*CagA* protein contains Glu-Pro-I1e-Tyr-A1a (EPIYA) motifs, including a tyrosine phosphorylation site. According to the sequences flanking the *EPIYA* motifs, four distinct *EPIYA* segments, *EPIYA-A, EPIYA-B, EPIYA-C, and EPIYA-D.* As regard to function of the repeated regions, initial demonstrations suggest that *H.pylori* strains that have a large number of EPIYA segments in their regions are less resistant to gastric acid. There is relation between strains*vacAs1m1* and *CagA* positive. It has association with most sever gastric diseases.Also *CagA* gene is associated with extra-gastric disease, such as relationship of *CagA*-positive strains with fatal cardiovascular events. And it is also has a relation with autoimmune thyroid diseases (Roesler*et al.*, 2014).

B. *VacuolatingCytotoxin* Gene (*vacA*)

VacA is a cytotoxin secreted from bacteria as a large 140-kDa poly peptide and latter trimmed at both ends to finally deliver it in active form to host cells, where it exerts its activity. *VacA* gene is present in all strains of *H.pylori* and it displays allelic diversity in three main regions,the *s* (signal), the *i* (intermediate), and the *m* (middle) regions, and the cytotoxic activity varies between strains. Different combinations of two major alleles of each region (*s1, s2, i1, i2, m1, m2*) may exist, which result in*VacA* toxins with distinct capability of inducing vacuolation in epithelial cells. Generally *VacA* has ability to activate a proinflammatory response. Strains with *s1* allele are highly associated with ulcers and GC; however *s1/s2* combination or *s2* genotypes are found in patients with GC. *M1*associated with an increased risk of developing gastric epithelial injury and GC, lastly *i1* is associated with gastric adenocarcinoma (Roesler,*et al*., 2014).

VacA toxin is composed of p33 and p55 domains that form an oligomeric structure. This complex can embed into host cell membrane, and also has the characteristic of an anion-selection channel. This channel can release bicarbonate and organic anions in the host cytoplasm. In this way, the channel might help *H.pylori* in growth by efflux of metabolic substrates. This complex can get into the endosome via endocytosis. The endocytosed*VacA* channel will allow anions to permeate into late endosome, which leads to accumulation of weak bases and thence to large vacuole formation by water influx. In addition to that *VacA* disrupts the balance of cell proliferation and death by affecting genes that regulate the cell cycle (Kao,*et al*., 2016).

c. *Duodenal Ulcer (DU) promoting* gene (*dupA)*

H.pylori DU promoting gene (*dupA*), located in the plasticity region of *H.pylori* genome, and has been initially described as a risk marker for DU development and a protective factor against GC. The function of *dupA* gene is not fully understood, but absent of *dupA* gene was associated with increased susceptibility to low pH (Roesler,*et al*., 2014).

d. *Induced by Contact with Epithelium* Gene (*iceA*)

iceA has two main allelic variants, *iceA1* and *iceA2* . The*iceA1* is regulated by the contact of *H.pylori* with gastric epithelial cells and exhibits sequence homology with a gene from *Neisseria lactamica*, *nlaIIIR*, which encodes a CTAG-specific restriction endonuclease. However, *iceA2* has no homology with known genes and its function remains unclear, although some researchers have related this allele to asymptomatic gastritis and non-ulcer dyspepsia. Some study show that *iceA1* genotype was linked with enhanced mucosal interleukin (IL-8) expression and acute antral inflammation (Roesler,*et al*., 2014).

e. Urease

H.pylori produce urease enzyme which hydrolyses urea into NH_3 and CO_2. This enzyme play an important role in colonization, thus bacteria which has mutants that lead to urease-defective are not able to colonize the gastric environment. Urease produce ammonia which causes damage to epithelium and in conjunction with neutrophil metabolism,forms carcinogenic agents that might participate in the development of gastric malignances. Ammonia is capable of causing different cells alteration; urease might also help to recruit neutrophils and monocytes in the mucosa and to produce proinflammatory cytokines (Roesler,*et al*., 2014).

f. OMPs

*H.pylori*has a large repertoire of *OMPs* encoded by a family of paralogous genes. This group is probably of remarkable importance for optimal adaptation of *H.pylori* to its host. *H.pylori* genome contains more than 30 *omp* genes, which have been divided in to *hop* (*Helicobacter OMPs*) and *hor* (hop-related groups) which are joined together in OMP family 1. The Hop subgroup is encoded by 21 genes and included the two best studied *H.pylori*adhesins: Lewis b (Le^b) blood group antigen-binding adhesion (*BabA*).Andsialyl Lewis X antigen-binding adhesion *(SabA)* (Roesler,*et al*., 2014).

g. *Blood group antigen-binding adhesion* (*BabA*)

BabA is the best-characterized adhesin and binds to ABO histo-blood group antigens and corresponding Le^b antigens, which are expressed on gastric human epithelial cells. There are three different alleles of *bab*have been discovered *(babB,*

babA1,babA2), only *babA2* gene product is needed for Le^b binding activity. A recent study with Iranian patients reported that *babA2* prevalence was significantly higher in GC patients (18.1%) and non-ulcer dyspepsia subjects (26.1%) (Roesler,*et al*.,2014).

h. *Sialic acid-binding adhesion* (*SabA*)

H.pylori infection induces expression of inflammation-associated ‘’sialylated’’ carbohydrate structures that are up regulated as part of complex gangliosides in inflamed gastric tissue. Therefore, adherence of bacteria to mucosa is dependent on *SabA* and cognate sialylated/fucosylatedglycan’s on the host cell surface.*H.pylori* also binds to red blood cells in gastric mucosal blood vessels in both infected human and rhesus monkeys. It was verified that *SabA* is the bacterial surface protein that mediates *H.pylori* binding to red blood cells. Another study has assessed the contribution of each *BabA, SabA* and the neutrophil-activating protein (HP-NAP) in the inflammation, using mutant strain of *H.pylori* (Roesler,*et al*., 2014).

i. *Outer inflammatory protein* (*OipA*)

OipA, a proinflammatory OMP, is called *HopH or OMP13*. It was correlated with mucosal IL-8 levels and that protein was present in 97.5% of patients with gastric or DU when compared with 70% of those with chronic gastritis. *OipA*isogenic mutants reduced the induction of IL-8 from gastric epithelial cell lines. Another study showed that *OipA* status was strongly correlated with *cagA, vacA, and iceA*genotyoes(Roesler,*et al*., 2014).

J. *HopZ*

Immunofluorescence studies have shown the presence of *HopZ* (74kDa) on *H.pylori*cells. *HopZ* appear to mediate adherence to gastric epithelial cell lines as bacterial binding is significantly reduced in *HopZ*knock-out strains. The exact function of HopZ is however still unclear. Also the host receptor for *HopZ* is yet unknown (Kalali ,*et al*., 2014)

K. Motility

Helicobacter pylori's motility is also involved in the infection's persistence colonization is possible even in the case of *Helicobacter pylori* variants that have mutations of the flagellins, but the preservation of the bacterial reserve in the mucus layer (chronic infection) necessitates that normal expression of both A and B flagellins. Helicobacter 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).

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 *bab A* gene, the *bab P* gene, the *alp A* and *Alp B* and later by the gene *sab A.* the genome's analysis has revealed that all these genes belong to a family of 32 genes that codify the external membrane's proteins(Zhong,*et al*.,2008).

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

Superoxide dismutase (SOD) – catalysis the transformation of superoxide and peroxide into hydrogen and oxygen ions; these are enzymes that allow *H.pylori* to resist the oxidative stress generated by phagocytes (Zhong ,*et al*.,2008).

2.5 Transmission

An oral-oral, fecal- oral or common environmental source have been proposed routes of transmission, with transmission of *H.pylori* primarily occurring in families. Resent finding suggest that mother-to-child transmission is most probable cause of intra-familial spread. Infection tends to cluster in families and people living in crowded or closed environments suggesting that person to person contact plays an-important parts in transmission, infection is associated with poor hygiene and it is commonly acquired during childhood. *H.pylori* colonizes the mucous layer of the antrum and fundus of the stomach but does not invade the epithelium(Forbes ,*et al* .,2007; Webb , *et al*., 1994).*H.pylori* has been detected in saliva, vomitus, gastric refluxate, and feces. But there is no conclusive evidence for predominant transmission via any of these products(Kusters,*et al*., 2006).

The majority recent studies have not found tobacco use or alcohol consumption to be risk factors for *H.pylori*infection. Adequate nutritional status, especially frequent consumption of fruits and vegetables and of vitamin C, appears to protect against infection with *H.pylori*. In addition to that food prepared under less than ideal condition or exposed to contaminated water or soil may increase the risk to get infection. Over all inadequate sanitation practices, low social class, crowded or high density living condition has relation with high prevalence of *H.pylori* infection. This finding suggests that poor hygiene and crowded condition facilitate transmission of infection among family members and has relation with intrafamilial and institutional clustering of *H.pylori* infection. Understanding the rout of *H.pylori* transmission is important if public health measures to prevent its spread are to be implemented. Iatrogenic transmission of*H.pylori* following endoscopy is the only proven mode (Brown .,2000). In case of feco-oral transmission and oral-oral transmission of bacteria is also possible. Contaminated water supplies in developing countries may serve as an environmental source of bacteria. Children who regularly swim in rivers, streams, pools, drinking stream water, or eat uncooked vegetables are more likely to be infected. Organisms have been identified in dental plaque, and the habit of feeding children with pre-masticated food commonly transmits the bacteria (Muhammed,*et al*., 2012).

2.6 Pathophysiology

H.pylori colonized and cause chronic active gastritis. This condition can be observed in all *H.pylori*-positive subjects. Many factors are needed for intragastricdistribution and severity of this chronic inflammatory process, such as characteristics of the colonization strain, host genetics and immune response, diet, and the level of acid production. As complication of chronic inflammation *H.pylori*-induced ulcer disease, gastric cancer, and lymphoma; ulcer disease and gastric cancer in particular occur in those individuals and at those site with the most sever inflammation (Kusters,*etal*., 2006).*Helicobacter pylori* colonize the mucus layer of the antrum and fundus of the stomach but do not invade the epithelium, it has ability to colonize the gastric mucosa, persist despite the host immune defense, and cause host tissue damage, and thus it is an effective and significant bacterial pathogen.*H.pylori* use motility to escape the acidity of

stomach and has urease enzyme which play a significant role in the survival and growth of *H.pylori* in the stomach by creating an alkaline microenvironment (Forbes,*et al*.,1998).

2.7 Pathology

H.pylori is curved, spirochete-like, in superficial mucus layer and along microvilli of epithelial cells, its invasion is unusual, and proton pump inhibitor use may increase risk of invasion. *H.pylori* usually not seen in areas of intestinal metaplasia, but it associated with chronic inflammatory infiltrate with germinal centers and plasma cells in lamina propria. Antibiotics may cause *H.pylori* to assume coccoid appearance and the present of follicles is strongly associated with *H.pylori*, the density of follicles is highest in angulus, the most common site of gastric lymphoma and lowest in the proximal greater curvature, where incidence of *H.pylori* induced gastric lymphoma is lowest. Chronic proton inhibitor use without antibiotics leads to relatively decreased inflammation in the antrum and increased numbers of micoorganisms. Acute infection associated with erosions, ulcers and hemorrhage (Weisenberg,*et al*.,2018).

2.8 Antigens and strain typing

Although various antigens are expressed by *H.pylori*, serotyping is of limited practical value. And it is considerable genetic diversity can be molecular typing based on DNA analysis(Greenwood,*et al*., 2003).

2.9 Associated disease

Despite the presence of chronic gastritis, most infections are symptomless, and endoscopic appearance of stomach is normal. Trace amount of persons develop peptic ulceration. However long standing infection is associated with an increased risk of developing gastric cancer,Moreover, gastric lymphoma although rare, is strongly associated with *H.pylori*infection(Greenwood,*et al*., 2000).

H.pylori is actively involved in the pathogenesis of what was once regarded as idiopathic peptic ulceration. Pathogenesis mechanism is unknown but several factors may operate e.g. the production of ammonia by urease which cause ionic changes in the mucus layer which lead to back-diffusion of hydrogen ions in the mucosa, production of toxin which active inflammatory cells, stimulation of auto-

immune response by production of antigens that cross-react with antral gastric antigens, and degradation of mucus by protease(Greenwood,*et al*., 2000).

2.10 laboratory diagnosis of *H.pylori* infection

Helicobacter pylori infection can be diagnosed by invasive techniques requiring endoscopy and biopsy(e.g. histological examination, culture and rapid urease test) and by non-invasive techniques, such as serology, the urea breath test, urinelblood or detection of *H.pylori* antigen in stool specimen,some non-invasive techniques can detect active infection as urea breath test and stool antigen test do, this is called 'active tests'. Also some non-invasive techniques can indicate exposure to *H.pylori*but do not indicate active infection is ongoing ''passive tests'' (e.g. serology, urine, near-patient tests) .the choice of appropriate test depending on pre-test probability of infection, the characteristics of the test being used and it's cost-effectiveness (Ricci , *et al* ., 2007).

2.10.1 Invasive tests:

H.pyolri can be detected in samples of gastric mucosa obtained by gastroscopy. For microscopy *H.pylori* was seen in histological section of gastric mucosa long before it was cultured(Greenwood,*et al* ., 2000).

2.10.1.1Culture

Culture is more sensitive than histology, it is only required for sensitivity tests or if the strain typing is needed for epidemiological studies, specimen must be kept moist and ideally should not be more than 2h old. Selective media should be used for isolation. Cultured media should be incubated at 37ºC for a week before being discarded as negative. *H.pylori* forms discrete domed colonies(Greenwood,*et al*., 2000).

2.10.1.2Biopsy urease test

H.pylori produces such abundant urease that it is detectable in biopsy tissue. A portion of tissue is put into a small quantity of urea solution with an indicator that detects pH change resulting from the formation of ammonia by the action of urease. Result will appear within minute up to 2h (Greenwood,*et al*., 2000).

2.10.1.3Molecular tests

DNAhybridization tests with DNA probes, usually with polymerase chain reaction (PCR) amplification (Greenwood,*et al* .,2000).

2.10.1.4Campylobacter-like organism (CLO) test

This testis use for detection of *H.pylori*(Campylobacter-like organism) in biopsy specimens by biopsy urease test, microscopy (Gram stained smears and histology) and culture. Media which used here is christensen's urea broth and christensen's urea broth modified by increasing the concentration of phenol red and omitting the nutrients, glucose and peptone. The specificity of this test is 100% unlike culture and histopathology. The modified broth was more sensitive (96% sensitivity compared with culture) than christensen's broth (92% sensitivity) but this difference was not statistically significant. This test is cheap and rapid alternative to diagnosis using gram stain or culture (Mcnulty ,*et al.*, 1989).

2.10.2Non-invasive tests:

2.10.2.1Serology

Most serological tests based on ELISA principles, can be used to detect antibodies to *H.pylori* or its products. Sensitivity is high but false-positive results may occur(Greenwood,*et al*., 2000). Non-invasive diagnostic tests include the urea breath test, serology, and stool antigen testing (HpSA) can be used. The clinical utility of serologic testing in both children and adults has been debated; IgG and IgA serologic tests are possibly less reliable in children than adults, but this is not established yet. Some investigators have supported that the use of IgM as an indicator of active disease, while others have found IgM to have little diagnostic value. IgG is more specific in children than in adult, while some investigator observed IgA to be equal to IgG in performance, others have found it to have no additional benefit. IgA yielded poor overall sensitivity and specificity, although it perform better for samples from children than adults. IgA and IgG has less specificity than HpSA. IgM has been found to have little diagnostic utility for *H.pylori* infections and is elevated only acutely after infection whereas *H.pylori* infections are generally chronic. IgM is lack of utility in either children or adults (She ,*et al*., 2009).

2.10.2.2Urea breath test

Urea containing an isotope of carbon (carbon-14 or -13) is fed to patient, and the emission of the isotope as carbon dioxide is measured in the breath. The test has excellent sensitivity and specificity (Greenwood,*et al*.,2000).

2.11 Epidemiology

The rout of transmission is unknown, but it is presumably by the oral-oral rout. *H.pyori* has been detected, with difficulty, in dental plaque, saliva and feces. infection is associated with poor living condition and overcrowding. *H.pyori* can be detected as nosocomial infection from inadequately disinfected endoscopes.Infection must be controlled by giving a cheap and effective vaccine to people in developing countries particularly for the prevention of gastric cancer (Greenwood,*et al*.,2000).

Generally *H.pylori* is sensitive to most Beta-lactam antibiotics, macrolides, tetracycline's and nitroimidazoles, but resist to trimethoprim. It is also sensitive to bismuth compounds (Greenwood,*et al*.,2000).

2.12 Treatment

Infection with *H.pylori* is elevated specially in developing countries it is more than in developed countries, *H.pylori* is associated with the development of gastrointestinal disorders as chronic gastritis, peptic ulcer, and gastric adenocarcinoma. Recommended treatment for eradication of *H.pylori* is the standard triple therapy, using a proton pump inhibitor or ranitidine bismuth citrate, combined with clarithromycin and amoxicillin or metronidazole (Brosek.,2018).

These triple therapies had a very high acceptance among clinicians but the efficacy of these triple regimens has decreased lately due to *H.pylori* resistance to key antibiotics, mainly clarithromycin, but also metronidazole and levofloxacin. Information about resistance for antibiotics should be taken for patient before starting the treatment to avoid repeated treatments(Goderska,*et al.*,2018).

Antibiotics can cause side effects, thus using natural treatment may be helpful in preventing these side effects, protecting the stomach, helping the body to better

fight infection, and promoting over all good health. There are eight natural treatments include (Brosek.,2018)

1\ Honey

Generally Honey has anti-bacterial properties, one study showed that Manuka honey suppressed the growth of *H.pylori* in gastric epithelial cells (Brosek.,2018). Other studies showed that honey has anti-*H.pylori* properties but more clinical trials are needed to assess honey's efficiency as complementary or alternative treatment (Brosek.,2018).

2\ Aloe vera

The gel from inside the leaves of aloe vera plant was effective in both inhibiting growth of and killing *H.pylori* strains, even those that were drug-resistant in the laboratory environment. This suggests that aloe vera could be effective against *H.pylori* infection when used with antibiotics in combination (Brosek .,2018).

3\ Broccoli sprout

Broccoli sprout contain sulforaphane which kill *H.pylori*(Brosek.,2018).

4\ Milk

Both human and cow's milk contain lactoferrin and a glycoprotein, those which inhibit the activity of *H .pylori,* one study used combination of antibiotics and lactoferrin from cow's milk, which result in a 100% eradication rate of *H.pylori*.There is compound called melanoidin formed by chemical reaction between the sugar lactose and a protein called casein in milk and dairy products which shows to inhibit the growth of *H.pylori* by suppressing it's colonization (Brosek.,2018).

5\ Lemongrass oil

This is used as part of an aromatherapy approach or as inhalation. Lemongrass essential oil inhibits the growth of *H.pylori* by reducing the colonization of bacteria in the stomach (Brosek.,2018).

6\ Green tea

It is contains many antioxidants and nutrients. Green tea decrease the number of bacteria as well as inflammation score of *H.pylori* infected mice. When mice received green tea before infection it can achieved better results(Brosek.,2018).

7\ Probiotics

These are live microorganisms that offer health benefits to people. Interest in probiotics as treatment for *H.pylori* is increasing. There are numerous types of probiotics. Many people use bifidobacterium, which is found in dairy and fermented products, to prevent gastrointestinal infection. Bifidobacterium exerts its effect against *H.pylori* by competing with the bacteria to stick to the mucous lining of the stomach (Brosek.,2018).

8\ Phototherapy

H.pylori is sensitive to ultraviolet light; during phototherapy an ultraviolet light source illuminates the whole stomach. Phototherapy has shown to reduce the number of bacteria in the stomach significantly. Phototherapy can become effective therapy for people who cannot take antibiotics(Brosek.,2018).

2.13 Prevention and control

As no one know the mechanism of spread of *H.pylori* infection, prevention on an individual level is difficult. Researchers are trying to develop vaccine to prevent, and cure from getting *H.pylori* infection. general following of good hygiene practice as washing hands with soap and water after using the bathroom and before eating, eating food that has been washed well and cocked properly, and drinking from clean, safe source (Broutet, *et al.*, 2001).

CDC, with partners in other government agencies, academic institutions, and industry, is conducting a national education campaign to inform health care providers and consumers of the link between *H.pylori*and stomach and duodenal ulcers. CDC is also working with partner to study routes of transmission and possible prevention measures, and to establish an antimicrobial resistance

surveillance system to monitor the change in resistance among *H.pylori* strain in United States (Broutet,*et al*., 2001).

Chapter three

Materials and Methods

Chapter three

3. Materials and Methods

3.1 Methodology:

3.1.1 Study type and design

Cross sectional descriptive study.

3.1.2 Study area and duration

Study was carried out in Khartoum State (Omdurman, Bahri and Khartoum) in the period from February to October 2018.

3.1.3 Sample size

A total of 100 samples were collected.

3.1.4 Sampling technique

Non probability convenience .

3.1.5 Ethical consideration:

Approval was taken from College of Graduate Studies, Sudan University of Science and Technology, department of medical microbiology; also averbal consent was taken from all householders from whose houses water samples were collected.

3.2 Methods:

3.2.1 Collection of water samples

Sampleswerecollected using sterile glass and plastic bottlesfrom different site (Collar, Zeer and tap water with and without filters) at different source of water in Khartoum state(32 samples from Omdurman, 45 samples from Bahri and 33 samples from Khartoum)and transported immediately to the laboratory for processing.100 samples were collected in sterile bottles(100 ml) contain 0.5g of sodium thiosulphate to dechlorinate the water specimens and were transported to the lab on ice chest, and processed within 2h of collection or store at 4ºC for further analysis.These specimens were taken from different site i.e. 60 samples of unfiltered tap water, 18 samples of coolers water 12 samples ofZeer water and 10 samples of tap water with filters. All samples were collected in aseptic conditions away from any cross-contaminations in separate tube(Ranjbar,*et al*., 2016 ; EL-Sarouny, *et al.*,2015).

3.2.2 Isolation of *H. pylori*

Samples were filtered through 0.45µm filter membrane. Each membrane was taken and immersed in 2 ml of Brain Heart infusion broth (BHI) (Himedia , India) for overnight. After that each 2 ml BHI was taken and cultured for *H.pylori*(Ranjbar,*et al.,* 2016).

Specimens were cultured in special Columbiaagar media (Himedia , India) contain trimethoprim (0.25 $mg.L^{-1}$) , colistinmethanesulfonate (30 $mg.L^{-1}$) , cycloheximide (100 $mg.L^{-1}$) , nalidixic acid (30 $mg.L^{-1}$) , vancomycin(10 $mg.L^{-1}$) (Sigma, St Louis, MO, USA), amphotericin B (10 $mg.L^{-1}$) , and Sheep blood(5%),to inhibit other bacteria and to allow only specimens that contain *H.pylori* to growth after incubation in closed jars contain specific kits to provide environment with the oxygen tension lowered to 5-15% and carbon dioxide raised to 1-10%. Incubation done at 37ºC for 3 days and incubated for a week before being discarded as negative(Greenwood ,*et al*., 2007 ; Ranjbar, *et al*., 2016).

3.2.3DNA extraction:

The*Helicobacterpylori*DNA was extracted from Brain Heart infusion Broth (BHI) using guanidine chloride extraction method. Each 2 ml of Brain Heart Infusion

(BHI) was centrifuged at 3000 rpm for 15-20 min. The pellet was collected and washed twice by phosphate buffer saline (PBS) to remove excess media.2 ml of lysis buffer, 10µl of proteinase K, 1 ml of guanidine chloride and 300µl of ammonium (NH_4) acetate were added to the pellet, vortexed, and incubate at 37ºC overnight. The mixture was cooled to room 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, Shacked, and kept at -20ºC 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 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 50µl of distilled water, genteelly vortetexed, and kept at -20ºC overnight. The extracted DNA integrity was assessed by ethidium bromide stained agarose gel electrophoresis(Tang , et al ,.2006).

3.2.4Target amplification by Polymerase Chain Reaction (PCR):

Multiplex PCR was done to detect *H.pyloriureC (glmM)* gene and *16sRNAH.pylori* specific gene, the amplification and detection of *ure C*(*glmM)*and *16sRNA* genes of *H.pylori* was carried out by PCR method in thermal cycler(Heal force, 960). Master Mix is Maximum 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).

Multiplex PCR was carried out in a 25µl volume of reaction mixture using the maxim PCR premix kit (iNtRON Biotechnology, Seongnam, Korea), dried and aliquoted premix were dissolved by 17µl of D.W, for each gene 0.5µl of forward primer, 0.5µl of reverse primer, 3µl of DNA were added. Multiplex PCR assay was performed to detect *ureC(glmM)* and *16sRNA* specific genes using specific primers (Macrogen, Corea) for *ureC(glmM)*gene(*H.pylori*-F:5'-GGATAAGCTTTTAGGGGTGTTAGGGG-3',*H.pylori*-R:5'-GCTTACTTTCTAACACTAACGCGC-3') and for *16sRNA* gene (*H.pylori*-F:5'-GCTAAGATCAGCCTATGTCC-3' , *H.pylori*-R:5'-TGGCAATCAGGTAAT-3')

The initial denaturation step was done at 94^{o}C Thermocycled for 40 cycles (3 min initial denaturing step at 94^{o}C, 30 seconds denaturing step at 94^{o}C, 30 Seconds annealing step at 53^{o}C , and 45 seconds elongation step at 72^{o}C) and final extension was done at 72^{o}C for 5 min (Essawi , *et al*., 2013).

3.2.5PCR Product sepration:

A total of 4µL amount of each PCR mixture were separated inagarose 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]. then the mixture cooled, and stained with 2µl ethidium bromide [ethedium bromide was prepared by taken 10mg of ethedium bromide and dissolved into 0.5ml D.W then kept into brown bottle]. 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 underUV Trans illuminators. A result was considered positive when a band of size 284 bpfor *Urease C* gene and 387bpfor *16sRNA H.pylori* specific genewere visible in the gel. Standard procedures for reducing contamination were strictly followed.

Chapter four

Results

Chapter four

4. Results

4.1 Frequency of *H.pylori* growth in special culture media from water samples

Out of 100 samples of water examined by cultural method no growth on columbia agar media for*H.pylori*.

Figure 1.No growth on columbia agar media

4.2Frequency of *16sRNA* gene of *H.pylori* in water samples

Out of 100 samples of water examined by conventional PCR, no sample was positive for *16s RNA* gen of *H.pylori.*

4.3 Frequency of *Urea C(glmM)*gene of *H.pylori* in water samples

Out of 100 samples of water examined by conventional PCR, no sample was positive for *Urea C*gen of *H.pylori.*

4.1 Number of positive and negative water samples

Water samples	No of Negative samples	No of Positive sample
Tap water without filters	**60 (60%)**	**0(0%)**
Coller water	**18 (18%)**	**0(0%)**
Zeer water	**12 (12%)**	**0(0%)**
Tap water with Filters	**10 (10%)**	**0(0%)**
Total	**100(100%)**	**0(0%)**

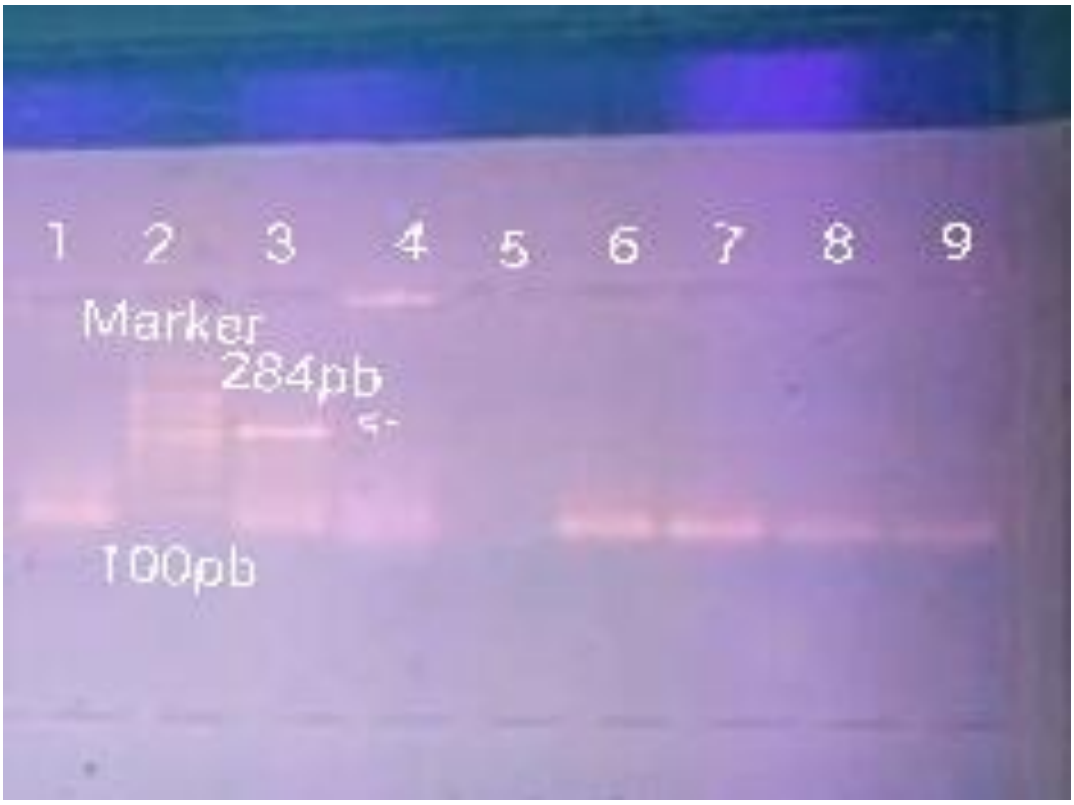

Figure 2. Multiplex PCR for amplification of *Helicobacter pyloriureC (glmM)* and *16sRNA* genes on 1.5% agarose gel electrophoresis.

Lane 2 represents 100pb fragments as Marker.

Lane3 representAmplicons of 284bp as control positive for *glmM* gene.

Lane 1,4,5,6,7,8,9 represent negative samples.

Out of 100 water samples no genes *(ureC(glmM) and 16sRNA)* detected in them.

Chapter five

Discussion and conclusion

Chapter five

5. Discussion and conclusion

5.1 Discussion

Several publications over the last decades have suggested that *H.pylori* may be transmitted through water, without showing that contaminated water is the source of*H.pylori*infections (Janzon*et al*.,2009).The original purpose of this study was to detect *H.pylori* from water samples using cultural methods and to detect *ure C* and *16sRNA* genes of *H.pylori* in water samples using conventional PCR technique.

In this study it was observed that *H.pylori* in water samples is not detected so we cannot consider that water is a vector for *H.pylori* infection and *H.pylori* is transferred by any way rather that water samples. And it is importance of *H. pylori* infection comes from fact that the prevalence rate of infection in some developing countries is more than 80% and in some developed countries the rate is below 20%. In addition to that long term infection with *H.pylori* is classified as a risk factor for gastric cancer. Published studies related to our study were many few to investigate the possible source of transmission of *H.pylori* and many of them contaminated water was the major cause(Aziz ,*et al*.,2005).

The present study revealed that all water specimens investigated were free from*H. pylori* contamination. This may be due to the addition of high chlorine percentage to water system in Khartoum,this finding agree with a study conducted by Abd Al Rahem S.A and her co-worker (2018) were conducted a study to detect *Helicobacter pyloriure C*gene in Khartoum drinking water and they found that out of 90 water samples, no sample (0%) was found positive for *H.pylori* DNA (AbdAlRahem ,*et al*., 2018).

This finding was similar to that reported by Janzon A and his colleagues(2009) in Dhaka (Bangladesh)(Janzon,*et al*., 2009).there was another study which was performed by EL-Sarouny E and her co-worker (2015) on Egyptian water system which showed that direct detection of *ureC* gene detected positive *Helicobacter pylori*DNA in only two samples (tap water and ground water) which is dissimilar to our study (EL-Sarouny, *et al*., 2015).

Also, a dissimilar study was conducted by Bahrami A.R and his co-authors to detect Helicobacter pylori *ureC* gene in a city water, dental units' water in Isfahan(Iran), they were able to detect this gene in 14 water samples out of 200 investigated specimens this may be due to using contaminated water as source of drinking (Bahrami, *et al*., 2013).

Abiri R, *et al* in (2017) conduct a study to detect *H.pylori*in drinking water using loop mediated isothermal amplification (LAMP) in Iran their results showed that out of 60 drinking samples, 16 were positive for *ure C* by, 20 were positive for *ureC* by LAMP, 37 were positive for 16s*rRNA* by PCR, that means loop mediated isothermal amplification (LAMP) is more sensitive than the PCR (Abiri,*et al* ., 2017).

5.2 Conclusion:

This study conducted that there is no *Helicobacter pylori* contaminatingdrinking water in Khartoum State (Sudan).

5.3 Recommendations:

1. Large amount of samples should be collected to insure that there is no water contamination with *Helicobacter pylori*.

2. Other techniques can be used to detect *H.pylori* DNA such as LAMP technique, DNA sequencing and phylogenetic analysis of *H.pylori* DNA in water.

3.Using of other *H.pylori* gene like *Urea A* can give specific result.

4. Same study can be carried in other state (Northern Sudan) to detect if there is any water contamination with *H.pylori.*

5. Other source of possible contamination with *H.pylori* should be examined (water from pit)

REFERENCE

Abd Al RahemS.A,andElhag W.I.(2018).Moleculer Detection of *Helicobacter pylori* in Drinking Water in Khartoum State (Sudan).*African Journal of Medical Sciences*, 3 (5) ajmsc.info.

Abiri R, *et al*.(2017).Detection of *H.pylori* in drinking water by Loop-Mediated Isothermal Amplification.doi: 10.5812/jjm.41859.

Aziz R.K, Khalifa M.M, and Sharaf R.R. (2005).Contaminated water as source of *Helicobacter pylori*infection.Journal of advanced Research; 6(4), 539-547.

Bahrami A.R, Rahimi E, Ghasemian S.H. (2013) .Detection of *Helicobacter pylori* in city water, Dental Units' Water, and Bottled Mineral Water in Isfahan, Iran. *The Scientific World Journal*.p 1-5.

Baker K.H, Hegarty J.P.(2001). *Presence of Helicobacter pylori in drinking water is associated with clinical infection. Scand J infect Dis.* 33(10):744-6. [Pub Med].

Blahd W, MD (6 December,2016). DIGESTIVE DISORDERS HELTH CENTER.[Online]Available from: https://www.webmd.com/digestive-disorders/h-pylori-helicobacter-pylori. [Accessed:8thmarch 2018].

Brooks G.F, Carroll K.C, *et al*. (2013) .Jawetz, Melinck and Adelberg's*medical microbiology*,chapter17,261-63.26thed.

Brosek S.(31 Jul 2018) What are the best natural *H.pylori* treatments?. Medical News Today.https://www.medicalnewstoday.com/articles/322627.php.

Broutet N, Marais A, *et al*. (2001). '' cag A Status and Eradication Treatment Outcome of *Anti-Helicobacter pylori* Triple Therapies in Patients with Nonulcer Dyspepsia '' J ClinMicrobiol 39(4): 1319-22.

Brown LM. (2000) .*Helicobacter pylori* : epidemiology and routes of transmission. 2000;22(2):283-97.

CHEESBROUGH M.(2008)**.***MEDICAL LABORTORY MANUAL FOR TROPICAL CONTRIES, chapter36, p 58-68.*volume2.

Cullen D J E , Collins BJ, *et al.* (1993). When is *Helicobacter pylori* infection acquired?. Gut chapter 34: p 1681-1682.

EL-Sarouny E, EL-Shazli H, and Olama Z.(2015). Detection of *Helicobacter pylori* DNA in Some Egyptian Water Systems and Its Incidence of Transmission to Individuals. *Iran Journal of public Health*, 44(2). p 203-210.

EssawiT,Hammoudeh W, *et al*.(2013). Determination of *Helicobacter pylori* Virulence Genes in Gastric Biopsies by PCR. ID 606258, 4pages .http :\\dx.doi.org\10.1155\2013\\606258.

FORBES B.A, SAHM D.F, and WEISSFELD A.S.(1998).DiagnosticMicrobiology BAILEY&SCOTT'S, chapter 45, 575_76 .10th ed .

FORBES B.A, SAHMD.F , and WEISSFELD A.S. (2007). *Diagnostic Microbiology BAILY&SCOTT'S,* chapter 36, 421-422 .12th ed .

Goderska K, Pena S.A.(2018) .*Helicobacter pylori* treatment: antibiotics or probiotics.ApplMicrobiolBiotechnol 102:1-7.DOI 10.1007/s00253-017-8535-7.

Goodwin C.S, Armstrong J.A ,*et al* . (1989). Transfer of campylobacter pylori and campylobacter mutelae to Helicobacter gen. nov., as *Helicobacter pylori* comb. Nov .and Helicobacter mustelaecomb.Nov ., Respectively. *National Journal of systemic bacteriology*, oc. p.397-405.

Graham DY, Malaty HM, *et al* .(1991). Epidemiology of *Helicobacter pylori* in an asymptomatic population in united states. Gastroenterology. 1991 Jun; 100(6): 1495-501.

Greenwood D,Slack R.C.B, Peutherer J.F.(2000).*MEDICAL MICROBIOLOGY,* chapter 30, p 294_95.15thed.

Greenwood D,SlackR.C.B.,John, Peutherer J.F.(2003) .MEDICAL MICROBIOLOGY, chapter 29, p 292_93.16thed.

Greenwood D,SlackR.C.B,PeuthererJ.F,*etal*.(2007).*MEDICAL MICROBIOLOGY,* chapter 29,304-08.17th ed.

Janzon A, Sjoling S, *et al*. (2009) .Failure to detect *Helicobacter pylori* DNA in drinking and environmental water in Dhaka, Bangladesh, using highly sensitive Real-time PCR assay.American society for Microbiology applied and Environmental Microbiology; 75 (10), 3039-3044.

Kalali B, *et al.* (2014). *H.pylori* Virulence Factors: Influence on Immune System and Pathology. Mediators of inflammation.ID 426309, 9pages. http:// dx.doi.org/10.1155/2014/426309.

Kao C.Y, Sheu B.S, *et al*. (2016). *Helicobater pylori*: An overview of bacterial virulence factors and pathogenesis. Biomedical Journal 39 (2016) 14-23.

Kusters J.G, van Vliet A.H.M, and Kuipers E.J.(2006) . Pathogenesis of *Helicobacter pylori* Infection. Clinical Microbiology Review, p. 449-490.

Marshall B.J, McCallum R.W and Guerrant R.L.(1991) .''*Helicobacterpylori* in peptic ulceration and gastritis.''Blackwell Scientific publications.24-25, 58.

McNULTYC.A,Dent J.C, *et al* . (1989). Detection of *Campylobacter pylori* by the biopsy urease test: an assessment in 1445 patients. Gut, chapter 30, p 1058-1062.

Mobley H.L.T, Mendz G.L. and Hazell S.L. (2001) .''*Helicobacter pylori* physiology and genetics''. ASM Press ; 69-71, 75-76, 300 .

Muhammed J.S, etal. (2012). Epidemiological Ins and Outs of Helicobacter pylori: areview. Vol.62, NO.9.

Ranjbar R, Khamesipour F, *et al*.(2016) . *Helicobacter pylori isolated from Iranian drinking water:VacA, cagA, iceA, oipA and babA2 genotype status and antimicrobial resistance properties*. P.434-435. DOI:10.1002/2211-5*463.12054*

Ricci C,*et al*. (2007) . BestPract Res ClinGastroenterol. Diagnosis of Helicobacter pylori: invasive and non-invasive tests.[online] . PMID:17382278[Indexed for MEDLINE]. Available from: https://www.ncbi.nlm.nih.gov/m/pubmed/17382278/.

Roesler B.M, *et al*.(2014).Virulence Factors of *Helicobacter pylori*. *Clinical Medical Insights:Gastoenterology* .p 9-14.

Rourke J.O, Bode G. (2001) **.**Morphology and Ultrastructure .In: Mobley HLT, Mendz GL, Hazell SL, editors. *Helicobacter pylori*: *Physiology and Genetics. Washington (DC)*: ASM Press. Chapter 6.

Rudnicka K, GraczykowskiM,*et al*.(2014).*Helicobacter pylori* morphological forms and their role in the transmission of infection. PostepyHig Med Dosw(online), 68:227-237.e-ISSN 1732-2693.

She R.C, Wilson A.R, and Litwin C.M.(2009). Evaluation of *Helicobacterpylori*Immunoglobulin G (IgG), IgA, and IgM Serologic Testing Cmpared to Stool Antigen Testing.*Clinical and Vaccine Immunology*.16(8).P.1253-1255.

Tang X.W, *et al*.(2006). Modified Guanidine hydrochloride method for DNA extraction from cord blood used in HLA genotyping. *Zhongguo Shi Yan Xue Ye XueZaZhi*.14(2), 363-5.

WebbP.M,Knight T, *et al*. (19 MARCH 1994) . Relation between infection with *Helicobacter pylori* and living conditions in childhood: evidence for person to person transmission in early life. 308. P.750-51.

Weisenberg E.(1 June 2018).*Helicobacter pylori* gastritis. [Online] Pathology Outlines.com(248)646-0325.Available from: www.pathologyoutlines.com/topic/stomachhelicobacter.html.

ZhongQ, *et al* .(2008).Type IV secretion system in *Helicobacter pylori:* a new insight in to pathogenicity. Chin. Med. J. (Eng1.) 120:2138-42.

Appendix 1

Preparation of culture media:

columbia agar medium:

To prepare about 35plates

Formula:

Columbia blood agar powder	15.9g
Distilled water	500ml

Method:

1- Prepare agar medium by manufacturer. Sterilize by autoclaving at 121ºC for15 minutes. Transfer to 50ºC water bath.

2- when the agar has cooled to 50ºC, added aseptically supplement antibioticsand 5% sterile blood and mixed gently but well avoid forming air bubbles.

Important: the blood must allowed to warm to room temperature before being added to the molten agar.

3- Dispense aseptically in 15 ml amount in sterile petri dishes.

4- Date the medium and give it a batch number .

5-Store the plates at 2-8ºC, preferably in sealed plastic bags to prevent loss of moisture.

Note: Before culturing, a few plates should be incubated first to make sure the blood is sterile.

:ssion brothbrain heart infu

To prepare about 250 ml

Formula:

Columbia blood agar powder	9.25g
Distilled water	250ml

Method:

1- Prepare broth medium by manufacturer. Sterilize by autoclaving at 121ºC for15 minutes.

3- Dispense media aseptically insterile glass bottle.

4- Date the medium and give it a batch number.

5- Store the media at 2-8ºC, preferably in sealed plastic bags to prevent contamination.

Note: Before culturing, a little amount of media should be incubated first to make sure the media is sterile.

Appendix 2

Chemicals and reagents:

1/ 70% alcohol: for sterilization.

2/ Sodium thiosulphate: for dechlorination of water.

3/ Phosphate buffer saline: for washing colonies from the residual media before extraction.

4/ Lysisbuffer: cell lysis.

5/ Proteinase K: breakdown of protein.

6/ Chloroform: phase separation in DNA extraction.

7/Absolute ethanol: DNA precipitation

8/70% ethanol: washing the precipitated DNA.

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

10/ Agarose gel: gel electrophoresis.

11/ 1X TBE buffer: gel preparation and running.

12/ Loading dye: Tracking DNA in gel.

13/ Ethidium Bromide: Visualizing DNA in gel.

14/ MasterMix: to perform PCR process.

Appendix 3

:Instuments

- Hot air oven: and drining of itSterilization of glass ware
- .Sensitive balance: Weight the culture media , chemicals and reagents
- sterilization of culture media and gla ssAutoclave: Used for bottles
- .Vacuum pump: Filtration of water samples
- .Incubater: Incubation of bacteria
- .Distiller: Production of distilled water for reagents prepration
- Centrifuge: For collectiong the cololnies from broth culture and for the .sepration of samples in DNA extraction
- .Refrigator: Preservation of extracted DNA and PCR product
- .Microwave: for agarose gel prepration
- .and PCR product Gel elecrophoresis system: to detect DNA
- .PCR machine: TO amplify the target sequence

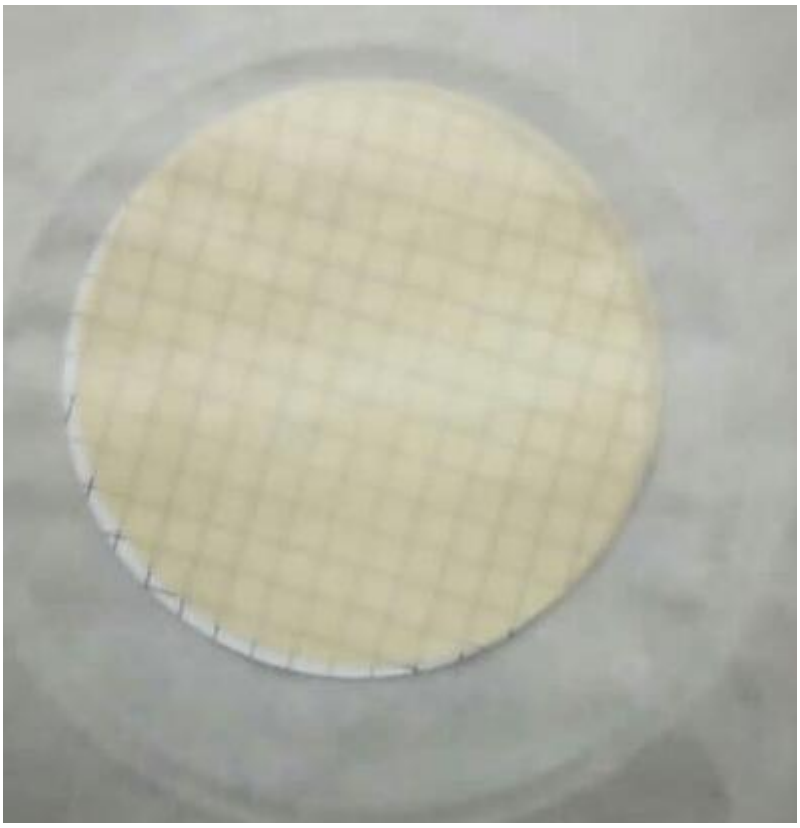

Figure 3.0.45µm Cellulose nitrate filter paper

Figure 4.Taq ready-to-use master mix (iNtRON) Biotechnology

Figure 5.Thermo cycler, PCR machine or DNA amplification

Figure 6.Microwave for agarose gel preparation

Figure7.Gel electorophoresis andUV Transilluminators