Sudan University of Science and Technology

Collage of Graduate Studies

Detection of 23SrRNA Gene Mutations Related to Clarithromycin Resistance of *Helicobacter pylori* Isolated from Dyspeptic patients in Khartoum state

الكشف عن الطفرات الجينيه الرايبوزي الرايبوسومي 23 إس آر المتعلقة بمقاومة الكلاريثرومايسين للبكتريا الملوية البوابية المعزولة من مرضى عسر الهضم في ولاية الخرطوم

A Thesis submitted for Partial Fulfillment for the Requirements of MSc Degree in Medical Laboratory Science (Microbiology)

Submitted by:

Duha Mahmoud Zeinalabdin

BSc. Medical Laboratory Science (Microbiology)

Supervisor:

Dr Husham NourAldaym

April/2021

الأية

بسم الله الرحمن الرحيم

(قُل لَّوْ كَانَ الْبَحْرُ مِدَادًا لِّكَلِمَاتِ رَبِّي لَنَفِدَ الْبَحْرُ قَبْلَ أَن تَنفَدَ كَلِمَاتُ رَبِّي وَلَوْ جِئْنَا بِمِثْلِهِ مَدَدًا)

صدق الله العظيم

سورة الكهف الآية 109

Dedication

To my mother and father the source of love and support

To my wonderful brothers, sisters and friends

To my beloved Husband

To the memory of my grandmother

Thank you

Acknowledgement

I would like to thank

Dr. Hisham Nour Aldayem Altayeb and U.Maram Alnosh

And gratitude to all Students and Teachers in

college of Medical Laboratory Science S.U.S.T and

Finally, I would like to appreciate and express my

deepest feelings to all my colleagues

Abstract

Helicobacter pylori infection is a major gastric infection and clarithromycin resistance is increasing worldwide, data regarding resistance are limited in developing countries.

This cross-sectional study was conducted in Khartoum-Sudan in the period from June 2018 until April 2019 to detect the clarithromycin resistance mutations in the 23SrRNA genes isolated from *Helicobacter pylori* among dyspeptic patients in Khartoum state.

A seventy-one gastric biopsies were collected from patients undergoing gastroduodenal endoscopy, DNA using the guanidine method then polymerase chain reaction to detect 16SrRNA and 23SrRNA genes Then all the positive results were subjected to sanger sequencing to detect mutations associated with clarithromycin resistance.

Out of seventy-one specimens, forty-two patients (59.2%) were positive for *16SrRNA* gene from which nineteen specimens (26.8%) were positive for the 23srRNA gene. the prevalence of mutations that is related to clarithromycin resistance was (7.1%) with only two Samples had the A2143G mutation (2.8%), two samples had the T2182C mutation and one Sample had both A2143G and T2182C mutations, but there is no significant correlation between the presence of these mutations and age, gender or disease.

This study concluded that Clarithromycin resistance of *Helicobacter pylori* in this study is low but there are mutations that can affect the eradication therapy.

•

ملخص البحث

عدوى الملوية البوابية هي من اهم انواع العدوى التي تصيب المعدة وتتزايد مقاومتها للمضادات الحيوية مثل الكلاريثروميسين في جميع أنحاء العالم ، والبيانات المتعلقة بالمقاومة محدودة في البلدان النامية.

أجريت هذه الدراسة المقطعية في السودان–الخرطوم في الفترة من 2018 إلى 2019 لقراءة التسلسل الجيني لي الجين الحمض النووي الرايبوزي الرايبوسومي 23 إس بين مرضى الجهاز الهضمي في ولاية الخرطوم.

تم جمع واحد وسبعين خزعة معدية من المرضى الذين خضعوا للتنظير المعدي الداخلي، وخضعت جميع العينات لاستخراج الحمض النووي باستخدام طريقة الجوانيدين ثم تفاعل البلمرة المتسلسل للكشف عن وجود جينات الحمض النووي الرايبوزي الرايبوسومي من فئة ١٦إس و٢٣ إس.

ثم تعرضت جميع العينات الإيجابية لوجود الجينين لقراءة التسلسل الجيني لي جين٢٣ إس للكشف عن الطفرات المرتبطة بمقاومة كلاريثروميسين.

من أصل واحد وسبعين عينة إثنان وأربعون عينه (59.2٪) كانت موجبة لجين ١٦إس و تسعه عشر عينه (26.8%) كانت موجبة للجين٢٣ إس و كان تواتر الطفرات المرتبطة بمقاومة الكلاريثروميسين (7.1٪) ، مع عينتان فقط (2.8%) تحتويان علي الطفرةA 2143G و عينتان تحتويان علي الطفره (2.8%) T2182Cعينه واحده فقط احتوت علي كل من الطفرتين A2143G,T2182C

خلصت هذه الدراسة إلى أن مقاومة كلاريثروميسين لبكتيريا الملوية البوابية في هذه الدراسة منخفضة ولكن هناك طفرات يمكن أن تؤثر على القضاء عليها نهائيا.

List of contents

List of tables

List of Figures:

Chapter One

Introduction and Litrature Review

CHAPTER ONE

1. Introduction:

Helicobacter pylori is the first formally recognized bacterial carcinogen and classified as a group I carcinogen by the International Agency for Research on Cancer (Testerman and Morris, 2014), it is the same category as smoking, radiation, and asbestos. It is a fastidious microaerophilic, gram-negative, slightly curved, with 2–6 distinctive unipolar flagella. It colonizes human gastric mucosa and causes gastritis, peptic ulcer, and gastric cancer, it can cause persistent and lifelong infection (Kusters, van Vliet and Kuipers, 2006). Clinical presentations can result from interactions between bacterial virulence (e.g. CagA, VacA, BabA), host genetics, and environmental factors. infection with *H. pylori* carrying Certain virulence factors can lead to more severe outcome (Chang, Yeh and Sheu, 2018).

H. pylori infection is universal public health concern with global prevalence (44.3%). higher infection rates can be found in developing countries (50.8%) and in Africa (70.1%) according to (Ngnameko et al (2021) cited in (Hooi et al., 2017)) compared to other regions about (85%) of the infected patients only have mild asymptomatic gastritis while (15%) of patients can develop to have peptic ulcer disease and less than (1%) can develop to have gastric cancer., (Chang, Yeh and Sheu, 2018). The prevalence of resistance is increasing worldwide and for primary resistance in adults in Northern Europe, the global prevalence was less that (5%) while in Southern Europe it was as high as up to (20%) or more. For children from all European countries a high prevalence has also been reported, ranging from (12.4% to 23. 5%).The essential risk factor for clarithromycin resistance is previous use of macrolides, and therefore, a trend for an increased prevalence is observed in most countries. The prevalence is even higher in children because they are more likely to be treated with macrolides when they have respiratory infections. The prevalence of secondary clarithromycin resistance is extremely high, in the range of (60%) (Mégraud and Lehours, 2007).

Two triple-therapy regimens considered to be first-line contain clarithromycin, a PPI, and either amoxicillin or metronidazole, Bismuth quadruple therapy also gives eradication rates similar to those of clarithromycin-based therapies. (Leena Myran and Zarbock, 2021).

In Sudan there are limited data that have been published concerning *Helicobacter pylori* resistance to clarithromycin. In 2014 a study reported a high prevalence of *H. pylori* (65.8%) and (Abdalazeem et al., 2012) also reported a high prevalence of resistance to clarithromycin (25%). Resistance mechanism can reduce the efficacy of clarithromycin and failure of treatment regimen, this is why the future of *H. pylori* treatment and management need to be recognized (Abdallah, Mohammed, Mohammed and Ali, 2014).

Clarithromycin is a bacteriostatic antibiotic used to prevent the translation process in ribosomes, recent knowledge regarding mechanisms of resistance to clarithromycin showed the significance of many point mutations predominantly at position 2142/43(A2142 to G/C/T; A2143 to G/C) within the peptidyl transferase region encoded in domain V of 23SrRNA in 50S subunit that cause detachment of peptidyl enzyme throughout elongation reaction. (versalovic *et al*., 1996), (Hamid Abdollahi, 2021). Till now two major mutations A2142G and A2143G were known as the major cause of resistance in clinical isolates. (Megraud and Lehours., 2007).

1.2. Rationale:

Due to increasing prevalence of clarithromycin resistance World health organization designated it as high priority for research also the future of *Helicobacter pylori* treatment and management need to be recognized especially in Africa which contain high infection rates also there are limited data that have been published concerning resistance of *H.pylori* to clarithromycin which is the best effective and well tolerated antibiotic and the success chance of regimen if

resistant bacteria existed will be less than 40% (Abadi, 2017).this data can be used to establish new guidelines to direct the empirical treatment regimen.

1.3. Objectives:

1.3.1 General objectives:

To determine the *Helicobacter Pylori* resistance to clarithromycin in dyspeptic Sudanese patients.

1.3.2. Specific objectives:

1-To determine *Helicobacter pylori among* dyspeptic patients undergoing upper gastrointestinal endoscopy using polymerase chain reaction by detecting 16SrRNA genes.

2-To detect the point mutations related to clarithromycin resistance in 23SrRNA, especially A2143G and A2142G mutations.

3-To determine the presence of significant correlations between the type of mutation and Gender, Age and clinical conditions.

Chapter Two

Rationale and Objectives

CHAPTER TWO

Literature review:

2.1. Genome, plasmids, and strain diversity:

The sequenced *H. pylori* genomes' size is approximately 1.7 Mbp, with a GC content of 35 to 40%. The genome contains two copies of the 16S, 23S, and 5S rRNA genes. Many strains possess one or more cryptic plasmids, which do not seem to carry antibiotic resistance or virulence genes. Some of these plasmids form the basis of *H. pylori-E. coli* shuttle vectors used in molecular cloning experiments, the existence of *H. pylori*-infecting bacteriophages has been reported, it can be found in humans and some nonhuman primates but no conclusive evidence for zoonotic transmission (Kusters, van Vliet and Kuipers, 2006).

2.2. Pathogenesis of *Helicobacter pylori*

H. pylori produce urease that increases the pH of the surrounding area to protect itself against gastric acid. The curved shape of H. pylori helps the polar flagella to penetrate through viscous mucus. Chemotaxis systems direct H. pylori towards some amino acids, bicarbonate, and cholesterol (Testerman and Morris, 2014).

"H. pylori glycosylate host cholesterol and add it into its outer membrane, H. pylori lacking cholesterol are extremely sensitive to environmental stress and reduces CagA-mediated activities and interactions with T cells. Lipopolysaccharide (LPS) was found in *H. pylori*, which may contribute to persistence by limiting the host inflammatory response. Unlike LPS from other species, *H. pylori* LPS is recognized by TLR-2 rather than TLR-4. In some strains, side chains on the LPS O antigen mimic the Lewis blood group antigens Le^x and Le^y. The membrane extends to form a sheath covering the flagella. The combination of sheathed flagella, hypo inflammatory LPS, and molecular

mimicry reduces the host response, allowing the organism to persist with minimal pathology (Testerman and Morris, 2014).

2.3. Diagnosis:

2.3.1. Noninvasive methods:

2.3.1.1. Urea breath test: The urea breath test consists of drinking carbon-13 labeled or carbon-14 labeled urea and it is converted to carbon dioxide and ammonia by the urease created by the *H. pylori*. A sample of expired air is analyzed for the presence of the labeled carbon. It's preferred for the establishment of active infection. The sensitivity and specificity for the urea breath test is 99% and 98%, respectively. (Testerman & Morris., 2014).

2.3.1.2. Stool antigen: analysis of *H. pylori* stool antigens using monoclonal or polyclonal antibodies. The sensitivity and specificity for the test is 94.1% and 91.8%, respectively. it is more economical than endoscopy for confirmation of eradication. Patient submission of stool samples may be a limitation of this test (Testerman & Morris., 2014).

2.3.1.3. Serology: Micro titer plates coated with *H. pylori* antigens, combined with a secondary antibody, and are used to detect *H. pylori*-specific IgG. Seropositivity is not evidence of active *H. pylori* infection and cannot be used to monitor the success of antimicrobial therapy. The sensitivity and specificity of serology for detection of initial infection compared to the gold standard of histology with the sensitivity of 85% and specificity of 79%. (Testerman & Morris., 2014).

2.3.2. Invasive methods: Endoscopy is needed to collect biopsy material and to assess the degree of pathology in the stomach. Giemsa, Warthin-Starry, and Diff-Quik and the standard hematoxylin and eosin staining can be used to identify *H. pylo*ri. (Testerman and Morris, 2014).

2.3.2.1 Rapid Urease test: involves the utilization of the *H. pylori* urease to identify active organisms. Samples of gastric tissue are placed on urea agar gel with pH-color indicator, Urea is converted to ammonia, causing a color change. Sensitivity greater than 93% and specificity of 98% is reported for rapid urease testing. (Testerman & Morris., 2014).

2.3.2.2. Culture: is highly specific but less sensitive compared to other methods. Also is technically challenging and costly, *H. pylori* is a fastidious, microaerophilic organism requiring 5-7 days to form visible colonies on solid media.it is recommended for situations requiring specific antimicrobial sensitivity testing. (Testerman & Morris., 2014).

2.3.2.3. Polymerase chain reaction: can be used to detect *H. pylori* DNA in biopsy samples, but saliva and feces have also been used. PCR sensitivity nearing 100% and specificity of 100% can be obtained. Contamination from improperly cleaned endoscopes may create false positive results. False negative results may occur due to PCR inhibitors within gastric tissue or feces. PCR is primarily used in research. (Testerman & Morris., 2014).

2.6.2.4. Fluorescence *in situ* hybridization (FISH): is a sensitive and specific method to detect *H. pylori* in gastric biopsies. A fluorescently labeled DNA probe hybridizes with the complementary sequence within the bacterial chromosome. FISH can detect coccoid *H. pylori.* The use of different fluorescent dyes can permit simultaneous detection of H. pylori 16SrRNA and gene sequences. (Testerman & Morris., 2014)

2.4. Treatment:

Triple therapy with metronidazole and either bismuth subsalicylate bismuth sub citrate plus either amoxicillin or tetracycline for 14 days eradicates *H. pylori* infection in 70–95%of patients. An acid-suppressing agent given for 4–6 weeks enhances ulcer healing. Proton pump inhibitors (PPIs)directly inhibit *H. pylori*

and appear to be potent urease inhibitors. The preferred initial therapy is 7–10 days of a PPI plus amoxicillin and clarithromycin or a quadruple regimen of a PPI metronidazole, tetracycline and bismuth for 10 days (Jawetz *et al.*, 2016).

2.5 Prevention and Control

Improved personal hygiene is important in the prevention of the disease (Jawetz *et al.*, 2016).

2.6. Previous studies:

many previous studies were conducted to detect point mutations in 23SrRNA genes in *H. pylori* with various methodology and findings. Due to the increasing prevalence of clarithromycin-resistant *Helicobacter pylori* in Vietnam. This study was carried out to detect point mutations in the 23S rRNA domain V of clinical isolates, estimate the prevalence of phenotypic clarithromycin resistance and to assess the association between 23SrRNA domain V genotype and clarithromycin resistant phenotype. 185 gastric biopsy specimens with *H. pylori*-positive chronic gastritis were subjected to sequencing, of which 104 samples were subjected to susceptibility testing to determine clarithromycin resistance. (Tran *et al*., 2019).

As a result of this point, mutations were detected. A2143G and A2142G mutations were observed in 40.5% and 4.3%, respectively. New point mutations were detected (C2041T, C2083T, C2191T, G2220A, G2225A, G2240A, C2273T, T2276C, G2287A, C2399T, A2445G and C2622T). 23S rRNA domain V genotypes were diversified, with combinations of two or more-point mutations as well as single point mutations. (Tran *et al*., 2019).

In Turkey, they aimed to detect the resistance of clarithromycin in *H. pylori* strains by phenotypic tests and molecular methods and to determine the mutations related to resistance. Forty-eight gastric biopsies from dyspeptic patients. Were

identified as *H. pylori* by culture and biochemical tests. Minimum inhibitory concentrations were determined using clarithromycin E-tests. To detect the resistance, then PCR to amplify 23SrRNA, then sequencing. The result is A2143G and A2144G point mutations are most frequent. Other mutations A2115G, G2141A, A2142G/C, T2182C, T2190Cand C2195T were found. According to the results of the sequencing, (37.5%) resistant strains were detected. Point mutations were A2142G/C (8.3%), A2143G (16.6%), T2182C (8.3%), A2144G (2.1%) and G2141A, A2142G/C, A2144G, A2115G (2.1%) multiple mutation in 1 sample. T2190C and C2195T were not detected. (Ergin *et al*., 2018)

Regarding African and developing countries this study was carried out in Tanzania where they reported the prevalence of mutations conferring resistance to clarithromycin and fluoroquinolones among dyspeptic patients. Between August 2014 and August 2016 patients undergoing upper gastrointestinal endoscopy at the Bugando Medical Centre were enrolled. Biopsies were taken for polymerase chain reaction (PCR) and sequencing to detect mutations conferring resistance to clarithromycin and fluoroquinolones, a total of 208 non repetitive biopsies were examined. of which (90.4%) tested positive for *H. pylori* specific 23SrRNA PCR. Clarithromycin resistance mutations were detected in (28.7%) of patients tested. The most frequently detected mutation was A2143G followed by A2142G. (Jaka *et al*., 2019).

The first study conducted in Sudan by (Abdalazeem *et al*., 2012) to address *Helicobacter pylori* resistance to clarithromycin aimed at characterizing the mutations in 23SrRNA gene related to Clarithromycin-resistant *Helicobacter pylori* strains. among dyspeptic patients in Khartoum State. They collected 200 gastric biopsies by endoscopy from patients with dyspepsia. DNA was extracted from culture isolated and relevant mutations in 23SrRNA gene were detected. They found that Out of the 200 biopsies, *H. pylori* were isolated from (24%)

biopsies. Twelve of them were found to be resistant to clarithromycin. Eight of the resistant strains had both A2143G and A2142G by using restriction enzymes Bsa1and Bbs1. Sequencing the remaining four isolates by PCR detected A2140G mutation. (Abdalazeem *et al*., 2012).

Chapter Three

Methods and Materials

Chapter Three

3. Materials and methods:

3.1. Study design:

A descriptive- cross-sectional hospital-based study.

3.2. Study Area:

The study was conducted in Khartoum state Hospitals-Gastroenterology and endoscopy units (Omdurman military hospital and Ibn sina hospital).

3.3. Study period:

The study was conducted from June 2018 to September 2019.

3.4. Study population:

Total of Seventy-one dyspeptic patients suspected of having gastric abnormality undergoing GIT endoscopy in Khartoum state hospitals from both genders and different age groups have an equal chance enrolled.

3.5. Inclusion criteria:

Dyspeptic Patients suspected of having gastric pathology undergoing GIT endoscopy and confirmed by endoscopy.

3.6. Exclusion criteria:

Patients with gastric bleeding history

3.7 Sampling technique:

Simple random sampling

3.8 Sample size:

It is obtained using the following formula

$N=t2p(1-p)/M2$

N= sample size

Z=normal standard deviation =1.96

P=frequency of occurrence of an event for unknown frequency=50%

D=degree of precision=0.04%

N= (1.96)2(0.5) (0.5)/ (0.04)2=

Due to cost and time we took 71 samples

3.9. Data collection tool:

Data was collected through a questionnaire.

3.10. Data analysis and presentation:

The IBM statistical package for social sciences (SPSS statistics version 25) was presented in the form of columns. Bio Edit program used for analysis of DNA sequences (GenBank accession number U27270) used as a reference sequence.

3.11. Ethical consideration:

Ethical approval was obtained from the ethical research committee from Sudan university of science and technology, the Khartoum ministry of health and the hospital where research has been conducted.

A written informed consent was taken from each patient who participated in this study also Privacy and confidentiality as well as the right to withdraw were explained to participants.

3.12. Laboratory methods:

3.12.1. Collection and preservation of specimens:

The specimens were placed in thioglycollate broth preserved in -20 C until processing. Manual grinding of biopsies took place using disposable material (Mégraud and Lehours, 2007).

3.12.2. DNA Extraction:

The *Helicobacter pylori* DNA was extracted using the guanidine chloride extraction method. Each 2 ml tryptic soya broth (TSB) 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 (NH4) acetate were added to the pellet, vortexed, and incubated at 37°C overnight or at 65°C for 2 hr. The mixture was cooled at room temperature, then 2 ml of pre-chilled chloroform were added, vortexed, and centrifuged at 3000 rpm for 5 min. The mixture's upper layer was transferred to a new tube, 10 ml of absolute cold ethanol were added, shaken, and kept at -20°C for 2hrs. Then the tube was centrifuged at 3000 rpm for 15-20 min, the supernatant was drained carefully, 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, briefly vortexed, and kept at -20^{o}C overnight (Roosendaal et al., 1995).

3.12.3 Polymerase chain reaction Technique:

Primers targeting the 16S rRNA gene were used to detect *H. pylori*, another primer to detect the 23S rRNA gene; primers were dissolved according to manufacturer guidelines.

(Table 1) Primer sets used for amplification of 16SrRNA and 23SrRNA genes in *Helicobacter pylori* strains:

Primer name	**DNA sequence**	**Primer designation**	**Amplicon size**	**Reference**				
16SrRNA forward	(5'-GCTAAGAGATCAGCCTATGTCC-3')	*16SHp F*	532 bp	(Engstrand, Nguyen, Graham and el-Zaatari, 1992)				
16SrRNA reverse	(5'-TGGCAATCAGCGTCAGGTAATG-3')	*16sHp R*	532 bp	(Engstrand, Nguyen, Graham and el-Zaatari, 1992)				
23SrRNA forward	(5'TCGAAGGTTAAGGATGCGTCAGTC-3')	*23SHp F*	320 bp	(Abd albagi, aldayem Altayeb and Khalil Abuzeid, 2019)				
23SrRNA reverse	(5'-GAC-TCC-ATA-AGA-GCC-AAA-GCC-TTAC-3')	*23SHp R*	320 bp	(Abd albagi, aldayem Altayeb and Khalil Abuzeid, 2019)				

Reaction mixture of 16SrRNA and 23SrRNA genes:

PCR was carried out in a 20µl volume using the Maxime PCR premix kit (iNtRON Biotechnology, Seongnam, Korea) were dissolved by 9 µl of D.W ,7 µl of the PCR mix ,1 µl of forward and reverse primer, 2 µl of DNA.

Protocols used for amplification:

The protocol used for amplification of 16SrRNA:

The PCR was done using a thermocycler (Convergys, Germany) with the following conditions, at 94°C for 3 min, followed by 35 cycles at 94°C for 30 sec (denaturation), annealing at 53°C for 30 secs, and 72°C for 45 secs and a final extension at 72°C for 5 min.

The protocol used for amplification of 23SrRNA gene:

At 94°C for 2min followed by 35 cycles of Denaturation 98°C for 10 secs, annealing at 68°C for 20sec, Extension for 72°C for 2 min.

3.12.4 Visualization of PCR products:

The amplicons were separated at 50 V for 1hr in 1.5 %(w/v) agarose gel containing ethidium bromide, bands were visualized under U. V transilluminator (UVitec-UK) to detect the specifically amplified by-product by comparing with 100 bp standard ladder (iNtRON biotechnology. Korea).

3.12.5 Sequencing:

It was done by Sanger-sequencing (BGI, China).

Interpretation of results was done using BLAST (Basic local alignment search tool) and Bio Edit program used for analysis of DNA sequences (GenBank accession number U27270) used as a reference sequence.

Chapter Four
Results

CHAPTER FOUR

4. Results:

4.1 Demographic data:

A seventy-one clinical isolates were collected. according to gender distribution thirty-seven patients (52.1%) were males and thirty-four patients (47.9%) were females. Regarding clinical presentations majority of patients which were fifty-eight patients (81.7%) were gastritis cases, five patients (7%) were from gastric ulcer cases and eight patients (11.3%) were from duodenal ulcer cases. They have been divided into three age groups (1-25) years were seven patients (9.9%), from (26-50) years were thirty-six patients (50.7%) and from (51-78) years were twenty-eight patients (39.4%).

Molecular detection of *H. pylori* showed that: forty-two patients (59.2%) were positive for the 16SrRNA gene (40.8%) from which nineteen samples (26.8%) were positive for the 23SrRNA gene.

Out of seventy- one clinical isolates, prevalence of clarithromycin resistance was (7.1%) only with Two specimens only had the A2143G mutation (2.8%), Two specimens (2.8%) had the T2182C mutation of these four resistant clinical isolates only one Sample had combination of A2143G and T2182C mutations.

There was no significant association of mutations with disease, Age or gender.

Figure (1): Distribution of gender among the study population:

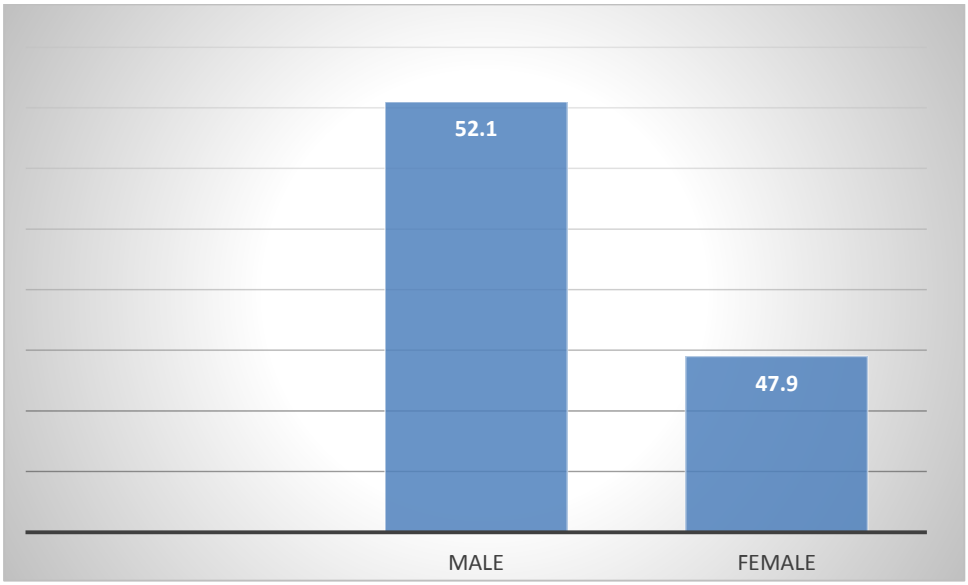


Figure (2): distribution of Clinical conditions among the study population:

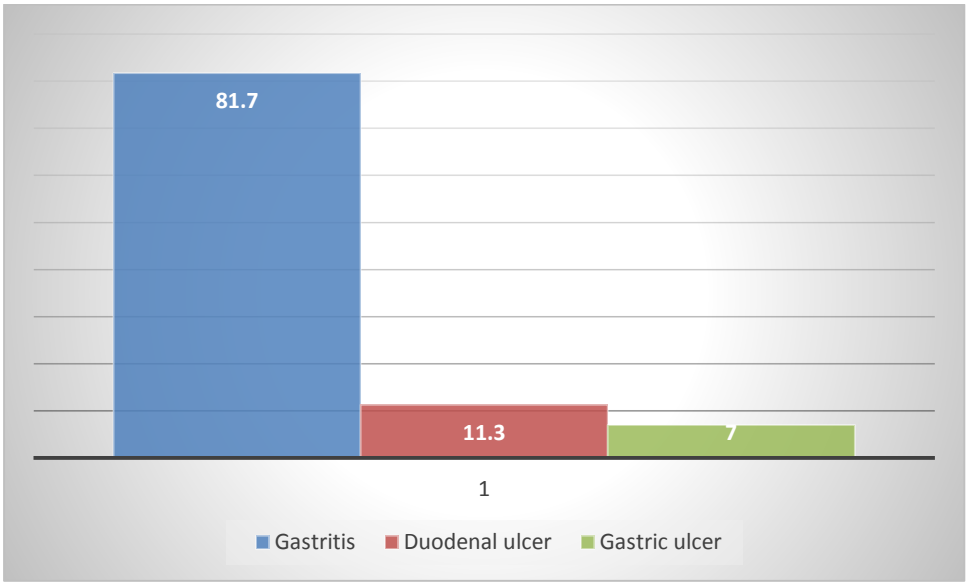


Figure (3): distribution of age groups among the study population:

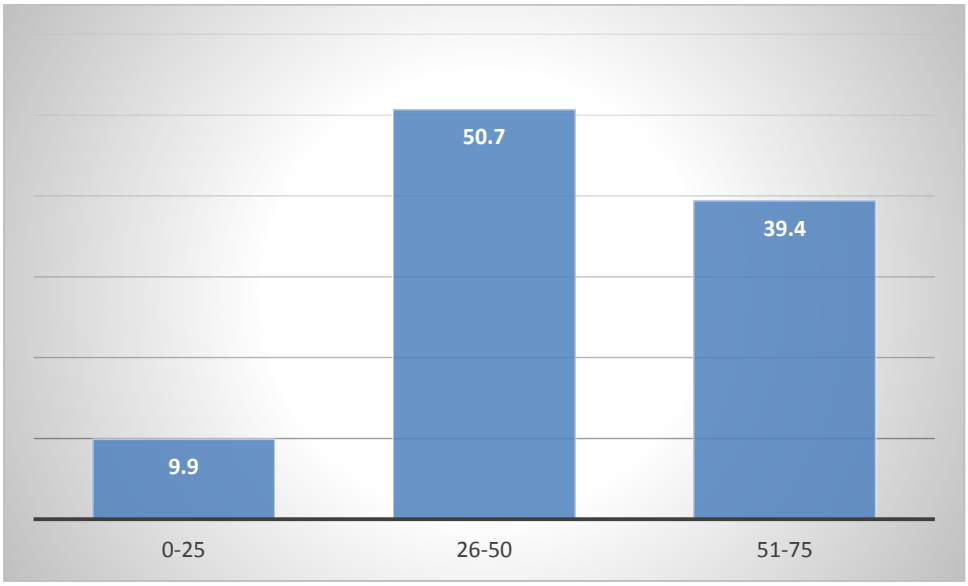


Figure (4): Distribution of *16SrRNA and 23srRNA* among study population:

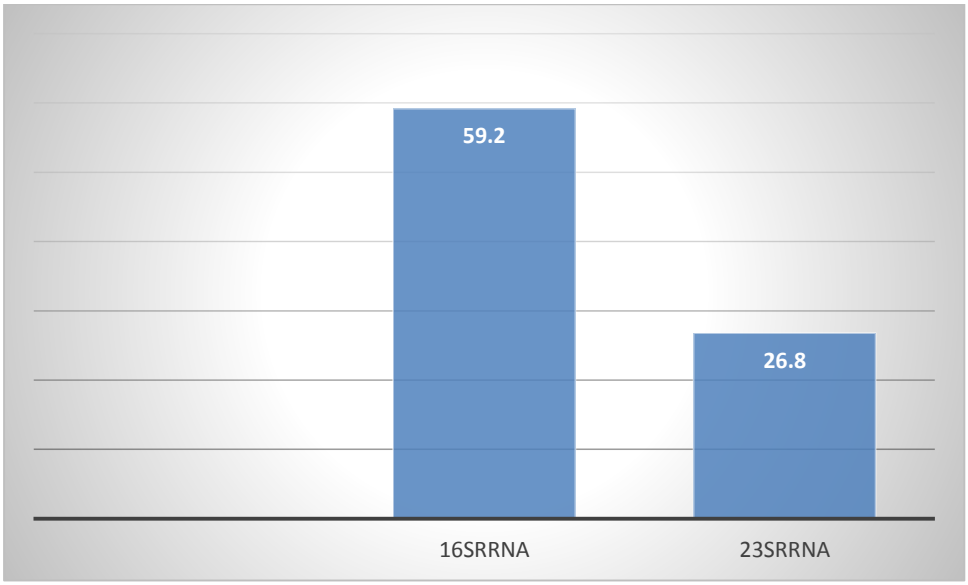


Figure (5): Distribution of genotypic clarithromycin resistant and sensitive *H. pylori* among the study population:

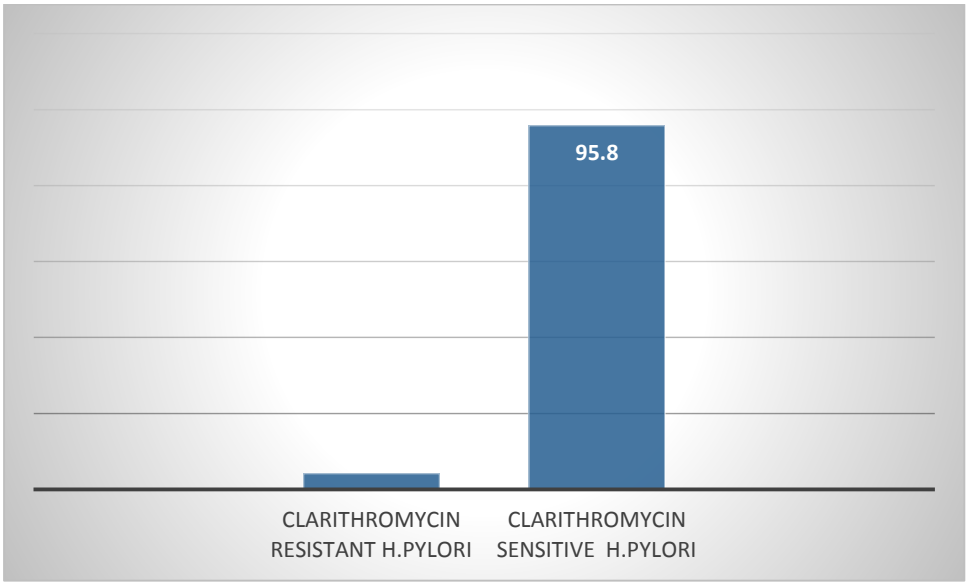
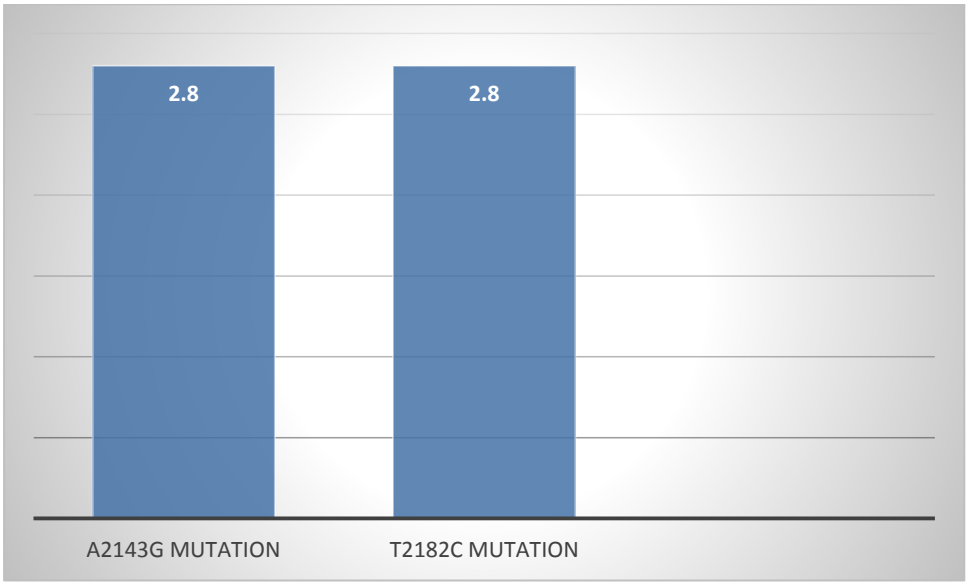


Figure (6): distribution of A2143G and T2182C mutations among the study population:



4.2 The associations between A2143G, T2182C mutations with gender, age or disease:

There was no significant association between the mutation and gender, age or disease.

Table (2): Association of the disease with A2143G and T2182C mutations:

		A2143Gmutation		Total	T2182C		Total
		Positive	Negative		Positive	Negative	
Disease	Gastritis	2(2.8%)	56(78.9%)	58(81.7%)	2(2.8%)	56(78.9%)	58(81.7%)
	Duodenal ulcer	0 (0.0%)	8(11.3%)	8(11.3%)	0(0.0%)	8(11.3%)	8(11.3%)
	Gastric ulcer	0(0.0%)	5(7%)	5(7%)	0(0.0%)	5(7%)	5(7%)
Total		2(2.8%)	69(97.2)	71(100%)	2(2.8%)	69(97.2%)	71(100%)

- P value 0.794
- Chi square test was used to calculate P value
- P value less than 0.05 considered significant

4.3 The association between A2143G, T2182C mutations with age groups and gender:

There was no significant association between mutations and disease or gender.

Table (3): The Association of Age groups and A2143G and T2182C mutations:

		A2143Gmutation		Total	T2182C	mutation	Total
		Positive	Negative		Positive	Negative	
Age groups	1-25	1(1.4%)	6(8.5%)	7(9.9%)	1(1.4%)	6(8.5%)	7(9.9%)
	26-50	1(1.4%)	35(49.3%)	36(50.7%)	1(1.4%)	35(49.3%)	36(50.7%)
	51-75	0(0.0%)	28(39.4%)	28(39.4%)	0(0.0%)	28(39.4%)	28(39.4%)
Total		2(2.8%)	69(97.2%)	71(100.0%)	2(2.8%)	69(97.2%)	71(100.0%)

- P value 0.124
- Chi-square test was used to calculate P-value
- P-value less than 0.05 considered significant

Table (4): The Association of gender with A2143G and T2182C mutations:

		A2143Gmutation		Total	T2182C	mutation	Total
		Positive	Negative		Positive	Negative	
Gender	Male	1(1.4%)	36(50.7%)	37(52.1%)	1(1.4%)	36(50.7%)	37(52.1%)
	Female	1(1.4%)	33(46.5%)	34(47.9%)	1(1.4%)	33(46.5%)	34(47.9%)
Total		2(2.8%)	69(97.2%)	71(100.0%)	2(2.8%)	69(97.2%)	71(100.0%)

- P value 0.732
- Chi-square test was used to calculate P-value
- P-value less than 0.05 considered significant

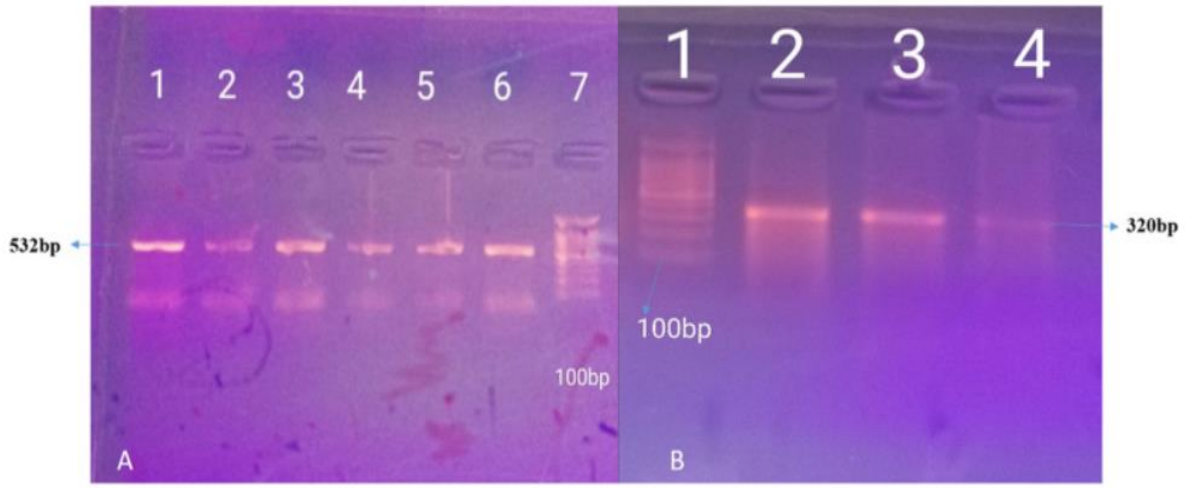


Figure (7): PCR amplification of *H. pylori* detection genes 16S RNA in addition to wild type 23SrRNA on 1.5% agarose gel electrophoresis. A: Lane 7 marker (100-1500 bp), lanes 1 to 6 contain amplicons of16 s RNA (532 bp). B. Lane 1 marker (100-1500 bp). Lanes 2, 3, and 4 include amplicons of 23 SrRNA 320 bp)

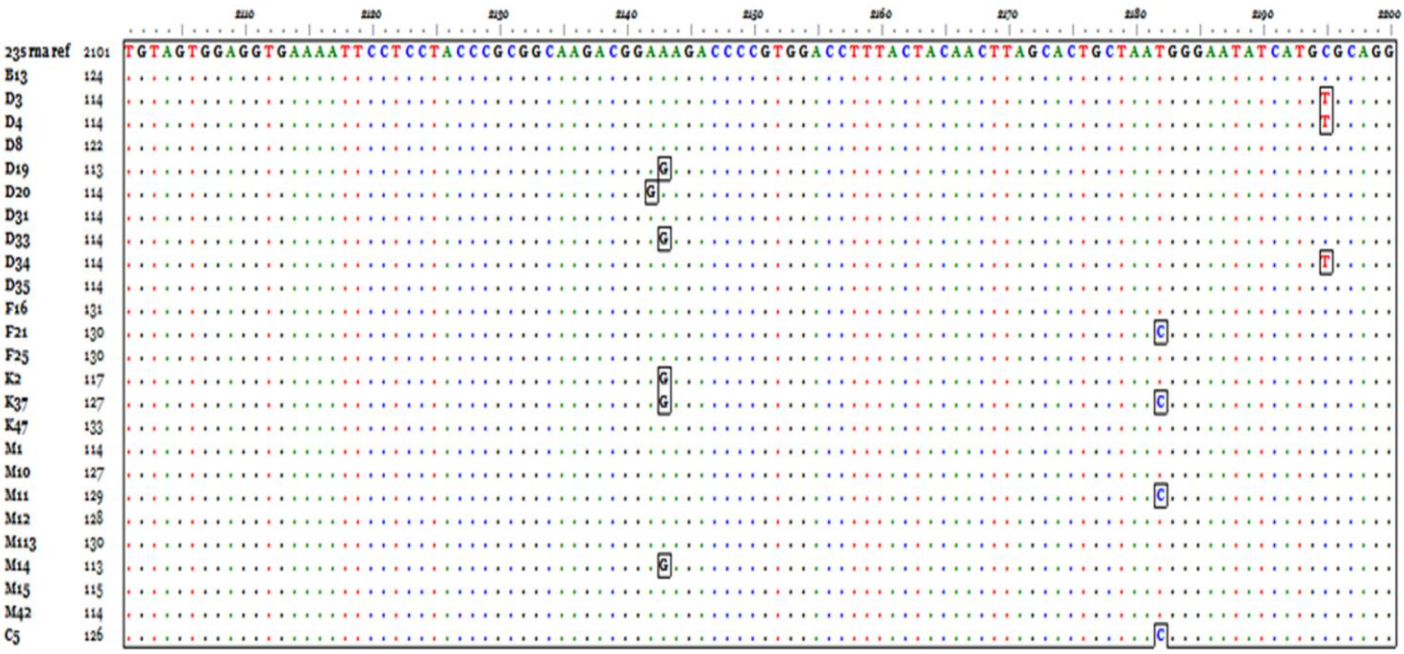


Figure (8): Multiple sequence alignment of 23S rRNA gene sequences compared to a reference gene, the mutant nucleotides appear in boxes.

Chapter Five
Discussion, Conclusion and Recommendations

CHAPTER FIVE

5. Discussion, conclusion, and recommendations

5.1 Discussion:

Globally there is increase in clarithromycin resistance mechanisms that caused by point mutations in the 23srRNA genes of *Helicobacter pylori* that leads to eradication therapy failure, especially in Africa where there is high prevalence of *Helicobacter pylori* infections and also high prevalence of clarithromycin resistance (28.2%) (Jaka et al., 2018) and scarce data that have been published concerning resistance mechanisms and whether there is necessity to implement new therapeutic approaches.

Distribution of gender in this study agree with (Idris et al., 2020) which carried out in different regions of Sudan and (Mohamed et al., 2020), and (Abdalazeem et al., 2012) however it doesn't agree with (Nyi, Soe and Htut, 2021) and (Mohmmed Elhassan Ali Noor, Mohammed Abdalla, Abd Alla and Ibrahim Hashim, 2020). regarding the distribution of the clinical conditions the present study confirmed that gastritis has the highest percentage compared to other clinical conditions this finding has been reported earlier by (Idris et al., 2020), (Nyi, Soe and Htut, 2021) and (Abdalazeem et al., 2012).

The results indicated that (59.2%) were infected by H. pylori , this observed prevalence agrees with (58%) (Abdalazeem et al., 2012) and (59.6%) (Nyi, Soe and Htut, 2021) but doesn't agree with (37.3%)(Idris et al., 2020), (23.2%) (Mohamed et al., 2020), (Mohmmed Elhassan Ali Noor, Mohammed Abdalla, Abd Alla and Ibrahim Hashim, 2020) using stool antigen detection ,(92.2%) (Jaka et al., 2019) and (Hosseini et al., 2021) It seems that the prevalence of *Helicobacter pylori* differs according to geographical location in different countries and even in the same country such difference could be explained by the various clinical differences of the patients and the diverse detection procedures

that vary in specificity and sensitivity and also various types of specimens that have been included and different clinical conditions that have been discussed .

the prevalence of mutations conferring clarithromycin resistance was (7.1%), this considered very low prevalence and it's in line with report from Mauritania (Khiddi et al., 2020) where the prevalence to clarithromycin was (5.26%) and India (6.5%) (Mahant et al., 2019) and Myanmar (6.1%)(Nyi, Soe and Htut, 2021) however its considered lower compared studies reported earlier from Sudan by (Abdalazeem et al., 2012 which was (25%) and from Tanzania (28.7%) (Jaka et al., 2019) and Korea (21.3%) (Seo et al., 2019) and Iran (57.2%) (Hosseini et al., 2021) .this could be attributed to the use of phenotypic methods in these reports combined with molecular methods to determine prevalence of resistance and also higher numbers of samples that have been included in these studies.

according to (Abdalazeem et al., 2012) clarithromycin resistance rate of more than 20% may change the treatment option to the quadruple therapy and substitution of clarithromycin with tetracycline.

In 23SrRNA genes, most of the known point mutations conferring resistance to clarithromycin are A to G transition (Hellmig *et al.*, 2003) A2143G mutation was detected in this present study (2.8%), it's also previously reported by (Abdalazeem et al., 2012) using the BbsI and BbaI restriction enzymes, and also reported in studies from India (Mahant et al., 2019) , Tanzania (Jaka et al., 2019) , (Seo et al., 2019) , Vietnam (Tran et al., 2019) , Turkey (Ergin *et al*., 2018) and (Nyi, Soe and Htut, 2021).T2182C mutation was also detected this study and reported earlier by (Ergin et al., 2018), (Quek et al., 2016)

A2143G and T2182C was detected in equal frequency and this doesn't agree with numerous studies where they reported that A2143G mutation had the highest prevalence and it's the most common mutation. (Abdalazeem et al., 2012), (Jaka et al., 2019), (Park et al., 2018), (Seo et al., 2019) and (Hosseini et al., 2021).

The presence of A2143G mutation have various prognosis; it significantly reduces the eradication rate of the *H. pylori* compared to other mutations. (Giorgio et al., 2013), (Park et al., 2018) and (Nyi, Soe and Htut, 2021)

In this study, resistance to clarithromycin from the individual's gender, age and clinical condition aspect was also examined. The results revealed that: There was no significant correlation of clarithromycin resistance due to A2143G mutation in relation with Gender, this finding correspond to report from (Eghbali et al., 2016), (Tran et al., 2018), (Nyi, Soe and Htut, 2021) and (Khashei et al., 2016)

Regarding the age there was no significant correlation of clarithromycin resistance due to A2143C mutation with age which is consistent with (Khashei et al., 2016), (Tran et al., 2018), (Eghbali et al., 2016), (Hosseini et al., 2021) and (Nyi, Soe and Htut, 2021)

Also resistance due to A2143G mutation was also examined in relation to clinical condition but as (Eghbali et al., 2016), (Hosseini et al., 2021) and (Nyi, Soe and Htut, 2021) report there was no significant association.

T2182C was the most common clinically insignificant mutation as analyzed via sequencing and it has less influence on the eradication rate according to (Seo et al., 2019).It is important to mention that T2182C association with clarithromycin resistance is still under debate and its clinical significance has not been determined (Hwang et al., 2010), (Gehlot et al., 2016) and (JM et al., 2021) ,according to (Marques et al., 2020) that T2182C mutation was found in susceptible isolate its clinical significance has not been determined.

5.2 Conclusion:

Clarithromycin resistance of *H. pylori* in this study is low, but there are mutations that can affect the eradication therapy.

5.3 Recommendations

More studies should be carried out and continuous surveillance of antimicrobial susceptibility tests to determine the exact prevalence of clarithromycin resistance

in Sudan is essential to generate data that can guide empirical treatment and design the best effective eradication therapy.

Antimicrobial Susceptibility tests by physicians can play an important role in reduction of secondary resistance to clarithromycin.

References

Abadi, A., 2017. Resistance to clarithromycin and gastroenterologist's persistence roles in nomination for *Helicobacter pylori* as high priority pathogen by World Health Organization. *World Journal of Gastroenterology*, 23(35), pp.6379-6384.

Abd albagi, S., aldayem Altayeb, H. and Khalil Abuzeid, N., 2019. Molecular detection and characterization of mutation on 23S rRNA gene associated with clarithromycin resistant in *Helicobacter pylori. bioRxiv*.

Abdalazeem, N., Abdul-Aziz, H., Adam, A., Hussein Omer, W. and Bolad, A., 2012. *Clarithromycin-Resistant Helicobacter Pylori Strains among Dyspeptic Patients in Sudan*. [online] Research gate. Available at: <https://www.researchgate.net/publication/267708921> [Accessed 19 September 2021].

Abdallah, T., Mohammed, H., Mohammed, M. and Ali, A., 2014. Sero-prevalence and factors associated with *Helicobacter pylori* infection in Eastern Sudan. *Asian Pacific Journal of Tropical Disease*, 4(2), pp.115-119.

Chang, W., Yeh, Y. and Sheu, B., 2018. The impacts of H. pylori virulence factors on the development of gastroduodenal diseases. *Journal of Biomedical Science*, [online] 25(1). Available at: <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6131906/> [Accessed 10 February 2020].

Eghbali, Z., Mojtahedi, A., Moien Ansar, M., Fakhrieh Asl, S. and Aminian, K., 2016. Detection of 23SrRNA Mutations Strongly Related to Clarithromycin Resistance in *Helicobacter pylori* Strains Isolated from Patients in the North of Iran. *Jundishapur Journal of Microbiology*, 9(2).

Engstrand, L., Nguyen, A., Graham, D. and el-Zaatari, F., 1992. Reverse transcription and polymerase chain reaction amplification of rRNA for detection of *Helicobacter species*. *Journal of Clinical Microbiology*, 30(9), pp.2295-2301.

Ergin, S., Kocazeybek, B., Caliskan, R., Demiryas, S., Dinc, O., Gülergün, R., Tokman, H., Ziver, T., Saribas, S., Yuksel, P., Kalayci, F., Demirci, M., Taşcı, İ., Erzin, Y. and Bal, K., 2018. Mutations in the 23S rRNA genes of *Helicobacter pylori* mediate resistance to clarithromycin (A preliminary study). *International Journal of Infectious Diseases*, 73, pp.127-128.

Gehlot, V., Mahant, S., Mukhopadhyay, A., Das, K., Alam, J., Ghosh, P. and Das, R., 2016. Low prevalence of clarithromycin-resistant *Helicobacter pylori* isolates with A2143G point mutation in the 23S rRNA gene in North India. *Journal of Global Antimicrobial Resistance*, 6, pp.39-43.

Gehlot, V., Mahant, S., Mukhopadhyay, A., Das, K., Alam, J., Ghosh, P. and Das, R., 2016. Low prevalence of clarithromycin-resistant *Helicobacter pylori* isolates with A2143G point mutation in the 23S rRNA gene in North India. *Journal of Global Antimicrobial Resistance*, 6, pp.39-43.

Giorgio, F., Principi, M., De Francesco, V., Zullo, A., Losurdo, G., Di Leo, A. and Ierardi, E., 2013. Primary clarithromycin resistance to *Helicobacter pylori*: Is this the main reason for triple therapy failure? *World Journal of Gastrointestinal Pathophysiology*, 4(3), p.43.

Giorgio, F., Principi, M., De Francesco, V., Zullo, A., Losurdo, G., Di Leo, A. and Ierardi, E., 2013. Primary clarithromycin resistance to *Helicobacter pylori:* Is this the main reason for triple therapy failure? *World Journal of Gastrointestinal Pathophysiology*, 4(3), p.43.

Hamid Abdollahi, M., 2021. *Detection of A2142C, A2142G, and A2143G Mutations in 23s rRNA Gene Conferring Resistance to Clarithromycin among Helicobacter pylori Isolates in Kerman, Iran*. [online] PubMed Central (PMC). Available at: <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3556758/> [Accessed 16 August 2021].

Hooi, J., Lai, W., Ng, W., Suen, M., Underwood, F., Tanyingoh, D., Malfertheiner, P., Graham, D., Wong, V., Wu, J., Chan, F., Sung, J., Kaplan, G. and Ng, S., 2017. Global Prevalence of *Helicobacter pylori* Infection: Systematic Review and Meta-Analysis. *Gastroenterology*, 153(2), pp.420-429.

Hosseini, R., Rahimian, G., Shafigh, M., Validi, M., Khaledi, M. and Gholipour, A., 2021. Correlation Between Clarithromycin Resistance, Virulence Factors and Clinical Characteristics of the Disease in Helicobacter pylori Infected Patients in Shahrekord, Southwest Iran". *Research square*.

Hwang, T., Kim, N., Kim, H., Lee, B., Nam, R., Park, J., Lee, M., Park, Y., Lee, D., Jung, H. and Song, I., 2010. Change in Antibiotic Resistance of *Helicobacter pylori* Strains and the Effect of A2143G Point Mutation of 23S rRNA on the Eradication of H. pylori in a Single Center of Korea. *Journal of Clinical Gastroenterology*, 44(8), pp.536-543.

Idris, A., Hassan, H., Ali, M., Eltaher, S., Idris, L., Altayb, H., Abass, A., Ibrahim, M., Ibrahim, E. and Hassan, M., 2020. Molecular Phylogenetic Analysis of 16S rRNA Sequences Identified Two Lineages *of Helicobacter pylori* Strains Detected from Different Regions in Sudan Suggestive of Differential Evolution. *International Journal of Microbiology*, 2020, pp.1-12.

Jaka, H., Rhee, J., Östlundh, L., Smart, L., Peck, R., Mueller, A., Kasang, C. and Mshana, S., 2018. The magnitude of antibiotic resistance to *Helicobacter pylori* in Africa and identified mutations which confer resistance to antibiotics: systematic review and meta-analysis. *BMC Infectious Diseases*, 18(1).

Jaka, H., Rüttgerodt, N., Bohne, W., Mueller, A., Gross, U., Kasang, C. and Mshana, S., 2019. *Helicobacter pylori* Mutations Conferring Resistance to Fluoroquinolones and Clarithromycin among Dyspeptic Patients Attending a Tertiary Hospital, Tanzania. *Canadian Journal of Gastroenterology and Hepatology*, 2019, pp.1-7.

JM, K., JS, K., N, K., YJ, K., IY, K., YJ, C., CH, L. and HC, J., 2021. *Gene mutations of 23S rRNA associated with clarithromycin resistance in Helicobacter pylori strains isolated from Korean patients*. [online] PubMed. Available at: <https://pubmed.ncbi.nlm.nih.gov/18852516/> [Accessed 23 September 2021].

Khashei, R., Dara, M., Bazargani, A., Bagheri Lankarani, K., Taghavi, A., Moeini, M., Dehghani, B. and Sohrabi, M., 2016. High rate of A2142G point mutation associated with clarithromycin resistance among Iranian *Helicobacter pylori* clinical isolates. *APMIS*, 124(9), pp.787-793.

Khiddi, F., Abdellahi, M., Horma, M., Billoet, A., Collobert, G., Amar, A., Nech, H., Vadel, E., Houmeida, A., Raymond, J., Dauga, C. and Gastli, N., 2020. Characteristics of Helicobacter pylori strains isolated from Mauritanian patients. *Helicobacter*, 25(5).

Kusters, J., van Vliet, A. and Kuipers, E., 2006. Pathogenesis of *Helicobacter pylori* Infection. *Clinical Microbiology Reviews*, 19(3), pp.449-490.

Leena Myran, W. and Zarbock, S., 2021. *Management of Helicobacter pylori Infection*. [online] Uspharmacist.com. Available at: <https://www.uspharmacist.com/article/management-of-helicobacter-pylori-infection> [Accessed 16 August 2021].

Mahant, S., Sharma, A., Gehlot, V., Mukhopadhyay, A., Chhawchharia, A., Dutta, S., Agarwal, A., Som, A., Das, K. and Das, R., 2019. Geographically Distinct North-East Indian Helicobacter pylori Strains are Highly Sensitive to Clarithromycin but are Levofloxacin Resistant. *Indian Journal of Medical Microbiology*, 37(3), pp.337-344.

Marques, A., Vítor, J., Santos, A., Oleastro, M. and Vale, F., 2020. Trends in *Helicobacter pylori* resistance to clarithromycin: from phenotypic to genomic approaches. *Microbial Genomics*, 6(3).

Mégraud, F. and Lehours, P., 2007. Helicobacter pylori Detection and Antimicrobial Susceptibility Testing. *Clinical Microbiology Reviews*, 20(2), pp.280-322.

Mohamed, A., Elhassan, N., Awhag, Z., Ali, F., Ali, E., Mhmoud, N., Siddig, E., Hassan, R., Ahmed, E., Fattahi, A., Ahmed, A., Muneer, M., Osman, H., Mohamed, N. and Edris, A., 2020. Prevalence of *Helicobacter pylori* among Sudanese patients diagnosed with colon polyps and colon cancer using immunohistochemistry technique. *BMC Research Notes*, 13(1).

Mohmmed Elhassan Ali Noor, R., Mohammed Abdalla, W., Abd Alla, A. and Ibrahim Hashim, A., 2020. Diagnosis of *Helicobacter pylori* infection using immunochromatography among patients attending Tamboul Hospital in Gezira State, Sudan: a cross-sectional study. *F1000Research*, 9, p.1054.

Ngnameko, C., Noubiap, J., Simo Nemg, F., Galdima, M., Takuissu-Nguemto, G., Ngo-Boumso, S., Njayou, F., Moundipa, P. and Bigna, J., 2021. Epidemiology and antibiotic resistance profile of *Helicobacter pylori* infection in Cameroon: a systematic review with meta-analysis.

Nyi, K., Soe, A. and Htut, Z., 2021. Molecular detection of genotypic clarithromycin- resistant strains and its effect on the eradication rate of concomitant therapy in Helicobacter pylori infection. *GastroHep*.

Park, C., Kim, S., Lee, E., Jeon, H. and Han, S., 2018. Clinical relevance of point mutations in the 23S rRNA gene in Helicobacter pylori eradication. *Medicine*, 97(33), p. e11835.

Quek, C., Pham, S., Tran, K., Pham, B., Huynh, L., Luu, N., Le, T., Quek, K. and Pham, V., 2016. Antimicrobial susceptibility and clarithromycin resistance

patterns of *Helicobacter pylori* clinical isolates in Vietnam. *F1000Research*, 5, p.671.

Seo, S., Do, B., Kang, J., Kim, H., Jang, M., Kim, H. and Shin, W., 2019. *Helicobacter pylori* Eradication According to Sequencing-Based 23S Ribosomal RNA Point Mutation Associated with Clarithromycin Resistance. *Journal of Clinical Medicine*, 9(1), p.54.

Testerman, T. and Morris, J., 2014. Beyond the stomach: An updated view of *Helicobacter pylori* pathogenesis, diagnosis, and treatment. *World Journal of Gastroenterology*, 20(36), p.12781.

Tran, V., Ha, T., Le, P., Nguyen, V., Phan, T. and Paglietti, B., 2018. *Helicobacter pylori* 23S rRNA gene mutations associated with clarithromycin resistance in chronic gastritis in Vietnam. *The Journal of Infection in Developing Countries*, 12(07), pp.526-532.

Versalovic, J., Shortridge, D., Kibler, K., Griffy, M., Beyer, J., Flamm, R., Tanaka, S., Graham, D. and Go, M., 1996. Mutations in 23S rRNA are associated with clarithromycin resistance in *Helicobacter pylori*. *Antimicrobial Agents and Chemotherapy*, 40(2), pp.477-480.

Appendices

Appendices

Questionnaire:

Specimens No:( )

1-Age: …….

2-Residence…….

3-Origin……

4-Do you have any of these symptoms:

nausea and vomiting that may include vomiting blood ……...

passing dark or tarry-like stools ……

Fatigue …….

low red blood cell count (anemia)……...

decreased appetite……

diarrhea……

5-Have had been diagnosed with H. pylori?

If answer is YES, which test have been used to confirm diagnosis:

Final diagnosis…….

Clinical remarks…….

Conergys Thermocycler