Sudan University of Science and Technology

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phenotypic and molecular detection of *Helicobacter pylori* in Fresh Milk Produced from Various Farms in Khartoum State

الكشف الظاهري والجزيئي للبكتريا الملوية البوابية في اللبن الطازج المنتج من مزارع مختلفة بولاية الخرطوم

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الآيـــــة

بِسَمِ اُللهِ الرَحمنِ الرَحِيم

قال تعالى:

(الَّذِينَ يَذْكُرُونَ اللَّهَ قِيَامًا وَقُعُودًا وَعَلَىٰ جُنُوبِهِمْ وَيَتَفَكَّرُونَ فِي خَلْقِ السَّمَاوَاتِ وَالْأَرْضِ رَبَّنَا مَا خَلَقْتَ هَٰذَا بَاطِلًا سُبْحَانَكَ فَقِنَا عَذَابَ النَّارِ).

صدق الله العظيم

سورة آل عمران: الآية (191)

I

DEDICATION

For my *Dad*, Moslih Mahjoub,

No one has ever been given more loving and unconditional support than I have been given by you. Thank you for teaching me to believe in God, in myself and in my dreams.

To my *Mum*, Awadia Hassan,

Whose affection, love, encouragement and prayers of day and night make me able to get such success and honor.

For my *husband*, Omer Kamal,

For his support, love and motivation. Thanks for always being there for me.

For my *brothers* and *sister*.

For my *best friends* Tasneem Azhari and Salsabeel Edrees.

I love you all.

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Whatever I have done is only due to such guidance and assistance, so thank you all.

ABSTRACT

The aim of this study was to detect the presence of *Helicobacter pylori* (*H. pylori*) in fresh milk samples in Khartoum State in the period between March and November 2018.

Total of 50 fresh milk samples were taken from cows (18 sample) , goats (18 sample) and sheep (14 sample). 39 sample were collected in the morning, while 11 were collected in the evening using sterile containers.

All milk samples were cultured on Columbia blood agar media for isolation.

DNA extraction by guanidine chloride method and multiplex PCR were applied to these samples to detect *H.pylori glmM* and *16s RNA* genes.

The results showed that out of 50 samples, no growth was seen on culture media, and there is no gene detected by PCR technique (0%).

It is suggested that *H. pylori* is not present in animal sources and transmission of *H. pylori* from animal sources such as milk is not a primary factor in infection related to this bacterium in humans.

More research work is needed to validate these results.

ملخص الأطروحة

كان الهدف من هذه الدراسة هو الكشف عن وجود بكتيريا الملوية البوابية في عينات اللبن الطازج المنتجة من ولاية الخرطوم في الفترة ما بين مارس ونوفمبر 2018.

تم أخذ 50 عينة حليب طازجة من الأبقار (18 عينة) والماعز (18 عينة) والأغنام (14 عينة).

تم جمع 39 عينة في الصباح ، بينما تم جمع 11 عينة في المساء باستخدام حاويات معقمة.

تم تزريع جميع عينات الحليب على آغار الدم من نوع كولومبيا لعزلها.

تم استخراج الحمض النووي بواسطة طريقة كلوريد الجوانيدين وتم تطبيق تقنية تفاعل البلمرة المتسلسل المتعدد على هذه العينات للكشف عن جينات بكتريا الملوية البوابية (glmM and 16s RNA).

أوضحت النتائج أنه من بين 50 عينة ، لم يظهر أي نمو في أوساط الاستنبات ، ولا يوجد أي جين تم اكتشافه بواسطة تقنية تفاعل البلمرة المتسلسل (0%).

نعتقد أن بكتيريا الملوية البوابية غير موجودة في المصادر الحيوانية وأن انتقالها من مصادر حيوانية مثل الحليب ليس العامل الرئيسي في العدوى المرتبطة بهذه البكتيريا لدى البشر. هناك حاجة لمزيد من البحوث للتحقق من صحة هذه النتائج.

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CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

INTRODUCTION

1.1. introduction:

Three decades have passed since Warren and Marshall described the successful isolation and culture of *Helicobacter pylori*, the gram negative bacterium that colonizes the stomach of half the human population worldwide (Dionyssios *et al*., 2015).

Helicobacter pylori (*H. pylori*) infection constitutes a public health concern in developed and developing countries (Hunt *et al*., 2011; Leonardo *et al*., 2014), since it was associated with chronic gastritis, gastric and duodenal ulcers, and gastric adenocarcinoma in humans, as well as mucosa- associated lymphoid tissue lymphoma (Bauer and Meyer, 2011; Meine *et al*., 2011; Marina and Christine, 2014; Neumeister *et al*., 2015).

H. pylori may also have a role in uninvestigated and functional dyspepsia, unexplained iron deficiency anemia and idiopathic thrombocytopenic purpura (William *et al*., 2017)

Although the rate of infection in the developed world has decreased to a minimum rate (Tajeldin *et al*., 2014) , Previous seroepidemiologic studies showed about 50% of the adult population in developed countries and nearly 90% of those in developing countries were seropositive for *H. pylori* (Nazar *et al*., 2016).

In Sudan, the prevalence of infection was estimated to be 80% among patients with gastritis and Barrett's oesophagus. However, there have been few published data considering the prevalence of *H. pylori* (Tajeldin *et al*., 2014).

For many years, the transmission dynamics of *H. pylori* largely remained unknown and has thus gained the interest of many researchers around the world (Mazaheri Assadi *et al*., 2015).

The Centers for Disease Control and Prevention (CDC) has documented that the bacteria are most likely spread from person to person through fecal-oral or oral-oral routes. Possible environmental reservoirs include contaminated water sources. Iatrogenic spread through contaminated endoscopes has been documented (CDC, 2018).

Literatures indicate the presence and survival of *H. pylori* in food samples, such as milk, vegetables and meat, and suggest these foods may play an important role in the environmental transmission of this pathogen (Zamani *et al*., 2017).

Good conditions for the survival of *H. pylori* in animal milk provides opportunities for its transmission to humans (Fan *et al*., 1998) .

Milk is usually consumed as human food, especially by children. Therefore, one of the suggested theories is the transmission of *H. pylori* through milk from animals to human beings. Some epidemiologic studies have reported the presence and survival of *H. pylori* in raw and pasteurized milk and milk products of cow, sheep, and goat directly or indirectly (Eyman *et al*., 2015).

1.1.2. Rationale:

More than half of the world's population is colonized with *H. pylori* in the gastric mucosa which is the major cause of chronic gastritis and peptic ulcer. Moreover, *H. pylori* has been associated with the development of gastric cancer, the second leading cause of cancer-related deaths worldwide, and has been linked to extra-intestinal diseases, with unsettled causal relationships (Lydia *et al*., 2010). The human host immune response is unable to clear the *H. pylori* infection, and the clinical phenotype is dependent on the interactions between the host immune response and the pathogenicity of the bacterium. The prevalence of *H. pylori* infection is greatly reduced in developed countries but can be as high as >90% in underdeveloped countries, and remains a great health problem world-wide (Yvonne and Rob, 2001).

In Sudan, the prevalence of infection was estimated to be 80% among patients with gastritis and Barrett's oesophagus (Tajeldin *et al*., 2014).

This increasing in infection rates requires complete knowledge about the routes of infection. for that, several studies reported the direct transmission of *H. pylori*, mainly fecal-oral, oral-oral and gastric-oral routes, but only few studies have mentioned the environmental transmission of this pathogen through food samples and some epidemiologic studies have reported the presence and survival of *H. pylori* in raw milk and milk products of cow, sheep, and goat, but there are still many lacking parts about the possibility of this bacterium to transmit into humans by this route.

Also the Sudanese population consume milk frequently as it is one of the main nutrition sources and sometimes without any heating process.

 For all those reasons, this study was conducted to detect the presence of *H.pylori* in raw milk samples.

1.1.3. Objectives:

1.1.3.1. General objectives:

To study the possibility for detection of *H. pylori* in milk samples collected from Khartoum State.

1.1.3.2. Specific objectives:

1- To isolate *H. pylori* from different milk samples using phenotypic method.

2- To detect the *H. pylori* isolated from milk samples using Gram's stain and biochemical tests.

3- To detect *H. pylori glmM* and *16s RNA* genes using multiplex PCR.

Literature Review

1.2. Literature Review

1.2.1. Background:

After a long history of discoveries on the pathology and bacterial colonization of the gastric mucosa starting in the beginning of the last century (Kidd and Modlin, 1998), the gastroenterologist Barry Marshall and the pathologist Robin Warren, in the 1980's, fulfilled Koch's postulates for the association between gastritis and the human gastric pathogen *Helicobacter pylori* (Warren, 1983; Marshall *et al*., 1985; Kidd and Modlin, 1998).

H. pylori was originally called *Campylobacter pyloridis*, but after correction to the Latin grammar the organism was renamed as *C. pylori*. In 1989, DNA sequencing data showed that the bacterium did not belong to the *Campylobacter* genus. Hence, it was placed in its own genus, *Helicobacter* (Marshall and Warren, 2001). The name pylori originates from the Latin word pylorus, which means gatekeeper, and refers to the pyloric valve between the stomach and duodenum.

It's the principal species of the genus *Helicobacter* that is responsible for a variety of gastro–duodenal pathologies in human in the developed and developing world (Perez-Perez *et al*., 1991; Abdulrasheed, 2005).

In many countries, the incidence of *H. pylori* infection has been decreasing in association with improved standards of living. Yet the prevalence of this bacterium is still ubiquitous, especially in the Far East (James *et al*., 2017).

The infection is generally asymptomatic, but it may cause a variety of gastrointestinal diseases, that are associated with significant morbidity and mortality (James and David, 2001).

In June 1994, the International Agency for Research on Cancer Working Group of the World Health Organization classified *H pylori* as a group I, or definite, human carcinogen (James, 2003).

1.2.2. Morphology:

H. pylori is a small, micro-aerophilic, curved or spiral shaped, highly motile, flagellated, Gram-negative bacillus (Yvonne and Rob, 2001; Jagadish, 2006; Jagadish and Nibedita, 2007; Tanih *et al*., 2010; Ananya *et al*., 2012; James *et al*., 2017), although its morphology is not constant. Under adverse conditions it becomes coccoid, but there is controversy about the nature of the coccoid form. Some researchers have stated that this form is either a contaminant or a dead bacterium (Kusters *et al*., 1996), but others consider it to be a metabolically active form that cannot be cultured in vitro (Bode *et al*., 1993; Nilius *et al*., 1993). It has also been suggested that some cocci can revert to their original spiral shape (Andersen *et al*., 1997).

1.2.3. Sources and modes of transmission:

Humans are the only known reservoir for *H. pylori* (Nakamura, 2001). While *H. pylori*'s main habitat is the stomach, some studies have reported the presence of this microorganism in saliva and dental plaque (Hooshmand *et al*., 2004; Gebara *et al*., 2006). Transmission is 'opportunistic' in that any method that allows the organism's access to the stomach is likely to be a mode of transmission. The evidence overwhelmingly supports person-to-person transmission as the predominant mechanism, although few investigations have been able to discriminate between direct transmission from one person to another, and common sources of infection associated with crowding (Graham *et al*., 1992).

Various routes of direct transmission have been described in the literature, mainly fecal-oral, oral-oral and gastric-oral routes.

H. pylori has been cultured from vomitus, diarrhoeal stools and dental plaque, demonstrating that the bacterium is transmissible by these 3 routes (Graham *et al*., 1992).

As for indirect modes of transmission, transient or persistent survival of *H. pylori* in environmental reservoirs has been hypothesized to promote the spread of the infection. In areas with lax sanitation, contaminated water or food may play a role in transmission; however, the culture of *H. pylori* from any of these sources is very rare (Brown, 2000). It has been suggested that *H. pylori* may exist in the environment in a dormant, spore like state that can be viable but not culturable. This hypothesis stems from the fact that, under stressful conditions, *H. pylori* undergoes a morphologic transformation from actively dividing and swimming spiral bacilli to inactive cocci (Amieva and El-Omar, 2008).

1.2.4. Genetic and geographical diversity:

To persist in the gastric niche, *H. pylori* must quickly and constantly adapt to new environments. *H. pylori* demonstrates a substantial heterogeneity and allelic diversity making it one of the most diverse bacterial species known. No two isolates are alike and despite being classified as the same strain, differences are found even in isolates from the same individual at different time points (Kersulyte *et al*. 1999; Kuipers *et al*. 2000; Israel *et al*. 2001; Falush *et al*. 2001; Gressmann *et al*. 2005; Salama *et al*. 2007). This diversity was noticed early using simple techniques, and modern developments of more elaborate genome sequencing have enabled detailed studies on the mechanisms that confer this variability (Gressmann *et al*. 2005; Fischer *et al*. 2010).

Sequence comparison of seven different genomes identified 4.9 to 12.4% of the total gene pool to be strain specific, and microarray analyses have found this number to be as high as 27% (Fischer *et al*. 2010; Gressmann *et al*. 2005). Diversification of *H. pylori* is conferred by two main processes. First, during replication the genome is subjected to changes such as point mutations, rearrangements, and slipped-strand mispairing. Second, these changes are further distributed between genetic variants within the strain, or between strains in a mixed infection, by natural transformation and recombination.

Because of inversions, many strains also demonstrate different syntenies (gene orders) (Lara-Ramírez *et al*. 2011). The outcome of this diversity is a quasipanmictic population structure where, in a changing environment, one genetic variant is superior and could surpass the other clones when they are challenged by changes in the environment (Suerbaum *et al*. 1998).

The microenvironment in which *H. pylori* lives is a constant source of mutagens such as free radicals and low pH. In addition, *H. pylori* also uses a polymerase that does not possess proof reading activity and results in a hypermutator phenotype when it is overexpressed (García-Ortíz *et al*., 2011). In line with having a small genome, the mechanisms to cope with such high mutation rate are limited and no homolog for the mismatch repair system has been identified (Dorer *et al*., 2011). Because of the lack of many conventional repair mechanisms, homologous recombination is important for DNA-repair. This is supported by the findings of Dorer *et al*. (2010) that upon DNA damage, *H. pylori* upregulates a lysozyme and genes involved in natural competence such as the unique comB Type IV secretion system to acquire new genes from lysed cells in the microenvironment (Hofreuter *et al*., 2001; Dorer *et al*., 2010).

The average length of imported gene fragments is estimated to be 417 base pairs and is much shorter than that seen in other bacteria (Falush *et al*., 2001). Interestingly, it appears that the uptake of external DNA consists of larger pieces that are then digested to smaller fragments before being imported into the chromosome to further increase the genetic diversity of the organism (kulick *et al*., 2008; Lin *et al*., 2009; Kennemann *et al*., 2011).

1.2.5. Pathogenesis:

Clinical sequelae are dependent upon bacterial virulence factors and host genetic diversity, particularly within immune response genes. The organism is able to evade the harsh acidic environment in the gastric mucosa and host immune response by elaborating a number of factors that aid in the achievement of its persistent colonization (Tanih *et al*., 2010).

H. pylori possess numerous virulence proteins (*cagA, vacA* and *iceA*) and enzymes (urease, catalase, lipase, phospholipase and proteases) with substantial genotypic diversity, which engenders differential host inflammatory responses that influence the pathologic outcome (Tanih *et al*., 2010).

1.2.6. Infections and complications:

1.2.6.1. Gastritis

Healthy stomach mucosa contains very few inflammatory cells. However, when *H. pylori* colonises the stomach, polymorphonuclear cells and neutrophils migrate to the area resulting in an acute inflammatory response (Atherton, 2006).

Hypochlohydira, or reduced gastric acidity, results from the acute infection. If these initial responses fail to clear the infection, neutrophils, T cells, B cells and macrophages accumulate in the gastric mucosa (Tham *et al*., 2001; Brandt *et al*., 2005), which is a characteristic histological picture of chronic active gastritis (Atherton, 2006). Most strains of *H. pylori* persist lifelong if not eradicated with antibiotics and all cause gastric inflammation (Atherton, 2006). The vast majority of patients stay asymptomatic without further progression. However, 15% of infections result in peptic ulceration and 0.5% - 2% in gastric adenocarcinoma (Goldstone *et al*., 1996; Graham, 1997; Hida *et al*., 1999; Dixon, 2000; Everett *et al*., 2002; Atherton, 2006). Three factors interact together to determine whether subjects develop such diseases; the virulence of the infecting *H. pylori* strain, the type and the extent of the host immune response to infection, and modulating cofactors such as smoking and diet (Graham, 1997; Atherton, 2006).

1.2.6.2. Gastric Ulcer

Gastric ulceration (GU) is associated with a pan-gastritic inflammation pattern and reduced or normal acid production (Dixon, 2000; Dixon, 2001; Atherton, 2006).

GU develops most commonly at the transitional zone between antrum and body on the lesser gastric curve. *H. pylori* is found in 80% of patients with gastric ulcer (Dixon, 2000; Dixon, 2001; Atherton, 2006).

In individuals with normal or high acid secretion, *H. pylori* does not normally colonize the corpus because of the low pH. However, when there is low acid secretion, the colonisation will be consistently spread throughout gastric mucosa (Dixon, 2000; Dixon, 2001).

Colonization by *H. pylori* leads to progressive inflammatory cell infiltration, and rising exfoliation of the epithelial cells (Dixon, 2000; Dixon, 2001; Atherton, 2006).

These changes lead to ineffective mucin and bicarbonate production, which weakens the mucus barrier and the tissue becomes more susceptible to ulceration (Dixon, 2000; Dixon, 2001; Atherton, 2006).

1.2.6.3. Duodenal Ulcer

Duodenal ulcer (DU) is associated with dense *H. pylori* infection and severe inflammation but only when inflammation is largely confined to the antrum, probably as a result of increased basal acid output and a heightened parietal cell response to stimulation. *H. pylori* is found in 95% of patients with duodenal ulcers (Dixon, 2000; Fikret *et al*., 2001; Dixon, 2001; Atherton, 2006). Excessive acid secretion into the duodenal lumen promotes the migration of gastric mucosa into the duodenum a condition which is called gastric metaplasia (Fikret *et al*., 2001).

The appearance of gastric epithelial cells in the duodenum allows colonisation by *H. pylori*, which will establish a chronic inflammatory response (Fikret *et al*., 2001).

The inflammation process and bacterial effect on the epithelial cells render the duodenal mucosa sensitive to gastric acidity, and thus predisposes it to ulceration (Dixon, 2000; Fikret *et al*., 2001; Dixon, 2001; Atherton, 2006).

1.2.6.4. Gastric Cancer

Gastric adenocarcinoma is the second highest cause of cancer deaths worldwide (Globocan, 2002; Atherton, 2006). A possible explanation is the high prevalence of *H. pylori* infection (Correa *et al*., 1990; Meining *et al*., 1998; Globocan, 2002; Correa, 2003). Distal gastric adenocarcinoma, which is *H. pylori* associated, is more common than the proximal form (Correa *et al*., 1990; Globocan, 2002; Correa, 2003).

In addition, *H. pylori* causes MALToma or B cell mucosa-associated lymphoid tissue (MALT) (Du and Atherton, 2006) lymphoma of the stomach (Bandipalliam, 2006; Iida *et al*., 2007; Moss and Malfertheiner, 2007).

Gastric cancer arises as an outcome of long term gastric mucosal infection and chronic inflammation (Goldstone *et al*., 1996; Asaka *et al*., 1997; Blaser *et al*., 2007; Egi *et al*., 2007). Atrophic gastritis, intestinal metaplasia and dysplasia are preceding conditions of gastric cancer (Fikret *et al*., 2001). The difference in prevalence of infection *versus* incidence in gastric cancer (approx. 1%) suggests a multifactorial etiology such as differences in bacterial strains, host genotypes, and environmental conditions (Peek and Blaser, 2002; Atherton, 2006). In particular, strains that carry the *cag* PAI and virulent types of the vacuolating cytotoxin gene (*vacA*) are significantly associated with disease (Atherton, 2006).

Individual differences in host responses are also of major importance, and polymorphism in the pro-inflammatory cytokine interleukin IL-1 was the first described host risk factor for *H. pylori* associated gastric cancer (El-Omar *et al*., 2000, El-Omar *et al*., 2001). IL-1 is the most powerful inhibitor of gastric acid secretion known, and individuals who are colonised by *H. pylori* and possess high expression alleles of IL-1 run an increased risk of developing hypochlorhydria, gastric atrophy and gastric adenocarcinoma (El-Omar *et al*., 2000, El-Omar *et al*., 2001).

On the other hand, there is some evidence against the association of *H. pylori* with gastric cancer. The strongest evidence comes from the unexplained African and Asian enigma where there is high *H. pylori* infection rate and low gastric cancer rate (Holcombe, 1992, Singh and Ghoshal, 2006).

In a study conducted in two regions in China where there is high and low prevalence of gastric cancer, the prevalence of *H. pylori* infection was similar (Hu *et al*., 1995). Similarly, studies in some countries, Italy (Palli *et al*., 1993) and Costa Rica (Sierra *et al*., 1992), did not show a higher prevalence of infection among people living in regions with a high prevalence of gastric cancer.

Thus, both host and bacterial factors contribute to development of disease. Immune response to *H. pylori* also plays a major role.

Furthermore, different environmental factors such as high salt intake and inadequate consumption of fruits and vegetables containing vitamin C has been regarded as risk a factor for development of gastric cancer (Asaka *et al*., 1997, Correa *et al*., 1998).

1.2.7. Pathophysiology:

H. pylori infection can disrupt the normal physiology of the stomach by having direct effects on the gastric epithelium, as well as exciting a host immune response that leads to an intense inflammatory reaction that results in further physiological change (James and David, 2001).

1.2.8. Diagnosis:

Diagnosis of *H. pylori* has improved vastly in recent years and many non invasive tests are now available. However, a combination of both invasive and non-invasive methods is still the gold standard for accurate identification of the bacteria (Vaira and Vakil, 2001; Dzierzanowska-Fangrat *et al*., 2006; Ricci *et al*., 2007). Each of the tests are outlined below:

1.2.8.1. Non –Invasive Methods:

1.2.8.1.1. Urea breath test: This is a rapid diagnostic test. Patients swallow urea labeled with an uncommon isotope- either radioactive carbon-14 or non radioactive carbon 13 (Surveyor *et al*., 1989).

Ten to thirteen minutes later, detection of isotope labeled carbon dioxide in exhaled breath indicates that the urea was split through the action of *H. pylori* urease, and thus presence of the bacteria is confirmed (Surveyor *et al*., 1989).

1.2.8.1.2. Stool Antigen Test: This test utilizes polyclonal anti-*H. pylori* antibody adsorbed to microwells. Diluted patient samples and a peroxidase conjugated polyclonal antibody are added to the wells and incubated for one hour at room temperature. A wash is performed to remove unbound material.

 Substrate is added and incubated for ten minutes at room temperature. Colour develops in the presence of bound enzyme. A stop solution is added and the results are interpreted visually or spectrophotometrically. Any laboratory can perform the test in less than 90 minutes, since no special equipment is needed (Koletzko, 2005).

1.2.8.1.3. Antibody testing: This is a similar principle to the stool antigen test, anti *H. pylori* antibodies are used in an ELISA and patient's blood is added to the wells to detect bacterial presence.

1.2.8.2. Invasive Methods:

1.2.8.2.1. Endoscopy: with subsequent biopsy. Testing of biopic samples can occur in numerous ways:

(a) Slide with a colour change indicator if urea is broken down indicating a positive reaction for *H. pylori*.

(b) Performing nested PCR with primers specific to conserved sequences only found in *H. pylori* strains.

 (c) Culturing the sample and testing using antibodies in a Western blot or immunocytochemical format (Ricci *et al*., 2007).

Invasive methods have the advantage of a definitive positive or negative result, and estimation of bacterial load. However the nature of endoscopy is a drawback due to its discomfort (Della Monica *et al*., 2002).

While non-invasive methods are quick, generally in-expensive and cause minimal discomfort, results can be hampered by factors such as smoking and alcohol, or other pharmaceuticals, which alter the pH of the stomach briefly and decrease the number of bacteria present. This can lead to incorrect estimation of bacterial load or even a negative test result (Della Monica *et al*., 2002).

1.2.9. Treatment:

The recommended *H. pylori* eradication therapy continues to be triple therapy with a proton pump inhibitor (PPI) and two antibiotics of clarithromycin, amoxicillin or metronidazole for seven days (Della Monica *et al*., 2002).

PPI's inhibit gastric acid secretion by blocking the hydrogen/potassium adenosine triphosphatase enzyme system (proton pump) of the gastric cell. Clarithromycin prevents bacterial protein synthesis, thus inhibiting growth. Amoxicillin inhibits the synthesis of bacterial cell wall and metronidazole selectively disrupts nucleic acid synthesis in anaerobic bacteria (Michetti *et al*., 1994).

However, in 20% of cases this therapy fails and hence the requirement exists for continued revision and updating of treatments. For this reason the top six treatment regimes have been identified. Failure of one to eradicate *H. pylori* means moving down the list to the next best and rigorous treatment.

1) Clarithromycin, amoxicillin, and PPIs for seven days. (This is the 1st line defense, and works in 80% of cases).

2) Longer duration of therapy.

3) A Quadruple therapy- PPI, Bismuth, tetracycline and metromidazole.

4) Sequential treatment: Five day PPI, amoxicillin, followed by five day PPI, clarithromycin and metromidazole.

5) Adjuvant therapy

6) New anti-microbial-based therapies: Seven day PPI triple regime with levofloxacin and amoxicillin (Michetti *et al*., 1994, Weltzin *et al*., 1997).

1.2.10. Prevention:

As with most diseases, prevention is preferential to curing an established infection.

For this reason, the development of a vaccine against *H. pylori* is underway and while none are commercially viable at the moment, clinical trials suggest it is only a matter of time. Immunization with *H. pylori* urease subunit in conjunction with the mucosal adjuvants cholera toxin (CT) or heat labile toxin of *Escherichia coli*, into oral (Michetti *et al*., 1994, Weltzin *et al*., 1997), nasal (Weltzin *et al*., 1997), or rectal (Kleanthous *et al*., 1998) passageways, can impart protection against *H. pylori* infection in mice and ferrets (Cuenca *et al*., 1996).

Moreover, due to gastric mucosal colonization by the bacteria, mucosal vaccinations have also been explored in humans. Unfortunately these studies revealed weak urease immunogenicity, although the safety of recombinant urease was confirmed (Kreiss *et al*., 1996, Michetti *et al*., 1999, Banerjee *et al*., 2002). The main conclusion taken from these studies was that it is necessary to increase our knowledge on the mechanisms by which *H. pylori* is cleared following vaccination, hence creating a better understanding of the host defense systems involvement, leading to improvements in future vaccine design (Kreiss *et al*., 1996).

CHAPTER TWO
MATERIALS AND METHODS

Materials and Methods

2.1. Study design

This was descriptive; cross sectional, community-based study.

2.2. Study area and duration

This study was conducted at Khartoum State (Omdurman, Soba and East Nile locals). The PCR was performed at research laboratory in Sudan University of Science and Technology, during the period from March to October, 2018.

2.3. Study population

Milk samples were collected from dairy cattle (cows and goats and sheep) in different farms in Khartoum State.

2.4. Sample size

A total of fifty (n=50) milk samples from dairy cattle (cows, sheep, goats) were included in this study.

2.5. Sampling technique

This Study was based on convenience non probability sampling technique.

2.6. Data collection

Data was obtained by non-self-administrated questionnaire (appendix III) from cowmen and goat and sheep herders.

2.7. Ethical approval

Approval to conduct this study was obtained from the College of Medical Laboratory Sciences, Sudan University for Science and Technology.

2.8. Laboratory Methods:

2.8.1. Isolation

The samples were cultured on Columbia blood agar (appendix II) and incubated at 37 $^{\circ}$C for 7 days. Growth of each sample was checked periodically and reported after 7 days.

Isolated and completely identified *H. pylori* was used as control positive was used to verify the validity of the method.

2.9. Identification of *H. pylori ureC* (*glmM*) and *16s RNA* genes by Multiplex PCR:

2.9.1. DNA Extraction

The *H. pylori* DNA was extracted from the milk samples using guanidine chloride extraction method. Each 2 ml of milk sample was centrifuged at 3000 rpm for 15 min. The pellet was collected and washed twice by phosphate buffer saline (PBS). 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 pellet,vortexed, and incubated at 37°C 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, shaked, and kept at -20°C for overnight. Then the tube was centrifuged at 3000 rpm for 15 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°C.

The extracted DNA integrity was assessed by ethidium bromide stained agarose gel electrophoresis.

2.9.2. PCR

2.9.2.1. PCR primers for identification of *ureC* (*glmM*) and *16s RNA* genes

The following primers were used for *ureC* (*glmM*) and *16s RNA* genes detection (Tamer *et al*., 2013) (Table 3.1).

Table 2.1: Primers sequences used for detection *H. pylori*:

Target gene	Primer name	Sequence(5´-3´)	Size (bp)
ureC (glmM)	Hp-F	GGATAAGCTTTTAGGGGTGTTAGGGG	284
	Hp-R	GCTTACTTTCTAACACTAACGCGC	
16sRNA	16sRNA-F	AGAGTTTGATCCTGGCTCAG	387
	16sRNA-R	TACGGTTACCGGTTACCGACTT	

2.9.2.2. Preparation of 10X Tris Base EDTA (TBE) buffer

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

2.9.2.3. Preparation of 1X TBE buffer

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

2.9.2.4. Preparation of ethidium bromide

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

2.9.2.5. Preparation of agarose gel

Amount of 0.75g of agarose powder were dissolved into 100ml of 10X TBE, then the mixture had been cooled, then 2µl of ethidium bromide was added, mixed well and poured in a casting tray that had been taped up appropriately and equipped with spacers and suitable comb to form wells, any bubbles were removed and the gel allowed setting at room temperature, after solidification the comb and the spacers were gently removed.

2.9.2.6. Master Mix

Maxime PCR PreMix kit (iNtRON Biotechnology, Seongnam, Korea) is a premixed reagent ready to use, contains all the reagent required for PCR (except water, template and primers) and additional components needed for direct loading onto agarose gel, tracking blue dye that allow monitoring progress during the electrophoresis.

2.9.2.7. Preparation of reaction mixture:

Multiplex PCR was carried out in a 25µl volume using the Maxime 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.

2.9.2.8. Detection of *ureC* (*glmM*) and *16s RNA* genes:

Multiplex PCR assay was performed to detect *ureC* (*glmM*) and *16s RNA* genes using the specific primers in table 3.1.

PCR amplification involved initial denaturation at $94°C$ for 3 min, followed by 40 cycles of denaturation at $94°C$ for 30 sec, annealing at $53°C$ for 30 sec, extension at $72°C$ for 45 sec, and a final extension at $72°C$ for 5 min (Tamer *et al*., 2013).

2.9.2.9. Visualization of PCR product:

The amplicons were separated at 30 V for 30 min in a 1.5% (wt/vol) agarose gel containing ethidium bromide, bands were visualized under U.V transilluminator(UVitec –UK) to detect the specific amplified products by comparing with 100 base pairs standard ladders (INtRON biotechnology. Korea).

2.10. Data analysis:

Data were analyzed by chi square test using Statistical package for the social sciences (SPSS) version 16.

CHAPTER THREE

RESULTS

RESULTS

3.1. Isolation

A total of 50 milk samples were cultured on Columbia blood agar. After 7 days of incubation at 37°C, no growth was seen.

3.2. Detection of *16sRNA* and *ureC (glmM)* genes

All 50 milk samples (18 cow milk samples (36%) , 18 goat's milk samples (36%) and 14 sheep milk samples(28%)) were investigated for the presence of *ureC(glmM)* and *16sRNA* genes by multiplex PCR, but none of the genes was detected in any of the samples as shown in table 3.1. and figure 3.1.

Table 3.1.: Detection of *H. pylori* according to the animal type

Samples		Result				Total
		UreC(glmM)		*16sRNA*		
		Negative	Positive	Negative	Positive	
Cows	Count	18	O	18	0	18
	% of Total	36%	0%	36%	0%	36%
Goats	Count	18	0	18	0	18
	% of Total	36%	0%	36%	0%	36%
Sheep	Count	14	0	14	0	14
	% of Total	28%	0%	28%	0%	28%
Total	Count	50	0	50	0	50
	% of Total	100%	0%	100%	0%	100%
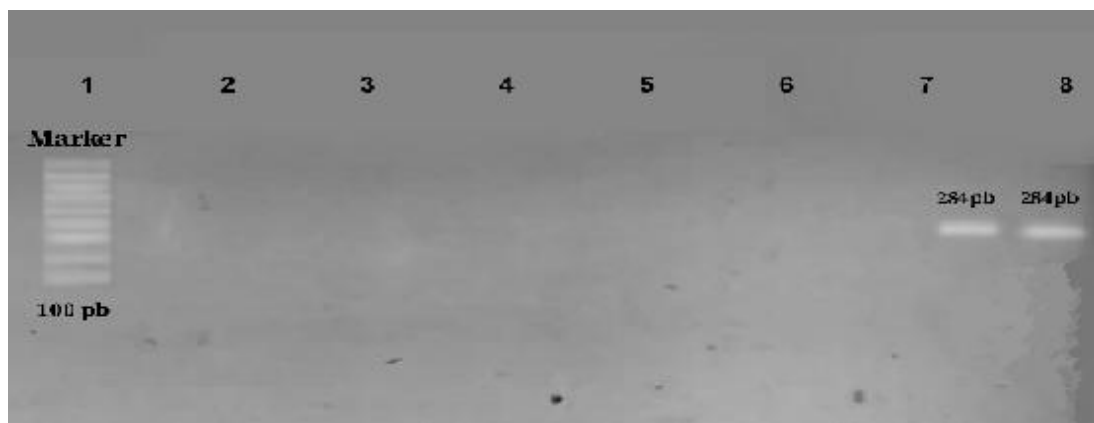
Figure 3.1.: Multiplex PCR for amplification of *H. pylori ureC* (*glmM*) and *16sRNA* genes on 1.5% agarose gel electrophoresis. Marker: 100bp fragments. Lane 1: Marker, Lane 2 – 6: negative samples, Lane 7 – 8: positive controls (Amplicons 284bp represent *glmM* gene)

Total of 39 milk samples were collected in the morning, while 11 milk samples were evening milk. All of them show no growth on blood agar and no genes (*ureC*(*glmM*) and *16sRNA*) detected in them.

Table 3.2.: Detection of *H. pylori* according to the milking time

Milk		Result		Total
		Negative	Positive	
Morning milk	Count	39	0	39
	% of Total	78%	0%	78%
Evening milk	Count	11	0	11
	% of Total	22%	0%	22%
Total	Count	50	0	50
	% of Total	100%	0%	100%

Total of 21 milk samples were from lactating animals, while 11 milk samples were from non lactating animals. All of them show no growth on blood agar and no genes (*ureC*(*glmM*) and *16sRNA*) detected in them.

Table 3.3.: Detection of *H. pylori* according to the lactation

Lactation		Result		Total
		Negative	Positive	
Lactating	Count	21	0	21
	% of Total	42%	0%	42%
Non lactating	Count	29	0	29
	% of Total	58%	0%	58%
Total	Count	50	0	50
	% of Total	100%	0%	100%

All animals included in this study were never examined by veterinarian, graze grass from local farms in wide spread areas and the water supplies wasn't treated with chlorine. All of them show no growth on blood agar and no genes (*ureC*(*glmM*) and *16sRNA*) detected in them.

The milking process of all animals included in this study was manual milking, also all of them show no growth on blood agar and no genes (*ureC*(*glmM*) and *16sRNA*) detected in them

CHAPTER FOUR

DISCUSSION

DISCUSSION

4.1. Discussion:

H. pylori infection is one of the most common infections worldwide. However, it is not well known how *H. pylori* is transmitted and where in the natural environment the organism resides. It is suggested that raw milk could be an intermediate transmission vehicle of *H. pylori* infection (Fujimura *et al*., 2002), so this study was formulated for the isolation of *H. pylori* and detection of *H. pylori glmM* (*ureC*) and *16s RNA* genes by multiplex PCR at Khartoum State, Sudan. A total of 50 milk samples were collected from different farms, but *H.pylori* wasn't grown on culture media or detected by PCR from any raw milk sample (0%).

On other study conducted by Fujimura *et al.* (2002) in Japan, *H.pylori* was found in 72.2% of cow raw milk specimens. Furthermore, Mousavi *et al.* (2014) reported that *H.pylori* existed in 1.4% of cow, 12.2% of sheep and 8.7% of goat's milk by PCR method. In addition, Eyman *et al.* (2015) were reported that the prevalence of *H.pylori* in cow's milk was 22%, and near to that study done in Iran by Safaei *et al.* (2011) who found that the prevalence of *H.pylori* was 16%. On other study conducted in Egypt, El-gohary *et al.* (2015) reported that the prevalence of *H.pylori* in cow's milk was 6.9%.

This apparent discrepancy could be due to cross contamination with animal and human sources during milking processes, or it could be due to the type of drinking water used, which is treated sewage water unlike Sudan, which uses well water.

On the other hand, my results was similar to another study in Iran done by Mohammed (2012) who failed to detect *H. pylori* from different raw milk samples.

And this may be due to considering Iran is an Arabic country geographically not far from Sudan with approximately similar environmental conditions and similar behaviors.

Thus, our data suggest that sheep, goat and cow's milk cannot play an important role in transmission of *H.pylori* infection in our study area.

4.2. Conclusion:

As a conclusion, it is suggested that *H. pylori* is not present in animal milk collected from different regions of Khartoum and transmission of *H. pylori* from animal sources is not a primary factor in infection related to this bacterium in humans.

4.3. Recommendation:

1. Further investigation with a greater number of samples is necessary to verify the ability of *H. pylori* to spread via milk consumption.
2. Good dairy farming practices to reduce possibility of milk contamination.

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APPENDIX (I)

COLOURED PLATES

Colour plate (I.1): milk samples and plates of Columbia blood agar.

Colour plate (I.2): Microwave.

Color plate (I.3): Thermo-cycler.

Color plate (I.4): Electrophoresis system.

Color plate (I.5): Tran-illuminator

APPENDIX (II)
REAGENTS AND CULTURE MEDIA

Appendix (II.1): Columbia blood agar medium:

To make about 35 blood agar plates:

Columbia blood agar powder. . . . . 15.9 g

Distilled water . . . . 500 ml

Preparation:

1- Prepare the agar medium as instructed by the manufacturer. Sterilize by autoclaving at 121°C for 15 minutes. Transfer to a 50°C water bath.

2- When the agar has cooled to 50 °C, add aseptically the antibiotic supplement (trimethoprim, cycloheximide, nalidixic acid, vancomycin, amphotericin B) and 5% sterile defibrinated blood and mix gently. Avoid forming air bubbles.
Important: The blood must be allowed to warm to room temperature before being added to the molten agar.

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

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

5- Store the plates at 2–8°C, 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.

Appendix (III)

Sudan University of Science and Technology

Collage Graduate Studies

Department of Medical Microbiology

Questionnaire Model

- ❖ farm: . . . .
- ❖ Date....\....\.......
- ❖ Sample No.(   )
- ❖ Type of animal:

a. cow   b. goat   c. sheep

- ❖ Examination by veterinarian:

a. monthly   b. annually   c. never

- ❖ housing:

a. local area   b. wide spread area

- ❖ food source:

a. grass from local farms

b. graze indiscriminately outside the boundaries of the house.

c. human food remainings.

- ❖ water supply:

a. treated with chlorine   b. not treated with chlorine

- ❖ Lactation:

a. lactating   b. not lactating

❖ milking:

a. machinery   b. manual

❖ personnel:

_suffer from H.pylori infection previously:

a. yes   b. No

_do they wash hands before milking:

a. yes   b. No

_do udder and teats are washed before milking:

a. yes   b. No

❖ time of collection of sample:

a. morning milk   b. evening milk