ACIBADEM MEHMET ALI AYDINLAR UNIVERSITY
INSTITUTE OF HEALTH SCIENCES

INVESTIGATION OF MOLECULAR MECHANISM OF MACROLIDE RESISTANCE IN LABORATORY MUTANT *HELICOBACTER PYLORI*

MELTEM AYAŞ

PH.D. THESIS

DEPARTMENT OF MEDICAL BIOTECHNOLOGY

SUPERVISOR
Prof. Dr. Yeşim GÜROL

ISTANBUL-2023

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Department:	Medical Biotechnology
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Thesis Title:	Investigation of Molecular Mechanism of Macrolide Resistance in Laboratory Mutant *Helicobacter pylori*
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Date of Defence:	20/01/2023

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DECLARATION

I declare that this thesis work is my own work, I had no unethical behaviour at any stages from the planning to the writing of the thesis, I obtained all the information in this thesis in accordance with academic and ethical rules, I cited all the information and comments that were not obtained with this thesis work, and I provided resources in the list of references. I also declare that there was no violation of any patents and copyrights during the study and writing of this thesis.

17.02.2023
Meltem AYAŞ

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LIST OF ABBREVIATIONS

A	Adenine
BABA	Blood-antigen binding protein A
BP	Base pair
C	Cytosine
CAGA	Cytotoxin-associated gene A
CLSI	Clinical and Laboratory Standards Institute
DMSO	Dimethyl sulfoxide
DUPA	Duodenal ulcer promoting gene A
EIA	Enzyme immunoassays
EUCAST	European Committee on Antimicrobial Susceptibility Testing
G	Guanine
ICEA	Induced by contact with epithelium gene A
MALT	Mucosa-associated lymphoid tissue
MIC	Minimum inhibitory concentration
µG	Microgram
µL	Microliter
µM	Micrometer
NAP	Neutrophil-activating protein
NGS	Next generation sequencing
OIPA	Outer Inflammatory Protein
OMP	Outer membrane protein
PCR	Polymerase chain reaction
pH	Power of Hydrogen
RT-PCR	Real time polymerase chain reaction
rRNA	Ribosomal ribonucleic acid
SABA	Sialic Acid-Binding Adhesin
SAT	Stool Antigen Test

T	Thymine
UBT	Urea breath test
UREB	Urease B
UREA	Urease A
VACA	Vacuolating cytotoxin A
WGS	Whole genome sequencing

LIST OF FIGURES

Figure 1. Examination of mucoid-coccoid *Helicobacter pylori* (A, AI, AII) and non-mucoid *Helicobacter pylori* (B, BI, BII) on Brucella Blood Agar using by Stereomicroscopy and light microscopy (43). Figure reproduced here under the terms of the Creative Commons Attribution License. 9
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ÖZET

Laboratuvar Mutantı *Helicobacter pylori* deki Makrolid Direncinin Moleküler Mekanizmasının Araştırılması

Helicobacter pylori (*H. pylori*) dünya nüfusunun yarısından fazlasını enfekte eden, gastrit, peptik ülser, mide mukozası ile ilişkili lenfoid doku (MALT) lenfoması ve mide kanseri gibi hastalıkların gelişmesine neden olan bir bakteridir. *H. pylori* enfeksiyonunun birinci basamak tedavisinde kullanılan makrolid grubu bir antibiyotik olan klaritromisine karşı oluşan direnç tedavi başarısızlığının en önemli nedenidir. *H. pylori*' de klaritromisin direnci çoğunlukla 23S rRNA' nın V domaininde peptidil transferazı kodlayan bölgesindeki nokta mutasyonları ile ilişkilendirilse de bu bölge dışındaki mutasyonların direnç ile ilişkisi güncel araştırmalardandır. Bu tez çalışmasında, dirençli suşlarda yeni nesil dizileme teknolojisi (NGS) kullanılarak olası yeni makrolid direnç mekanizmalarının araştırılması hedeflenmiştir. Ayrıca dirençli olgularda klaritromisine alternatif olabilecek josamisinin *H. pylori* üzerindeki etkinliğinin araştırılması da çalışmanın hedeflerindendir. *H. pylori* G27 suşundan, agar dilüsyon metodu ile, Minimum inhibitör konsantrasyonu (MİK) değerleri sırasıyla 128 µg/ml ve >256 µg/ml olan iki adet dirençli mutant suş elde edilmiştir. Klaritromisin direncinden sorumlu olabilecek aday mutasyonlar (A2143G, T1495A, T1494A, T1490A, T1476A, G1472T) mutant 1 suşunda 23S rRNA bölgesinde gösterilmiştir. Ayrıca dirençli suşta ilk kez saptanan Sulfite exporter TauE/SafE ailesi proteinini kodlayan gendeki mutasyonun klaritromisin direnci veya çapraz direnç ile ilişkili olduğu düşünülerek ileri çalışmalar için hedef olarak gösterilmiştir. Ayrıca makrolid dirençli *H. pylori* şuşlarında josamisine çapraz direnç ilk kez bu tez çalışması ile gösterilmiştir.

Anahtar Sözcükler: *H. pylori,* Makrolid direnci, Klaritromisin, Josamisin, Yeni nesil dizileme

ABSTRACT

Investigation of Molecular Mechanism of Macrolide Resistance in Laboratory Mutant *Helicobacter Pylori*

Helicobacter pylori (*H. pylori*) is a bacterium that infects more than half of the world, causing diseases such as gastritis, peptic ulcer, gastric mucosa-associated lymphoid tissue (MALT) lymphoma, and gastric cancer. Resistance to clarithromycin, a macrolide antibiotic used in the first-line treatment of *H. pylori* infection, is the most important cause of the treatment failure. Although most of the clarithromycin resistance in *H. pylori* is associated with point mutations in the peptidyl transferase-encoding region of the 23S rRNA, the relation between other mutations outside of the region and resistance is under investigation. In this thesis study, it was aimed to investigate possible new macrolide resistance mechanisms in resistant strains using new-generation sequencing technology (NGS). Investigation of the effectiveness of josamycin which may be an alternative to clarithromycin in resistant cases is also one of the study's aims. Two resistant mutants which minimum inhibitory concentration (MIC) values were 128 μg/ml and >256 μg/ml, respectively, were isolated from *H. pylori* G27 by agar dilution method. The candidate mutations (A2143G, T1495A, T1494A, T1490A, T1476A, G1472T) that may be responsible for clarithromycin resistance were demonstrated in 23S rRNA region of mutant-1 strain. In addition, the mutation in the gene encoding the Sulfite exporter TauE/SafE family protein, which was detected for the first time in the resistant strain, was considered as associated with clarithromycin resistance or cross-resistance and showed as a target for further studies. In addition, cross-resistance to josamycin in macrolide-resistant *H. pylori* strains was demonstrated for the first time in this thesis study.

Key words: *H. pylori,* Macrolide resistance, Clarithromycin, Josamycin, New generation sequencing

1 INTRODUCTION AND AIM

Approximately half of the world's population is colonized by *Helicobacter pylori* (*H. pylori*), a Gram-negative, spiral-shaped bacterium that can survive in an acidic environment in the stomach. The International Agency for Research on Cancer (IARC) has identified *H. pylori* as a Group 1 carcinogen since causing gastric cancer (1). Further, it has been reported that eradicating *H. pylori* infection is an effective strategy for treating peptic ulceration and lymphoid tissue lymphoma of the gastric mucosa and preventing gastric cancer (2).

It was reported that developing countries have a higher prevalence of *H. pylori* than developed countries. (3,4). A prevalence of approximately 82.5% is observed in our country for *H. pylori.* (5). The standard triple therapies comprising a proton pump inhibitor, clarithromycin, and amoxicillin or metronidazole have been the first-line treatment for *H. pylori* infection. In regions with a low clarithromycin resistance rate (<15%), triple standard therapy has become the most prevalent approach to treating *H. pylori* infections. First-line therapy is now preferred to use bismuth-containing therapies for areas with >15% clarithromycin resistance and second-line therapy for the treatment of *H. pylori* when the classical triple therapy has not been successful (6,7).

Clarithromycin, a macrolide antibiotic, is the most potent bacteriostatic antibiotic used to treat *H. pylori* infection. Since many international guidelines recommend clarithromycin-containing bismuth quadruple therapies as treatment for *H. pylori*, clarithromycin maintains its importance in treating the *H. pylori* infection (8-10). In recent years, clarithromycin resistance has increased throughout the world. The resistance rate in Europe increased from 9% to 17,6% between 1998 - 2008 years, and in Japan, it increased from 7% to 27,7% between 2000-2006 (11). It has been proposed that Turkey's clarithromycin resistance rates have reached approximately 40% (12). World Health Organization (WHO) has recently published a list of bacteria that require new antibiotics urgently, and clarithromycin-resistant *H. pylori* was categorized on the high-priority list (13).

Other macrolides such as josamycin have been considered as alternatives in the case of possible resistance to clarithromycin. Josamycin is an inexpensive alternative to clarithromycin but has not been tested for effectiveness in resistant cases (14, 15). A study showed that josamycin has an eradication rate of 85,6% and josamycin and bismuth have more than 90% eradication rate (16). Josamycin is not present in our country and, therefore, has not been used in the treatment regimen of *H. pylori.*

Inhibiting protein synthesis is the main mechanism of action of clarithromycin, which binds to receptors located in the 23S ribosomal subunit of the 50S ribosome. It has been shown that point mutations in the peptidyl transferase encoded in domain V of 23S rRNA are responsible for clarithromycin resistance (17). Several point mutations in the 23S rRNA gene have been reported to decrease ribosome affinity for the drug, causing the bacteria to become resistant. The most common mutations found in clinical isolates are A2142G, A2142C, and A2143G. Except for these, other point mutations in the 23S rRNA region are also shown in different studies (18,19). Another mechanism of resistance to clarithromycin has been reported in five efflux pump systems. The current findings suggest that four groups of genes (HP0605–HP0607, HP0971–HP0969, HP1327–HP1329, and HP1489–HP1487) have the potential to function as efflux pumps in *H. pylori* (20-22).

Despite the major molecular mechanisms of clarithromycin resistance that have been distinctly shown in various studies, it is still unclear whether other gene mutations outside of the 23S rRNA and efflux pump gene clusters are associated with resistance in *H. pylori.*

To the best of our knowledge, there are limited studies about josamycin activities on *H. pylori*. The primary goal of our study is to characterize the mechanisms of macrolide resistance by obtaining the laboratory mutants of the *H. pylori* G27 strain. Using the reference G27 strain and its laboratory-derived macrolide-resistant mutants in the project allows the changes to evaluate all putative resistance mechanisms that can come up in the higher MICs of the clarithromycin and josamycin by sequencing methods.

1.1. Aim of the Study

The presented thesis study aimed to find possible new clarithromycin resistance mechanisms in *H. pylori*. Since there is a need for more information about the clarithromycin resistance mechanism in *H. pylori*, this study is essential in contributing to the literature. It is predicted that the candidate mutations found also contribute to developing molecular diagnostic tests by regarding the new resistance mechanisms as the target regions. Moreover, the second objective of this thesis was to investigate the presence of possible cross-resistance to josamycin in mutant strains to suggest josamycin as an alternative to clarithromycin in *H. pylori* treatment. To the best of our knowledge, this study is the first that evaluates the in vitro activity of josamycin on clarithromycin resistance strains.

2 BACKGROUND

2.1. History of *Helicobacter pylori*

In the nineteenth century, the discovery of *Helicobacter pylori* (*H. pylori*) started with researchers observing the presence of spiral-shaped microorganisms in animal stomachs. Subsequently, the same spiral bacteria have also been detected in the guts of people with peptic ulcers and gastric cancer (23-24). In 1889, a Polish researcher, W. Jaworski, was the first to suggest the possible pathogenic role of this bacteria in gastric diseases for humans. Initially, the first culture was isolated from a gastric biopsy belonging to a patient with a duodenal ulcer. It was referred to as a Campylobacter-Like Organism (CLO), assuming it was a Campylobacter species (25).

It was subsequently named *Campylobacter pyloridis* and then changed to *Campylobacter pylori,* but ribonucleic acid sequencing studies determined that the organism did not belong to the genus Campylobacter. Despite considering there was a relationship to the genus Wolinella, phenotypic differences weigh against that. Recently, the bacteria were renamed *Helicobacter pylori,* standing for the species of a suggested new genus Helicobacter (26).

Warren and Marshall carried out the first isolation of H. pylori in 1982. Consideringly Koch's postulates, to show his colleagues that there is a relationship between bacteria and gastritis, Marshall drank *H. pylori's* culture, developed gastritis, and reisolated the bacteria from the lining of his stomach. Afterward, Warren and Marshall showed that antibiotics effectively treat *H. pylori* infection (27). The discovery of Robin Warren and Barry Marshall was considered worthy of the Nobel prize in the field of physiology or medicine for "the discovery of *H. pylori* and its role in gastritis and peptic ulcer disease" in 2005. Based on this knowledge, it was demonstrated that gastric colonization of *H. pylori* could cause various gastrointestinal disorders, from chronic gastritis to gastric cancer (GC) (28). *H. pylori* has been defined as a Class I carcinogen by the "International Agency for Cancer Research (IACR)" (1).

2.2. Microbiology of *Helicobacter pylori*

2.2.1. Morphology

H. pylori is a Gram-negative bacterium that measures 2 to 4 mm in length and 0,5 to 1 micron in width. Although it is commonly spiral-shaped, it may appear as a rod, while coccoid shapes may be observed after prolonged in vitro culture or antibiotic treatment. In vitro culture of coccoid forms is impossible, and they are considered dead cells (29). Some studies indicated that 5-20 days cultured coccoid form of *H. pylori* could not be re-cultured in vitro (30). On the other hand, this bacterium is thought to be alive by having a membrane and polyphosphate energy sources. Simultaneously, it is considered that this coccoid form act as responsible for the transmission of the infection and the retake of antimicrobial therapy (23, 31).

H. pylori has 2 to 6 unipolar, sheathed flagella approximately 3 mm in length and frequently carries a characteristic bulb at the end. The bacterium has rapid movement in the mucus layer superjacent to the gastric epithelial cells thanks to the flagella (32).

2.2.2. The genome of *Helicobacter pylori*

The genome structure of *H. pylori* was first determined in strain 26695 and J99 by sequencing. The size of the *H. pylori* genomes is approximately 1.7 M bp, which contains 35 to 40% of GC. While the genome of strain 26695 includes 1,587 genes, the J99 strain includes only 1,491 genes (33). On the other hand, it was shown that 16S, 23S, and 5S rRNA genes are present in two copies in both genomes. Furthermore, the presence of one or more plasmids that do not carry antibiotic resistance and virulence genes has been reported in many strains (34).

2.2.3. Growth conditions

H. pylori is a microaerophilic organism that can grow at certain O2 levels (2 to 5%) and needs of 5 to 10% CO2 and high humidity. Microaerobic conditions suggested

for *H. pylori* culture contain 85% N2, 10% CO2, and 5% O2. While the optimum temperature for growth is 37°C, growth can also occur at 34-40°C. Although it is found on acidic gastric mucosa and can survive at pHs under 4, the optimum growth temperature occurs only at neutral pHs (35, 36).

Due to the fastidious nature of *H. pylori*, it is often necessary to supplement the growth media with blood or serum (37). These supplements may act as extra nutrients for the bacterium and protect it from fatty acid toxicity. Especially using supplements such as b-cyclodextrins in the medium may protect from the toxic effects (38). Usually, the routine isolation and culture of bacteria are performed using Columbia or Brucella agar that includes horse or sheep blood supplements. To prevent possible contamination, particular antibiotic mixtures such as Dent supplement (vancomycin, trimethoprim, cefsoludin, and amphotericin B) or Skirrow supplement (vancomycin, trimethoprim, polymyxin B, and amphotericin B) are used for primary isolation and routine culture (38, 39). *H. pylori* can also be grown in liquid media such as Brain Heart Infusion or Brucella broth which is supplemented with 10% heat-inactivated fetal bovine serum (FBS) (40). For liquid culture, it is also suggested to add calf serum or b-cyclodextrins to the broth medium within 2 to 10% and 0,2 to 1% rates, respectively (41).

Isolation of *H. pylori* from gastric biopsy samples is complex and needs to be consistently successful. Cultures should be exanimated from day 3 to day 14. After incubation, small, translucent, smooth colonies are identified as *H. pylori* (42). Subsequently, good growth can occur at 1 - 3 days of incubation when passages of *H. pylori* are used (40). It was shown that the *H. pylori* colonies could be seen differently depending on the coccoid form and bacilli form (Figure 1) (43).

It is possible to store *H. pylori* at -80°C in a broth medium such as brain heart infusion or brucella added 15 to 20% glycerol or 10% dimethyl sulfoxide. To ensure optimal viability, new cultures that contain spiral-shaped cells should be used (28).

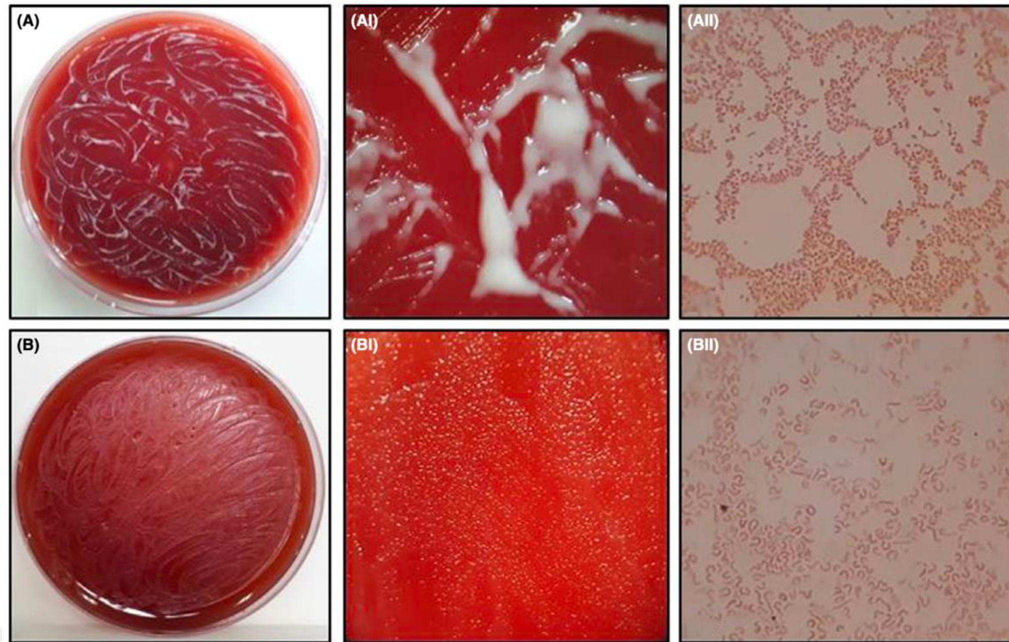

Figure 1. Examination of mucoid-coccoid *Helicobacter pylori* (A, AI, AII) and non-mucoid *Helicobacter pylori* (B, BI, BII) on Brucella Blood Agar using by Stereomicroscopy and light microscopy (43). Figure reproduced here under the terms of the Creative Commons Attribution License.

2.3. Epidemiology of *Helicobacter pylori*

H. pylori has been demonstrated worldwide in all ages of individuals. Studies including genetic analysis propose that since humans first migrated from Africa around 58000 years ago, they have been infected with *H. pylori*. It is approximated that more than half of the world population is infected with *H. pylori*, with a notable variation in the prevalence among countries and within different areas of the same country (44-47).

Several factors such as socioeconomic status and environmental conditions have been associated with the risk of getting *H. pylori* infection at early ages (48-51). Therefore, even though the incidence of *H. pylori* infection is decreasing in industrialized countries, it remains stable in developing countries (52).

H. pylori is acquired early in childhood. While the prevalence of *H. pylori* infection is raised in the first five years of life in developing countries, the decline of

the infection via improved hygiene and sanitation is reported. On the other hand, the acquisition of infection is quite low in adulthood (52-54).

According to current estimates, approximately 4.4 billion individuals worldwide are infected with *H. pylori*. In European countries, the prevalence of *H. pylori* has decreased since 2000. The prevalence, however, remains unchanged in Asia. According to reports, Africa had the highest prevalence (70.1%), while Switzerland had the lowest (18.9%). Among the countries in Southern Asia, Pakistan and India have the highest prevalence of *H. pylori* (81% and 63.5%, respectively). As far as Western Asia is concerned, Turkey represented the highest prevalence (77.2%), (55).

2.3.1. Transmission of *Helicobacter pylori*

The presence of *H. pylori* in saliva, puke, and stool has been observed, but no evidence has been presented for using one of these products as a predominant mode of transmission. Person-to-person transmission through either gastro-oral, oral-oral, or fecal-oral exposure seems quite likely. Since *H. pylori* has a limited host range, new infections are arisen because of person-to-person transmission or environmental contamination. It is unclear which of the mentioned transmission routes is frequently seen (56-59). Hereditary susceptibility to *H. pylori* infection is also considered, but this has not yet been proven (60).

2.4. Pathogenesis of *Helicobacter pylori*

Helicobacter pylori can chronically colonize the stomach and infect almost half of the world's population. Pathogenesis of *H. pylori* infection may cause the development of chronic gastrointestinal diseases due to colonization (61). Bacterial virulence, host genetics, immune response, and environmental factors are reported as related to pathogenesis that emerges with colonization (62).

Once *H. pylori* has entered the stomach at the beginning of the infection, it uses its urease activity to neutralize the acidic environment (63). It was reported that

motility is needed for the initial colonization of the stomach mucosa (64). As soon as *H. pylori* enters the stomach, it uses its flagella-mediated motility to move toward the gastric epithelium cells (63). Following, interactions between bacterial adhesins and host cell receptors cause successful colonization and lasting infection (65). Eventually, *H. pylori* causing host tissue damage by secreting specific effector proteins/toxins that include cytotoxin- associated gene A (CagA), and vacuolating cytotoxin A (VacA). Briefly, four steps are critical for *H. pylori* colonization and pathogenesis: Survival under acidic conditions in the stomach; movement towards epithelium cells thanks to flagella motility; attaching to host receptors with adhesins; the incurrence of tissue damage via toxin secrete (Figure 2), (63).

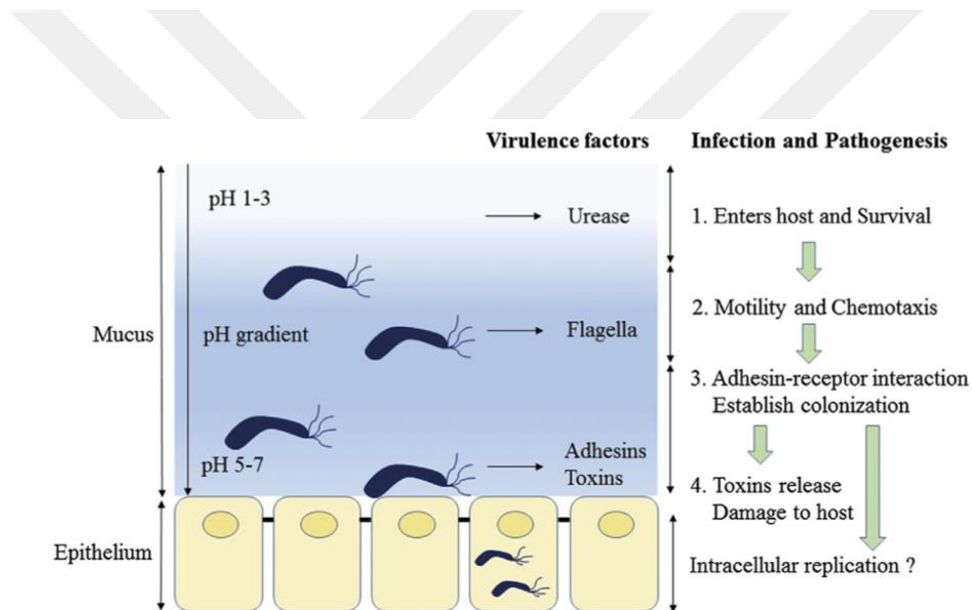

Figure 2. The Schematic diagram of *H. pylori* infection and pathogenesis. Reproduced from (63) under the terms of the Creative Commons CC-BY license, which permits unrestricted use.

2.5. Virulence Factors of *Helicobacter pylori*

A bacterium's ability to lead and develop a disease is defined as a virulence factor. In patients with severe clinical indications, strains possessing these virulence factors are frequently isolated (66). *H. pylori*-related infections are associated with many virulence factors (67).

2.5.1. Urease

Urease is one of the most important virulence factors for *H. pylori* and is crucial for bacterial metabolism and colonization of the gastric mucosa (68). There is a urease gene cluster that includes the urea A/B, ureI, and ure E-H that encodes to produce the urease enzyme in *H. pylori*. Urease neutralizes the stomach's acidic environment by hydrolyzing urea into NH3 and CO2 (69, 70). It was proved that the urease is responsible for *H. pylori* colonization by showing that bacteria mutants not possessing urease did not colonize the gastric environment (71).

The bacterial urease is classified as internal or external, depending on it is location, whether in the cytoplasmic compartment or on the surface of the bacteria (68). In the initial phase of cell lysis, external urease is produced and functions most effectively at 5,0- 8,5 pH levels, whereas internal urease is active at 2,5 and 6,5 pH levels (72).

Urease converts urea into ammonia and carbamate, increasing gastric pH by decomposing into ammonia and carbonic acid (67). It was demonstrated that there was higher urease activity in the spiral form than in the coccoid form of the bacteria (73). The possible relation between high urease activity and gastric carcinogenesis was reported. The modulation of the urease activity is probably performed by the flbA gene that takes a role in flagellar biosynthesis (74). It is considered that the inhibition of urease is a strategy for treatment by preventing *H. pylori*-associated diseases (75).

2.5.2. Flagella

The presence of flagella in *H. pylori* is a necessity for colonization. It was demonstrated that strains not posing flagella cannot colonize gnotobiotic piglets (76). There are two-six polar flagella described by two types of proteins encoded by flaA and flaB that are essential for motility and permanent infection (77). The number of flagella belonging to the *H. pylori* strain was related to the speed of the bacteria movement and showed variety among the species (78). Besides the colonization, it was

also demonstrated that flagella act in biofilm formation (79). Furthermore, it was reported that *H. pylori* strain with high motility could lead to high releasing IL-8 (80).

2.5.3. Adhesins

The bacterial adhesins in *H. pylori* interact with cellular receptors in the gastric epithelium and prevent the bacteria from being dislodged from the stomach. Then using their toxin, they injure the host cells and get nutrients for growing. Blood-antigen binding protein A (BabA), sialic acid-binding adhesin (SabA), and OipA are well-defined adhesins. Other adhesins have also been described in *H. pylori* that can adapt to different hosts and tissues (63).

2.5.3.1. Blood group antigen binding adhesion

Blood group antigen-binding adhesin (Bab A) promotes *H. pylori* adhesion to the gastric epithelium as well as the delivery of toxins (such as CagA, VacA) into host cells, causing damage to either the inflammatory or immune system of the host, depending on whether the infection is direct or indirect. A recent study suggested that BabA expression during *H. pylori* infection might be used as a biomarker (81). As well as causing paraneoplastic gastric lesions, co-expression of babA, cagA, and vacA might cause other digestive conditions (82).

2.5.3.2. Sialic acid binding adhesion

Associated with the outer membrane protein (OMP) family, sialic acid-binding adhesins (SabA) facilitate the adherence of *H. pylori* to the gastric epithelium through binding to the Lex antigen. The SabA enzyme is found in almost 40% of strains of *H. pylori*. There is a significant increase in SabA expression within the gastric microenvironment when bacterial-induced inflammation occurs. Increased expression of OMPs such as SabA, BabA, and OipA has been shown to modulate gastrointestinal disorders (83). The expression of SabA is associated with severe intestinal metaplasia, gastric atrophy, and gastric carcinoma development (84).

2.5.3.3. Outer inflammatory protein A

Outer inflammatory protein A (OipA), encoded by *hopH* gene, belongs to the OMP family. As one of the virulence factors of *H. pylori*, it is associated with bacteria adhering, colonizing, initiating, and progressing gastrointestinal disorders, as well as possibly affecting the patient's clinical outcome (85). Compared with OipA-negative strains of *H. pylori*, OipA-positive strains are more likely to induce much more severe inflammation in the gastric mucosa (86). It is believed that the expression of OipA regulates the synthesis of CagA and VacA (87). OipA stimulates the release of several pro-inflammatory cytokines, including IL-1, IL-6, IL-8, IL-11, IL-17, matrix metalloproteinase 1 (MMP-1), and tumor necrosis factor α (TNF-α) (88, 89).

2.5.4. Ice A

Induced by contact with epithelium gene A (IceA) is a recently defined virulence factor of *H. pylori* that needs to be thoroughly described. The gene encoding IceA has been defined in strains isolated in patients with peptic ulcers. The levels of IceA significantly increase while *H. pylori* binding to gastric epithelial cells (67).

2.5.5. Vacuolating Cytotoxin A

One of the most critical virulence factors defined for *H. pylori* is vacuolating Cytotoxin A (VacA). The *vacA* gene is present in all *H. pylori* strains, and 50-60% express a virulent cytotoxin that inhibits the intracellular membrane of target cells, causing vacuolar degeneration (75). The key types of receptors that VacA binds include receptor-like protein tyrosine phosphatases α and β, LRP-1 (low-density lipoprotein receptor-related protein 1), and sphingomyelin (90,91). There are currently several vacA genotypes, including s1, s2, m1, m2, s1m1, s1m2, s2m2, and s2m1. Peptic ulcer disease is associated with the VacA s1 genotype, one of the most common in *H. pylori*-infected patients (92).

2.5.6. Cytotoxin Associated Gene A

Generally, *H. pylori* strains can be divided into two subtypes: cytotoxin associated gene A (CagA) positive and negative. CagA is expressed when *H. pylori* adheres to gastric epithelial cells, and the process is mediated by the Fur protein (93). It has been suggested that *H. pylori* strains that are CagA positive stimulate the production of IL-8 and IL-12 in the blood of people infected with these strains (94). Bacterial strains that express CagA seem more motile than those that do not express CagA; therefore, CagA is also involved in the motility of bacteria, as indicated by this observation (95).

CagA expression in conjunction with OipA, VacA, and BabA significantly increases the inflammatory response, resulting in poorer clinical outcomes and an increased likelihood that gastrointestinal diseases will occur (96).

2.5.7. Duodenal ulcer promoting gene A

Duodenal ulcer promoting gene A (Dup A) is a virulence factor of the *H. pylori* strain whose pathogenicity is associated with developing duodenal ulcers and gastritis; however, it's expression is negatively related to gastric cancer risk (97). DupA expression is estimated to be present in approximately 45% of *H. pylori* strains and varies significantly by geographical region and ethnic group (98). In addition to stimulating urease and IL-8 secretion primarily in the antrum, DupA plays a role in the development of gastritis. It is also responsible for increasing the risk of duodenal ulceration following stimulation of IL-8 secretion by DupA. It has not been found that DupA expression is associated with CagA or VacA expression (99).

2.5.8. Neutrophil-activating protein

The neutrophil-activating protein (*napA*) gene encodes a 150 kDa activating protein called HP-Nap, which comprises ten identical subunits that activate neutrophils and increase neutrophil adherence to endothelial cells. A recent study reported that NAP promotes the adhesion of neutrophils to endothelial cells during infection with *H.*

pylori by stimulating a high level of oxygen radical production from neutrophils (100). As well as stimulating reactive oxygen species production, NAP induces neutrophils to express and release IL-8, macrophage inflammatory protein (MIP)-1a, and MIP-1b (101).

2.6. *Helicobacter pylori* Associated Diseases

It was demonstrated that there is a strict association between the presence of *H. pylori* and inflammation in gastric biopsy in many studies by various researchers. All these studies reported that *H. pylori* infection is associated with gastroduodenal diseases (102). Although the infection of *H. pylori* is often observed in childhood, related diseases appear in adulthood. *H. pylori* infections cause active gastritis and stay asymptomatic in infected individuals throughout their life. *H. pylori*-associated diseases can also be seen as more serious, such as gastric mucosa-associated lymphoid tissue (MALT) lymphoma, gastric cancer, non-cardia gastric adenocarcinoma, and duodenal or gastric ulcers. Development of these complications is usually induced by several factors, including environmental, host genetic, and bacterial virulence factors (103). It was reported that *H. pylori* infection is responsible for 92% of gastric cancers, the fourth most common cancer in the world (104). Due to the close relationship of *H. pylori* infection with cancer, *H. pylori* was defined as a type I carcinogen in 1994 by the World Health Organization (105).

2.7. Diagnosis of *Helicobacter pylori* Infections

H. pylori infection always is a significant health issue; therefore, several diagnostics methods are developed to detect the infection. In clinical practice, diagnostic tests should exceed 90% with sensitivity and specificity to accurately diagnose *H. pylori* infection. Diagnostic methods for H. pylori infections are divided into invasive and non-invasive tests (106).

2.7.1. Invasive tests

2.7.1.1. Endoscopy

Examination of conventional endoscopy is usually performed to diagnose *H. pylori*-associated diseases such as peptic ulcer, MALT lymphoma, and gastric cancer. The accurate diagnosis can be increased by carefully observing the gastric mucosa pattern using standard endoscopy; however, this method may take a prolonged period and may not achieve more accurate results than other invasive procedures (107). There are also novel endoscopic techniques used in several studies. In a study that utilized novel endoscopic methods (magnifying endoscopy), sensitivity and specificity were reported as 85.4 and 81.7% for gastric inflammation, 71.8 and 95.2% for gastric intestinal metaplasia, and 80 and 98.9% for gastric carcinoma, respectively (108). Additionally, magnifying an object is time-consuming and may cause more difficulty for patients than other biopsy-based tests (106).

2.7.1.2. Histology

In the direct detection of *H. pylori* infection, histology is commonly regarded as the standard method of diagnosis. On the other hand, the diagnostic exactness of this method is influenced by various factors such as size, site, number of biopsies, methods of staining, PPI, antibiotics, and knowledge of the examining pathologist (45).

2.7.1.3. Rapid urease test

Rapid urease tests (RUTs) are the most useful invasive tests in routine laboratory practices due to their low cost, rapidity, ease of use, and high specificity. The urea test mechanism depends on the activity of the urease enzyme, if it is present in the biopsy specimen, it converts the urea into ammonia, causing a pH increase, and the pH monitor changes color due to this increase. There are various commercial urease tests, and generally, they have specificity above 95%-100% and sensitivity above 85%-95% (109).

2.7.1.4. Culture

Culture is utilized as a gold standard diagnostic test for most bacterial infections. The performance of culture is influenced by some factors such as poor quality of specimens, late transport, unsuitable incubation conditions, or unpracticed microbiologist. On the other hand, due to the delicate and fastidious nature of *H. pylori*, culturing is difficult and time-consuming (110, 111). Despite these disadvantages, cultures have the advantage of being able to detect bacterial drug sensitivity. Moreover, as the culture allows for the isolation of *H. pylori* for further analysis, this facilitates a better understanding of the pathogens, which can then be used to evaluate therapies (106).

2.7.1.5. Molecular methods

The use of molecular techniques for detecting *H. pylori* infection or drug resistance has become increasingly popular in recent years.

2.7.1.5.1. Polymerase chain reaction

Based on its high sensitivity and specificity of over 95%, PCR has been widely used to detect H. pylori in various clinical samples. Several target genes, such as *UreA, UreC, glmM, 16S rRNA, 23S rRNA, HSP60,* and *VacA* genes, are used to detect *H. pylori.* Furthermore, samples except for gastric biopsy specimens using two different target genes may increase specificity. The advantages of PCR include the reduced number of bacteria required in the sample, the absence of unique processing supplies or transportation, and faster results, allowing clinicians to make an accurate and timely decision regarding treatment. Moreover, PCR detects antimicrobial resistance and virulence factors by detecting the current mutations (106).

2.7.1.5.2. Quantitative real time PCR

Real-Time PCR (RT-PCR) is extensively preferred for detecting *H. pylori* and antibiotic resistance. In the literature, various studies are published reporting the sensitivity and specificity of RT-PCR in different clinical samples compared with other diagnostic methods (112).

2.7.1.5.3. Fluorescence insitu hybridization

In-situ hybridization has usually detected the presence of *H. pylori,* the current virulence markers, and clarithromycin resistance. It was reported that the sensitivity and specificity for the diagnosis of *H. pylori* infection via in-situ hybridization are 95% and 100% (113).

2.7.2. Noninvasive tests

Due to endoscopic tests being expensive, discomfort, and unsuitable for some special patient groups, several alternative diagnostic methods have been used in routine for diagnosing *H. pylori.*

2.7.2.1. Urea breath test

Since the urea breath test (UBT) is the most popular and accurate noninvasive test, it is widely used to diagnose H. pylori infection. The procedure of the test is based on the urease activity of H. pylori; the patient takes the 13C- or 14C-labeled urea and hydrolyzes to labeled CO2 in the stomach absorbed in the blood exhaled by breathing in which labeled CO2 can be measured. Even though several factors affect the results of UBT, high sensitivity (96%) and specificity (93%) were reported for this test in the diagnosis of *H. pylori* infection (106,114).

2.7.2.2. Stool antigen test

Stool antigen test (SAT) is another non-invasive method that possesses high sensitivity (94%) and specificity (97%) in the diagnosis of *H. pylori* infection. It is possible to detect *H. pylori* using two different tests called enzyme immunoassays (EIA) and immunochromatographic assays (ICA). Two types of antibodies are used in these methods: polyclonal and monoclonal. On the other hand, it has been reported that tests based on monoclonal antibodies provide more accurate results than tests based on polyclonal antibodies (115). Although several factors, such as using antibiotics, PPI, or bleeding, affect the results of SAT, properties of the tests, such as easy to perform and cheap, make the test superior to other non-invasive tests (106).

2.7.2.3. Antibody based test

In diagnosing *H. pylori* infection, various serological tests are based on the detection of anti-*H. pylori* IgG antibodies were extensively used. EIA is the most common among these tests. Furthermore, the serological test is used to appraise *H. pylori* infection in children. Since the serological tests are not influenced by antibiotics, PPI, ulcer bleeding or gastric atrophy, these tests' properties make it superior compared to other invasive or non-invasive tests. On the other hand, serological tests are unreliable for evaluating eradication therapy since antibody levels may persist in the blood even if therapy is successful. Moreover, serological tests cannot distinguish active infection from previous exposure to *H. pylori*, so more tests should be conducted before treatment is recommended (45, 106).

2.7.2.4. Stool PCR

Detection of *H. pylori* also can be performed from stool samples by using PCR. As a non-invasive method, this method is especially preferred for children. Stool PCR also has the advantage of identifying specific genotypes and antibiotic resistance of *H. pylori* (106).

2.8. Treatment of *Helicobacter pylori*

Treatment of *H. pylori* is critical in managing gastrointestinal disorders such as gastric cancer and peptic ulcer. Depending on increasing antibiotic resistance, new approaches have been proposed, such as new antibiotics, combinations of existing antibiotics, or probiotics, to combat *H. pylori* (116).

2.8.1. Standard triple therapy

According to current international guidelines on *H. pylori* treatment, first-line therapy includes PPI, clarithromycin, and amoxicillin/metronidazole. Even though this therapy is considered standard, treatment failure due to increased resistance to the antibiotics used in treatment, especially clarithromycin, is observed. Therefore, the guidelines do not suggest using the standard therapy in regions where clarithromycin resistance rates are over 15% (8,117,118). As a result of increased resistance rates reported, it may no longer be possible to recommend standard triple therapies for use as empiric therapy. The high level of resistance to clarithromycin and metronidazole, as well as the various patterns of resistance across populations, makes it imperative that standard triple therapies are designed to address local resistance patterns. It is advisable to base treatment decisions on strain culture susceptibility information whenever possible. Various alternative treatment strategies are currently being used in clinical practice to treat strains of *H. pylori* that are resistant to antibiotics. Consequently, novel and more effective treatments have been developed and used, as well as probiotics, to improve eradication regimens and reduce antibiotic-associated side effects (116).

2.8.2. Bismuth quadruple therapy

Bismuth-containing therapy is now the preferred first-line treatment for areas with high clarithromycin resistance and as second-line therapy when the classical triple therapy against *H. pylori* failed (6,7). This therapy includes two antibiotics (tetracycline and metronidazole), plus bismuth and PPI, for 14 days (119). As far as

antibiotic resistance is concerned, this therapy is entirely independent of clarithromycin. Even in regions with high levels of antibiotic resistance, metronidazole at high doses and prolonged durations can significantly reduce the likelihood of infection by metronidazole-resistant strains (120). Bismuth has potential toxicity, and in some countries, bismuth salts or tetracycline are unavailable, thus limiting its use. Some studies have shown that amoxicillin can replace tetracycline (121). In a meta-analysis study, it was shown that there are similar eradication rates between the quadruple and triple therapy for eradicating *H. pylori* infection as the primary therapy (122). In another meta-analysis, this therapy was found to be effective as a second-line treatment, with an eradication rate of 77% after standard triple therapy was ineffective (123).

2.8.3. Sequential therapy

The results of 36 randomized clinical trials were analyzed in a recent meta-analysis. Eradication rates for sequential and standard triple therapy were 84.1% and 75,1%, respectively. Patients with one clarithromycin-resistant strain responded better to sequential therapy, with eradication rates of 80,9% compared to 40,7% with standard triple therapy (124, 125).

2.8.4. Non bismuth quadruple therapy

It is also possible to use a non-bismuth quadruple therapy for ten days in areas with a high level of clarithromycin resistance. This therapy contains a PPI [but does not contain bismuth], clarithromycin, amoxicillin, and metronidazole. Compared to other treatments, this treatment has the disadvantage of requiring many pills (6,7). As reported in a meta-analysis carried out in 2012, while non-bismuth quadruple therapy had a 90% eradication rate, standard triple therapy had a 78% eradication rate (123).

2.8.5. Hybrid therapy

In the literature, few studies are comparing hybrid therapy with other approaches, such as sequential or standard therapies; however, in the available studies, it was reported that no superior hybrid therapy when compared with the sequential and concomitant therapies (116). In Hybrid therapy, PPI and amoxicillin are given for seven days, followed by seven days of quadruple therapy with a PPI, amoxicillin, clarithromycin, and metronidazole. Based on a review, five studies compared hybrid therapy with sequential therapy, and three compared hybrid therapy with concomitant therapy. In conclusion, in both studies, no significant differences were reported between hybrid therapy and sequential therapy or hybrid therapy and concomitant therapy (126).

2.8.6. Levofloxacin based therapies

Due to the increased clarithromycin resistance, levofloxacin-based therapy is also preferred for *H. pylori* eradication to replace clarithromycin in triple or sequential regimens (127). The eradication rate of this therapy was reported as more than 90%, especially in regions where the levofloxacin resistance rate was less than 10%. Since secondary quinolone resistance may rapidly develop, levofloxacin in first-line treatment is not suggested in such a situation drug is reserved for second-line treatment after failure of standard triple therapy (128).

2.8.7. Probiotics

Although antimicrobial therapies for *H. pylori* are highly effective, due to the resistance of commonly used antibiotics, there is an increasing interest in using probiotics in conjunction with antibiotic regimens to treat the *H. pylori* infection. Numerous studies, it was investigated the favorable effect of different probiotics (6,7,116).

2.8.8. Adjuvant agents used in *Helicobacter pylori* treatment

2.8.8.1. Bismuth

Bismuth salts are topical antimicrobial agents that affect the bacteria cell membrane by collapsing along the cell membrane and periplasmic region. As a result of this effect, the integrity of the cell is broken. In *H. pylori* treatment, bismuth has been used combined with certain antibiotics. The resistance of bismuth in *H. pylori* has yet to be reported (129, 130).

2.8.8.2. Proton pump inhibitors

Proton pump inhibitors (PPI) inhibit the parietal cell H+ /K+ adenosine triphosphatase (ATPase) enzyme that secretes from gastric parietal cells have a part in gastric acid secretion. It was reported that there is a synergistic activity between PPIs and antibiotics used in the treatment depending on the antimicrobial effect of PPIs and the high pH provided by PPIs (131, 132).

2.9. Antimicrobial Susceptibility Tests for *Helicobacter pylori*

For managing *H. pylori* treatment, it is crucial to choose precise antibiotics by applying suitable antimicrobial susceptibility tests. The Maastricht V/Florence Consensus Report recommends that standard triple therapy without prior susceptibility testing should not be preferred when the clarithromycin resistance rate in the region is over 15%. The guideline also recommended that culture with susceptibility testing or molecular determination of genotype resistance should be performed after second-line treatment failure (8). To detect *H. pylori* antimicrobial susceptibility tests, several methods can be employed, including phenotypic and genotypic tests.

2.9.1. Phenotypic tests

Agar dilution, broth microdilution, E-test, and disc diffusion methods are culture-based methods that are usually performed for antimicrobial susceptibility testing of *H. pylori* (133).

2.9.1.1. Agar dilution

Agar dilution methods provide the chance to determine the MICs of the antibiotics by testing through two-fold serial dilutions of different concentrations of them (129). The agar dilution method, regarded as a reference method, has been offered by Clinical Laboratory Standard Institute (CLSI). In Europe, the European Helicobacter pylori Study Group has also published guidelines about antimicrobial susceptibility testing of *H. pylori* (Table 1) (134, 135).

Table 1. Agar dilution methods suggested by the guidelines (adapted from 136)

Method	**Conditions recommended by (reference):**	
	CLSI, United States (134)	European *H. pylori* Study Group (135)
Medium	Mueller-Hinton agar plus sheep blood (5% vol/vol)	Mueller Hinton agar horse blood (10% vol/vol)
Inoculum	$1x10^7$–$1x10^8$CFU/ml	$0.5x10^9$–$1x10^9$ CFU/ml
Incubation	35°C, microaerobic atmosphere	37°C, microaerobic atmosphere
Reading	3 days	3 days
Control strain(s)	*H. pylori* ATCC 43504	*H. pylori* CCUG 38770, *H. pylori* CCUG 38771, *H. pylori* CCUG 38772
Quality control availability	Amoxicillin, ciprofloxacin, clarithromycin, metronidazole, tetracycline	Amoxicillin, clarithromycin, metronidazole

2.9.1.2. Broth dilution method

The broth dilution method has an advantage, such as being adaptable to automation that determines the MICs of the antibiotics quickly. On the other hand, it has hardly ever been used for *H. pylori* since it is challenging to grow this bacterium in broth (136).

2.9.1.3. Breakpoint susceptibility testing

The breakpoint susceptibility testing method is a simple form of the agar dilution method. In this method, an agar plate containing constant antibiotic concentration equal to the breakpoint (e.g., 1µg/ ml for clarithromycin) or consisting of two different concentrations (0,25 and 1µg/ ml) is used to categorize the strains as susceptible/intermediate/ resistant. Although this method is simple to perform since there is no commercial form of agar, it is not widely used in routine laboratory practice (136).

2.9.1.4. E test

E-test (Biomerieux; Basingstoke, United Kingdom) is a quantitative kind of disc diffusion method which provide determines the MIC of the related antibiotics easily. This method is adapted to *H. pylori* and good correlation has been achieved when compared with the agar dilution method. EUCAST recommends the E tests strips for the antimicrobial susceptibility testing of *H. pylori* (137, 138).

2.9.1.5. Disc diffusion

The disc diffusion method is feasible and cost-effective for routine clinical laboratory practices. On the other hand, this method is not recommended for slow-growing bacteria such as *H. pylori* that require specific growth conditions. Although this method is not validated for *H. pylori*, some studies reported a good correlation between disc diffusion and other susceptibility tests (139).

2.9.2. Genotypic methods

Molecular tests to diagnose antibiotic resistance in *H. pylori* are available for clarithromycin, levofloxacin, and tetracycline. The phenotypic methods are time-consuming as they require complex growth conditions; nucleic acid-based methods are considered alternatively for detecting resistance in *H. pylori*. These molecular methods

provide the opportunity for rapid analysis, regardless of whether the bacteria live or not, by giving results on the same day (133). No molecular tests are defined to detect resistance for other antibiotics used in treating *H. pylori* since the mechanisms involved still need to be outlined (140, 141).

2.9.2.1. Detection methods of clarithromycin resistance in *Helicobacter pylori*

There are several methods are PCR-based and non-PCR-based (FISH), to detect the mutations that cause clarithromycin resistance in *H. pylori*. The most used methods are PCR-RFLP and real-time PCR.

2.9.2.1.1. PCR RFLP

Based on mutation detection, this method reveals restriction sites inside the amplicon obtained via primers specific to H. pylori. This method uses specific enzymes called BsaI, BsbI, and BceAI to recognize A2142G, A2143G, and A2142C mutations, respectively (142).

2.9.2.1.2. Real time PCR

In the first application of detection of clarithromycin resistance in *H. pylori* strains using real-time PCR was performed in 1999. In the study, following FRET (fluorescence resonance energy transfer), melting curve analysis was performed and for detecting the mutation caused clarithromycin resistance in *H. pylori*; the quencher SYBR green 1, a fluorophore specific for double-stranded DNA, was used, and it transferred its energy to the second fluorophore (Cy5) fixed on the specific probe (143). Detection of clarithromycin resistance in direct biopsy specimens using the same method mentioned above is reported (144). Another method is proposed for detecting clarithromycin resistance in *H. pylori* by using real-time PCR. In the method using specific primers, a 267-bp fragment of the 23S rRNA gene is amplified, and two probes (a sensor and an anchor) are used for hybridization. After then, a melting curve analysis is performed via LightCycler. The melting peak differences can separate the

wild-type and mutant strains (145,146). There is also a commercially available kit called GenoType HelicoDR (Hain Lifescience; Nehren, Germany) which can detect both clarithromycin and fluoroquinolone resistance. The sensitivity and specificity of the test for clarithromycin were 94%-100% and 86%-99%, respectively (147).

2.9.2.1.3. FISH

Detecting clarithromycin resistance in *H. pylori* except for the PCR is also possible by FISH. It provides rRNA-based whole-cell in situ hybridization using a set of fluorescents labeled oligonucleotide probes. It is possible to detection of resistance mutants of H. pylori using a 23S rRNA probe labeled with fluorescein (green) by fluorescence microscopy. It was reported that this method is sensitive and specific compared to culture susceptibility tests. On the other hand, a limitation depending on the observer (difficulty in reading) may occur (136).

2.9.2.1.4. Next Generation Sequencing

Although current PCR assays detect the known mutations for clarithromycin resistance in *H. pylori* (A2142C/G and A2143G), a limited number of nucleotide positions can be analyzed using this tool. On the other hand, NGS provides a more inclusive characterization of resistance patterns in isolates and determines new mutations causing clarithromycin resistance (148).

2.10. Mechanisms of Action and Resistance of the Antibiotics used in *Helicobacter pylori* Treatment

2.10.1. Macrolides

Macrolides show their bacteriostatic effect by inhibiting the protein synthesis via reversible binding to the peptidyl transferase loop of domain V of 23S rRNA in the 50s subunit of the bacterium (149). Clarithromycin is the most preferred one among macrolides for *H. pylori* treatment thanks to its pharmacokinetic advantages containing

acid stability and better absorption in the gastric mucus layer (150). The World Health Organization reported clarithromycin-resistant *H. pylori* as a high-priority pathogen that requires attention in treatment (151).

Generally, there are three mechanisms by which Gram-negative bacteria can develop resistance to macrolides: methylation of the target cells or mutations of the genes, efflux mechanisms, and enzymatic deactivation of the drug. On the other hand, in *H. pylori*, specific mutations in the 23S rRNA region were reported as associated with clarithromycin (149). There are two primary 23S rRNA mutations: adenine to guanine transitions at positions 2142 (A2142G) and 2143 (A2143G) and adenine to cytosine transitions at position 2142 (A2142C). The prevalence of these mutations was reported as 11,7%, 69,8%, and 2,6%, respectively. Numerous other point mutations have also been identified, such as A2115G, T2117C, G2141A, T2182C, G2224A, C2245T, T2289C, C2611A, and T2717C. Alongside their low frequency, these mutations are still unproven in terms of their clinical relevance.

On the other hand, a correlation was reported between the T2182C, C2611A, and T2717C and low resistance levels (152,153). Furthermore, a recent study showed that additional resistance mutations in experimentally induced clarithromycin resistance phenotypes affect the ribosomal protein L22p and the translation initiation factor IF-2. However, the mutations have not been reported yet in clinical isolates (154,155).

2.10.2. Fluoroquinolones

Various fluoroquinolones, such as levofloxacin and moxifloxacin, have been used as an alternative in first, second, or third-line therapies for *H. pylori* (8). As they are protonated and dissolved in gastric juice before maximum absorption in the intestines, their topical activity in the stomach is due to their favorable pharmacokinetic properties at acidic pH (156). As a result of their bactericidal activity, fluoroquinolones inhibit two essential bacterial type II topoisomerases, DNA gyrase and topoisomerase IV, which are responsible for chromosomal supercoiling necessary for DNA synthesis, transcription, and division of the cell (157,158). The resistance to fluoroquinolones

commonly occurs because of the mutations in DNA gyrase/ topoisomerase IV, plasmid-mediated resistance, and efflux systems (111, 112). Fluoroquinolone resistance in *H. pylori*, which lacks the topo IV genes, is mainly driven by mutated *gyrA* and *gyrB* genes (159,160).

2.10.3. Nitroimidazoles

A member of the nitroimidazole family, metronidazole is actively released into gastric juice, and it has been widely used for preventing the growth of *H. pylori* (161). Due to its nature as a prodrug, metronidazole exhibits bactericidal activity only after being reductively activated by cellular electron acceptors (162). Metronidazole activates transient derivatives such as nitro-anion free radicals, which cause cytotoxic effects by damaging subcellular structures and DNA directly and may also inhibit proton motive force inhibition by reducing the production of ATP in the cell (162). Generally, the resistance to metronidazole in *H. pylori* occurs due to the reduced activity of electron transport proteins (especially RdxA). It also reported that other mechanisms, such as the efflux pump, cause metronidazole resistance in *H. pylori* (45). Since there are various mutations of rdxA, it is impossible to perform antibiotic susceptibility testing for metronidazole using molecular methods (113). No comprehensive study has yet examined all the pathways related to resistance in identical isolates to determine the extent to which they are clinically relevant (163).

2.10.4. Tetracyclines

Tetracycline is a bacteriostatic antibiotic that has been used in the treatment of *H. pylori*. The mechanism of the action of tetracycline is based on inhibiting bacterial growth and protein synthesis. The primary mechanism of tetracycline resistance in *H. pylori* has been related to gene mutations in the target region (binding side of the drug) (164). Various regimens have been tested for eradicating *H. pylori* using tetracyclines, such as tetracycline and minocycline (8). In addition to their ability to be stable at low pH, these agents can achieve high concentrations in the gastric juice and mucosa of the stomach by acting as topical agents (165). The bacteriostatic and bactericidal

effects of tetracyclines are induced by their reversible binding to a pocket containing 16S rRNA in the 30S subunit of bacterial ribosomes (166). Several tetracycline resistance mechanisms are mediated by the family of resistance determinants referred to as ‘tetracycline proteins’ (Tet) in bacterial species, including tetracycline-cation complex efflux through proteins like TetA, ribosomal protection proteins like TetO, and tetracycline inactivation by enzymes like TetX. Additionally, tetracycline resistance can be caused by genetic mutations of the 16S rRNA gene that binds tetracycline (167). It was also reported that additional mechanisms, such as efflux pumps, may contribute to the development of tetracycline resistance in *H. pylori*, which is yet to be identified (168).

2.10.5. β Lactams

Amoxicillin is (a β-lactam antibiotic) widely used in *H. pylori* treatment. Amoxicillin exerts its topical intraluminal activity in the gastric juices and mucosa. However, this activity requires increased dosages of PPIs to enhance its stability and efficacy in an acidic environment (8). Amoxicillin binds to penicillin-binding proteins (PBPs) in the periplasmic space, disrupting the transpeptidase activity required for peptidoglycan crosslinking. As a result of this process, the mechanical stability of the bacterial cell walls is compromised, and the bacteria lose their viability and are lysed (169). Generally, amoxicillin resistance occurs in Gram-negative bacteria by producing β-lactamases or depending on alterations in PBP sites and efflux mechanisms. However, in *H. pylori* species, amoxicillin resistance developed primarily by reducing the affinity for a specific PBP, PBP1A, without generating a significant amount of β-lactamase activity (170).

2.10.6. Rifamycins

Rifabutin (a member of Rifamycin antibiotics) is frequently used to rescue *H. pylori* infection when the first treatments fail. Despite the stomach's acidic environment, the drug's chemical stability allows it to maintain antibacterial activity across a wide pH range. Rifabutin presents its bactericidal activity by binding to the

subunit of DNA-dependent RNA polymerase encoded by the *rpoB* gene. Although the relationship between mutations in *rpoB* and rifabutin resistance was reported, many rifabutin-resistant strains were absent of *rpoB* mutations was also shown; therefore, it is considered that additional yet-unknown mechanisms exist in *H. pylori* (171,172).

2.11. The prevalence of the antibiotic resistance in *Helicobacter pylori*

In a study performed in 65 countries included in WHO regions, primary and secondary resistance rates to clarithromycin, metronidazole, and levofloxacin were reported as 15%. However primary clarithromycin resistance in the Americas and South-East Asia region was 10% while primary levofloxacin resistance was reported as 11% in the European region (173).

Kocazeybek *et al.* reported the prevalence of primary antibiotic resistance of *H. pylori* strains in different geographical regions of Turkey between 1999 and 2015yr. Their systematic review presents 21 Turkish studies including 1,059 *H. pylori* strains in Turkey. The primary antibiotic resistance rates had been reported as follows: amoxicillin, 0.9%; clarithromycin, 24.8%; metronidazole, 33.7%; tetracycline, 3.5%; and levofloxacin, 23.7% (174).

2.12. Alternatives Antibiotics for *Helicobacter pylori* treatment

Regarding the increasing resistance of the antibiotics used in treating *H. pylori*, other antibiotics such as josamycin, nitazoxanide, and furazolidone have been considered alternatives (175). In the same manner as metronidazole, nitazoxanide and furazolidone act as antimicrobials by catalyzing intracellular activation steps by bacterial flavoproteins acting as electron acceptors. Moreover, these drugs exhibit high clinical potency in vivo and in vitro against *H. pylori* without being cross-resistant to metronidazole (176). The use of nitazoxanide and furazolidone in eradication regimens for *H. pylori* is therefore considered an alternative to metronidazole (177).

Considering the possible resistance of *H. pylori* to clarithromycin in the first-line therapy, other macrolides, such as josamycin, an inexpensive macrolide, have been considered an alternative in the treatment of *H. pylori* (8).

2.12.1. Josamycin

Josamycin was produced by *Streptomyces narbonensis var josamyceticus.* As with other macrolides, josamycin is readily absorbed through the gastrointestinal tract and may accumulate in different tissues and bodily fluids. The acute toxicity of josamycin is relatively low, with gastrointestinal disturbance as the most reported side effect. Josamycin is used to treat respiratory and suppurative infections (178). The usage of josamycin in the treatment of *H. pylori* infections is evaluated only in limited studies. A study showed that josamycin has an eradication rate of 85.6%, and josamycin and bismuth have a more than 90% eradication rate (179). Currently, in our country, josamycin is not used for treating *H. pylori* since the drug is absent in the country.

3 MATERIALS AND METHODS

3.1. Materials

3.1.1. Bacteria

3.1.1.1. *Helicobacter pylori*

Helicobacter pylori G27, which has been used extensively in *H. pylori* research since its relatively straightforward genetics and reliable growth, was used for this study. Prof. Dr. Anne Müller from the Institute of Molecular Cancer Research at Zurich University kindly supplied this strain. It was kept at -80°C in Brucella broth containing 25% (v/v) glycerol.

3.1.2. Mediums used for culture processes

3.1.2.1. Columbia blood agar

Before the susceptibility tests, to recover the bacteria from the frozen environment and ensure the growth of the bacteria, the frozen stocks were sub-cultured twice on Columbia agar plates consisting of Columbia agar base, sterile defibrinated horse blood, antibiotic mixture, and β-cyclodextrin. 1000 ml Columbia blood agar consists of 50 ml defibrinated horse blood and 1 ml 1000X antibiotic cocktail [Trimethoprim (100 mg), Amphotericin B (160 mg), and DMSO (20 ml)] and 5 ml of 200X antibiotic cocktail [vancomycin (100 mg), Cefsulodin (50 mg), polymyxin B (3.3 mg), ddH2O (50 ml)] and 5 ml of β-cyclodextrin (in DMSO).

3.1.2.2. Mueller Hinton Agar

Mueller Hinton agar added with 5% defibrinated sheep blood was used for susceptibility tests.

3.1.2.3. Brucella Broth

Brucella broth, with 10% Fetal bovine serum (FBS) and vancomycin, was also used for the culture process. 100 ml Brucella broth consisted of 90 ml Brucella broth, 10 ml FBS, and 10 ml vancomycin.

3.1.3. Antibiotics and solvents

Antibiotics and specific solvents were used for *H. pylori* culture and agar dilutions assays. The antibiotics, solvents, and supplier companies are given in Table 2.

Table 2. Antibiotics and their solvents that are used in the thesis study

Antibiotic	**Usage area in the study**	**Solvent**	**Supplier company**
Clarithromycin	Agar dilution tests	Deionized water	Abbott
Josamycin	Agar dilution tests	Deionized water	ChemCruz
Trimethoprim	Primary isolation of *H. pylori*	DMSO	ChemCruz
Amphotericin B	Primary isolation of *H. pylori*	DMSO	Bristol-Myers Squibb
Vancomycin	Primary isolation of *H. pylori*	Deionized water	Koçak Pharma
Cefsulodin	Primary isolation of *H. pylori*	Deionized water	Koçak Pharma
Polymyxin B	Primary isolation of *H. pylori*	Deionized water	ChemCruz

3.1.4. Commercial kits

The High Pure PCR template preparation kit (Roche, Germany) was used for genomic DNA isolation of *H. pylori.* Gram staining kit (Nova Lab, Belgium) was used for confirmation of the microscopic morphology of *H. pylori.*

3.1.5. Medium biological and chemical materials

Medium, biological, and chemical materials used in this thesis study, with their supplier companies were indicated in Table 3.

Table 3. Medium, biological, and chemical materials that were used in the thesis

Medium / Biological or Chemicals Materials	**Supplier company**
Columbia Blood Agar Base	Oxoid
Mueller Hinton II Agar	BD
Brucella Broth	Remel
Sterile Defibrinated Sheep Blood	GBL (Gul Biology Laboratory)
Sterile Defibrinated Horse Blood	Ant Medical
β-Cyclodextrin	Sigma
Glycerol	Sigma Life Science
DMSO (Dimethyl sulfoxide)	Sigma
Fetal Bovine Serum (FBS)	GIBCO

3.1.6. Equipment

The general types of equipment that were used in this thesis study and their supplier companies were shown in Table 4.

Table 4. Equipment that used in the thesis and their supplier companies

Equipment	**Supplier company**
Biosafety Class II Cabinet	Thermo Scientific
Water purification system	Merck Millipore, Milli-Q® Advantage A10

Table 4. Equipment that used in the thesis and their supplier companies (Cont'd)

Equipment	Supplier company
NanoDrop	Thermo Scientific One^{C}
Dry Block Heating Thermostat	BIOSAN, Bio TDB-100
Microcentrifuges	Thermo Scientific, MICROCL 21R Thermo Scientific, MICROCL 17
PCR tubes (1,5 ml)	Axygen
Electrophoresis system	MS Measure Science, BIO-RAD, Mini Protean
Imaging System	BIO-RAD, ChemiDocTM MP
Light microscope	Leica DM500
Vortex	bioSan Combi-Spin FVL-2400N
Incubator	Thermo Scientific, HERATHERM
Shaking Incubator	Thermo Scientific, MAXQ 4450
Autoclave	Witeg, Nüve steam Art
Dry heat sterilizator	Nüve FN120
Pipettes	CappAid, Thermo Scientific
Pipette Tips	RatioLab®
Serological Pipette Tips	Greiner bio-one
Water Bath	EMCO, ESM-3710
Fridges	Arctiko/ Kirsch
Polypropylene conical tubes (15 ml, 50 ml)	Falcon
Power supply	BIO-RAD, Power™ Pac Basic
Jar Gassing System	Donwhitley Scientific
DensiCHEK Plus	Biomerieux
Sterile Petri dishes (90mm)	FıratMed
Thermo Cycler, PCR	BIO-RAD, T100

3.1.7. Bioinformatics tools

Bioinformatic analyses were carried out by Trioscience company using the specific tools outlined in Table 5.

Table 5. Bioinformatics tools used for analyzing of the WGS results

Tools	Intended purpose	References
BBtools	Quality trim, filter	(180)
Spades 3.15.4	Genome assembly	(181)
Dfast	Annotation of the Assembled contigs	(182)
BBmap	Mapping of *H. pylori* reads to reference G27 genome	(183)
Sam files	Variant calling from sam files with callvariants.sh.	(184)
EMBOSS Transeq	SNP bearing contigs and regions converted for phenotypic change	(185)

3.2. Methods

3.2.1. Subculture of *Helicobacter pylori*

Firstly, the subculture of *H. pylori* on the Colombia blood agar was performed to recover the bacteria from the frozen environment. The Colombia blood agar included 5% defibrinated horse blood and antibiotic cocktails. Columbia agar was dissolved in distilled water according to the manufacturer's directions and then autoclaved at 121^0C, 15 minutes in liquid sterilization mood. After that, it was kept in a water bath at 50°C for one hour to achieve a suitable temperature for adding antibiotics and blood. Antibiotic cocktails and freshly prepared β-cyclodextrin were added to the media to prevent contamination and contribute to the growth. Finally, horse blood was added, and 20 ml medium was poured into each Petri dish. The streaking of *H. pylori* was performed on the prepared agar plates and incubated at 37°C for three days in microaerophilic conditions (O2 5%, CO2 10%, and N2 85%) provided by an automated closed system, was performed. After incubation, Gram staining was

performed to eliminate the contamination possibility. All processes on *H. pylori* were carried out in a biosafety level II cabinet in the microbiology laboratory.

3.2.2. Agar dilution susceptibility tests

Agar dilution susceptibility tests were performed according to CLSI guidelines. According to the guideline, the resistance breakpoint for clarithromycin is > 0.5 µg/ml (186). On the other hand, the resistance breakpoint for josamycin is > 0.25 µg/ml, as previously described in the studies (187).

3.2.2.1. Preparation of stock solution for clarithromycin

Clarithromycin injectable flacon was solved in 10 ml sterile deionized water. In the beginning, the concentration of the prepared stock solution contained 50 mg/ml clarithromycin, and to get the targeted concentration of clarithromycin, further dilution in sterile deionized water was performed by following the formula M1. V1= M2.V2. This process is summarized below:

2560 µg/ml was targeted in 4 ml.

Then;

$$M1 \times V1 = M2 \times V2$$

$$50000\ \mu g/ml .\ V1 = 2560\ \mu g/ml .\ 4ml$$

Therefore, 0,2048 ml (205µl) of the 50000 µg/ml stock solution was suspended in 3795 ml of sterile deionized water. Small portions of the stock solutions were stored at -80 °C deep freeze until use.

3.2.2.2. Preparation of stock solution for josamycin

To prepare the stock solution, Josamycin powder (with 90% potency) was used, and 5 mg of josamycin was dissolved in 9 ml of sterile deionized water by regarding the formula below:

$$\frac{\text{weight (mg)x antibiotic potency (µg/mg)}}{\text{desired concentration (µg/ml)}} = \text{volume (ml)}$$

$$\frac{\text{5 mgx900 µg/mg}}{\text{500 µg/ml}} = 9 \text{ ml}$$

Therefore 500 µg/ml stock solution was achieved in 9 ml. Small portions of the stock solutions were stored at -80 °C deepfreeze until use.

3.2.2.3. Preparation of the Mueller Hinton agar

The Mueller Hinton agar was prepared according to the manufacturer's instructions and sterilized by autoclaving at 121°C for 15 minutes. A water bath was used to equalize the prepared medium to 50°C before adding sheep blood and antibiotics.

3.2.2.4. Preparation of agar dilution

Firstly, the two-fold serial dilutions of clarithromycin were prepared in 2 ml broth. Then 1 ml sterile defibrinated sheep blood was added to each tube (to get 5 % horse blood concentration in 20 ml). Finally, 17 ml of the Mueller Hinton agar was added. Therefore, the initial concentration of the antibiotic was diluted to 1/10. Final concentrations between 256- 0,015 µg/ml were achieved on Petri dishes. All steps are shown in Figure 3. Agar dilution steps performed for josamycin are shown in Figure 4.

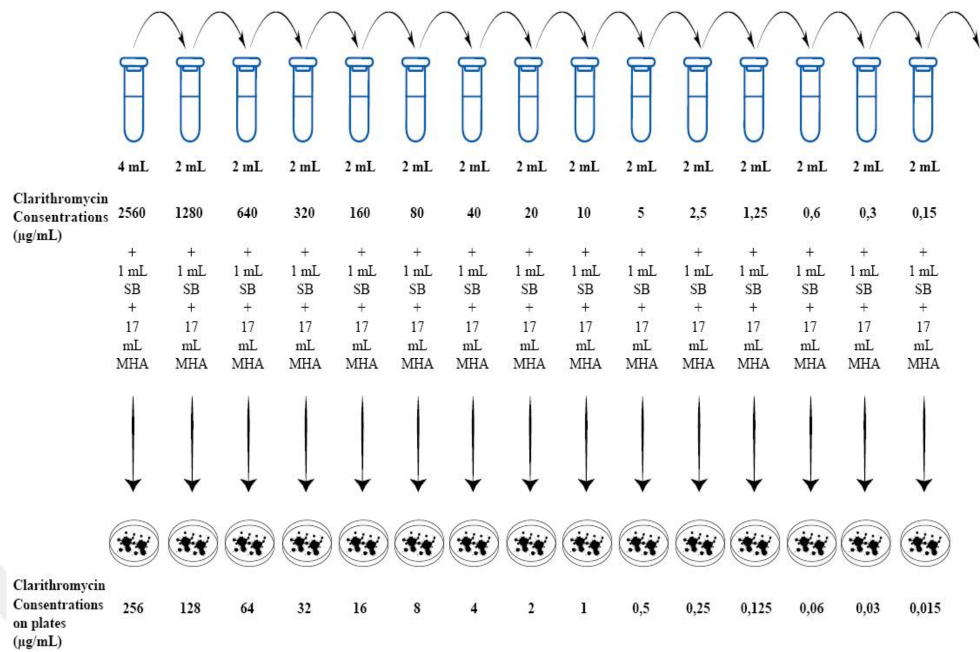

Figure 3. Schematic demonstration of agar dilutions steps performed in the study for clarithromycin. SB: Sheep blood, MHA: Mueller Hinton Agar.

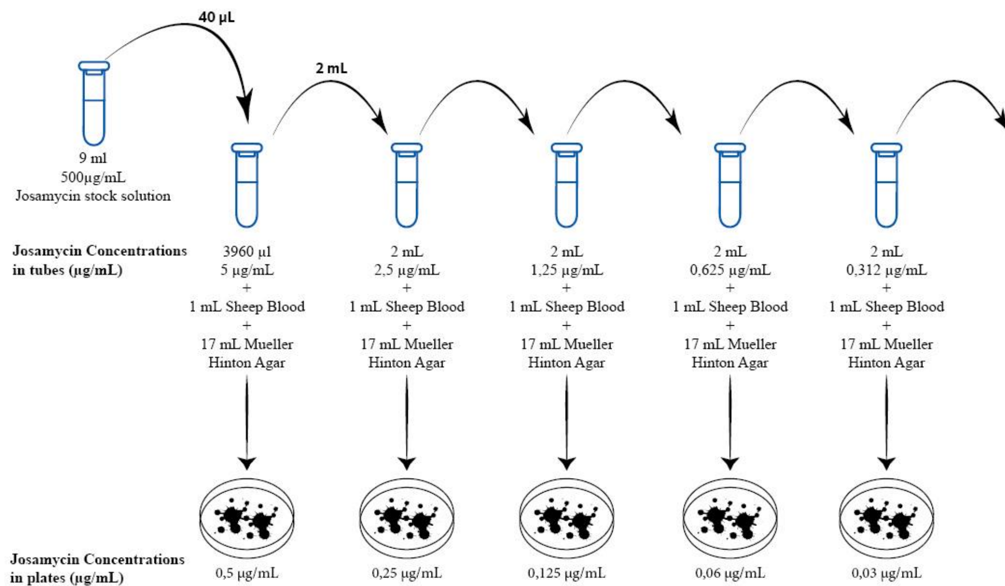

Figure 4. Schematic demonstration of agar dilutions steps performed in the study for josamycin.

3.2.2.5. Inoculation of bacteria and Interpretations of the results

The bacterial suspension with 2.0 McFarland turbidity standard was prepared in sterile saline, and 3 ml of the suspension was inoculated on each plate at different antibiotic concentrations. After 3-day incubation in microaerophilic conditions, the lowest concentration of related antibiotic that prevented the visible growth of the bacterium was defined as MIC (186). According to EUCAST (European Committee on Antimicrobial Susceptibility Testing) and CLSI guidelines, the resistance breakpoint for clarithromycin is > 0.5 mg/l (186,188). As previously described, the resistance breakpoint for josamycin is >0.25 mg/l (187).

Agar dilution susceptibility tests for clarithromycin and josamycin were reperformed three times in the thesis study.

3.2.2.6. In vitro selection of clarithromycin resistant strains

H. pylori G27, which is susceptible to clarithromycin, was used as the parent strain in the study. Clarithromycin-resistant strains were tried to construct from strain G27 by following exposure to low concentrations of clarithromycin in vitro using the agar dilution method. A single colony of G27 was inoculated with Mueller–Hinton agar medium supplemented with 5% defibrinated sheep blood without antibiotics. The plate was incubated at 37°C under microaerophilic conditions (10% O2, 5% CO2, and 85% N2) for 72 h. Colonies on agar plates were harvested, put into sterile saline, and exposed to serially doubling concentrations of clarithromycin (0,03- 256 µg/ml) via an agar dilution method. Then, colonies from the highest concentrations they could grow were obtained and repeatedly transferred to the same clarithromycin concentration five times before being exposed to the higher clarithromycin concentrations. Individual colonies on the plates containing a clarithromycin concentration greater than the MIC were isolated (155). In other words, in this step of the study, the determination of clarithromycin MIC and the selection of the mutants were performed simultaneously.

3.2.2.7. Determination of josamycin MIC for mutant strains

To determine the josamycin MIC values for mutant strains, an agar dilution plate that contains 0,03- 16 µg/ml josamycin was prepared. The bacterial suspension of mutant strains with 2.0 McFarland turbidity standard was prepared in sterile saline, and 3µl of the suspension was inoculated on each plate at different antibiotic concentrations. After 3-day incubation in microaerophilic conditions, the lowest concentration of related antibiotic that prevented the visible growth of the bacterium was defined as the MIC for josamycin.

3.2.2.8. DNA isolation of *Helicobacter pylori* G27 and mutant strains

Using the High Pure PCR template preparation kit, the genomic DNA of *H. pylori* was isolated following the manufacturer's instructions:

- The dry heat block was set to 70^0C
- Proteinase K was suspended in 4.5 ml distilled water
- Inhibitor Removal Buffer was dissolved in 20 ml absolute ethanol and mixed.
- The washing buffer was mixed with 80 ml of absolute ethanol.
- Elution buffer was portioned in Eppendorf and placed in a dry heat block at 70^0C.
- A sterile Eppendorf tube was filled with 200 µl of bacteria suspension, followed by 40 µl of proteinase K.
- After preparation, the sample was incubated at 70^0C for five minutes and then at 103^0C for ten minutes.
- After the incubation steps, 100 µl isopropanol alcohol was added to the tube and vortexed.
- Pipettes were used to transfer prepared samples into collection tubes containing filter tubes.
- It was centrifuged at 8000 rpm for one minute.
- The filter tube was removed and placed new collection tube.
- 500 µl of Inhibitor Removal Buffer was added to the filter tube.

- One minute of centrifugation at 8000 rpm was performed.
- The filter tube was removed and placed new collection tube.
- 500 µl of Washing Buffer was added to the filter tube.
- One minute of centrifugation at 8000 rpm was performed.
- The filter tube was removed and placed new collection tube.
- It was centrifuged at 13000 rpm for 10 seconds.
- The filter tube was removed and placed in a sterile 1.5 ml Eppendorf.
- 100 µl of the elution buffer that was heated at 70^0C was added to the filter tube placed in Eppendorf.
- One minute of centrifugation at 8000 rpm was performed.
- DNA was achieved as supernatant.

3.2.2.9. Determination of amount and purity of the isolated DNA

Quantification and quality control of DNA samples were completed immediately after isolations using NanoDrop One/OneC Spectrophotometer (Thermo Fisher Scientific, USA). The ratio of 260/280 (1.8-2.0 nm) and 260/230 (2-2.2 nm) of DNA samples were considered for including DNA sequencing analysis. All steps of molecular studies were carried out in a laminar flow cabinet in the molecular microbiology laboratory.

3.2.2.10. Whole genome sequencing and bioinformatic analysis

Whole Genome analysis was carried out with Illumina NGS Platforms (USA) by Eurofins Genomics (Germany). Candidate mutations for clarithromycin resistance were obtained by comparing the reconstructed genomes of the Mutant-1 with that of the *H. pylori* G27 strain. Bioinformatic analysis was carried out using the special bioinformatic tools outlined in Table 5 by Trioscience company.

3.2.2.11.Sanger sequencing

Mutations in the target region of clarithromycin were confirmed by using PCR-based sequencing to eliminate the possibility of misreading of the next-generation sequencer. Specific primers covering all putative mutations associated with clarithromycin resistance were used for the target regions. The primers and PCR conditions are described in Table 6 (189). PCR amplicons were sent to Eurofins firm for the Sanger Sequencing process. The DNA sequences were edited with BioEdit version 7.2.5 and aligned with BLAST (190, 191).

Table 6. Primers and PCR conditions used for getting PCR amplicons

<table>
<tr><th>Primers</th><th>PCR conditions</th></tr>
<tr><td>23S-F 5′-AGCACCGTAAGTTCGCGATAAG-3′</td><td rowspan="2">• Initial denaturation: 94^0C for 4 min.
• 30 cycles of 94^0C for 1 min.
• 56^0C for 45s
• 72^0C for 10 min</td></tr>
<tr><td>23S-R 5′-CTTTCAGCAGTTATCACATCC-3′</td></tr>
</table>

4 RESULTS

4.1. Cultivation of *Helicobacter pylori*

Typical morphology of *H. pylori* G27 colonies were observed on blood agar after incubation. As a result of Gram staining, Gram-negative curved bacilli were observed under a light microscope (Figure 5).

Figure 5 - The appearance of *H. pylori* colonies on Columbia sheep blood agar (at left) and in a light microscope at 100X magnification as Gram-negative curved bacilli (at right).

4.2. Agar Dilution Susceptibility Tests

4.2.1. Clarithromycin MIC value for *Helicobacter pylori* G27

The agar dilution tests were evaluated visually. The growth of bacteria was seen at a concentration of 0,015 and 0,03 µg/ml clarithromycin and on growth control media, however, no growth was seen at 0,06 µg/ml or upper concentrations. As a result, the MIC value of clarithromycin was determined to be 0,06 µg/ml (Figure 6).

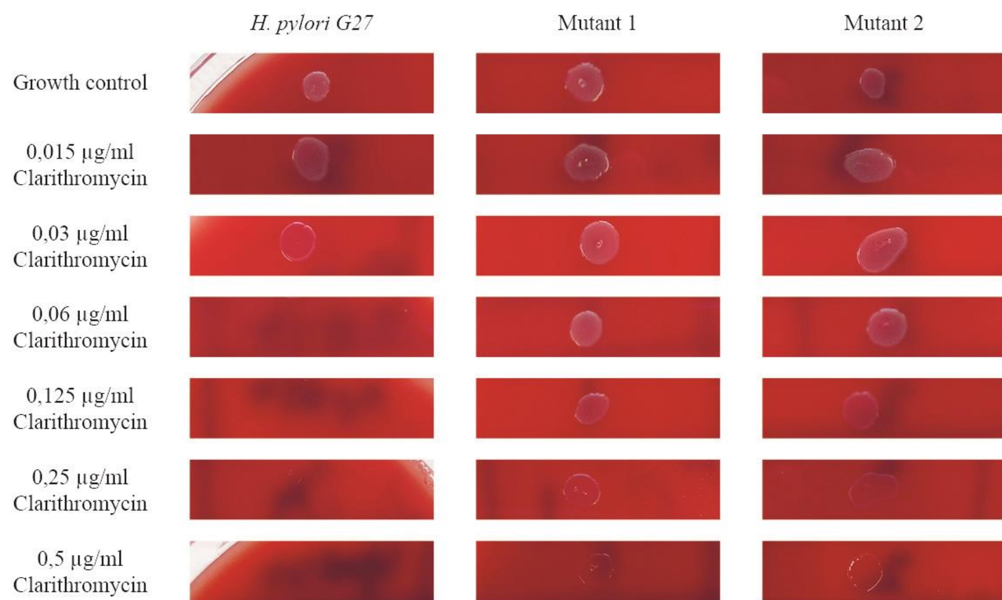

Figure 6. Agar dilution assays for clarithromycin susceptibility of the strains

4.2.2. Josamycin MIC value for *Helicobacter pylori* G27

As a result of incubation, growth was observed at 0,03 µg/ml, 0,06 µg/ml, and growth control media. The MIC value of josamycin was defined as the lowest concentration that did not result in growth on the plate. Consequently, MIC value was determined as 0,125 µg/ml (Figure 7).

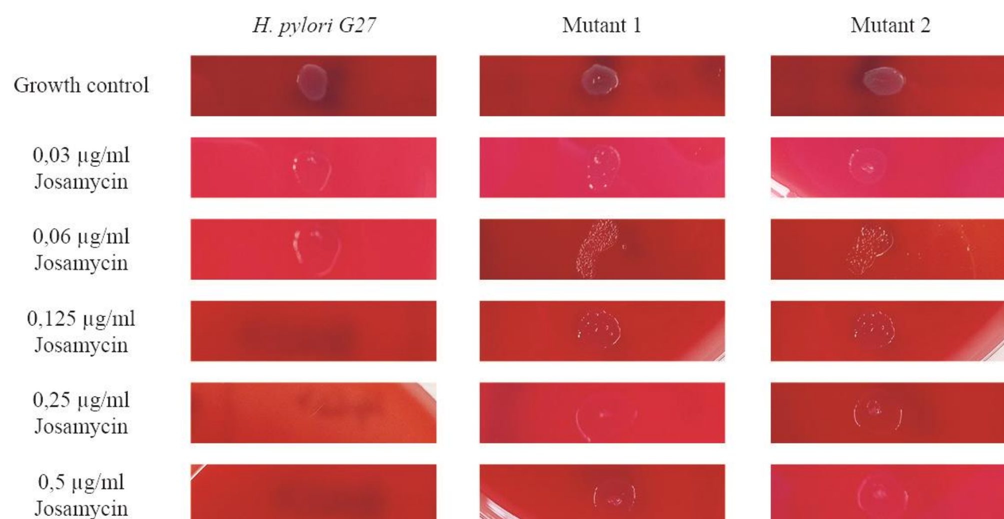

Figure 7. Agar dilution assays for josamycin susceptibility of the strains

4.3. Establishment of resistant strains

The reference strain *H. pylori* G27, which is susceptible to clarithromycin, was exposed to low concentrations of clarithromycin, and two resistant mutant strains were observed. Accordingly, mutant strain1 and 2 exhibited clarithromycin MIC values of 128 mg/ml and >256 mg/ml, respectively. Josamycin was found to have a MIC value of 8 and >16 mg/ml for mutant strains 1 and 2, respectively (Table 7).

Table 7. MIC values for clarithromycin and josamycin for parent and mutant strains

Strain	Clarithromycin MIC (µg/ml)	Josamycin MIC (µg/ml)
H. pylori G27	0, 06	0,125
Mutant-1	128	8
Mutant-2	>256	>16

4.4. Next Generation Sequencing for Detection of Mutations in Resistant Strain

The genome DNAs of *H. pylori* G27 and the mutant-1 strain were sequenced. The resulting genome sequences of the mutant-1 were compared with the *H. pylori* G27 genome. In the 23S rRNA region, a transformation of thymine to adenine at positions 1476, 1490, 1494, and 1495, and a transformation of guanine to thymine at position

1472 was observed. In addition, mutation A2142G was detected in the mutant-1 strain. Furthermore, a mutation in a gene that encodes for sulfite exporter TauE/SafE family protein was also observed. Due to this mutation, changes in the structure of the proteins occurred by the way Leucine converted to Proline (Table 8).

Table 8. Candidate mutations associated with clarithromycin resistance in Mutant-1 strain detected by whole genome sequencing

Strain	MIC (µg/ml)	CLA susceptibility	SNP	Gene	Amino acid change
Mutant-1	128	Resistant	A2142G	23S rRNA	None
			T1490A T1494A T1495A T1476A G1472T		
			A694384G	sulfite exporter TauE/SafE family protein	L>P

CLA: Clarithromycin; SNP: Single nucleotide polymorphism; A: adenine, T: thymine, G: guanine; L: leucine; P: proline

4.5. Sanger Sequencing

PCR results belonging to the amplicons are presented in Figure 8. As a result of sequence analysis, the A2142G mutation was detected in both Mutant-1 and Mutant-2 strains (Figure 8).

Figure 8. PCR amplification of *H. pylori* G27, mutant-1 and mutant-2 on the 1% agarose gel electrophoresis.

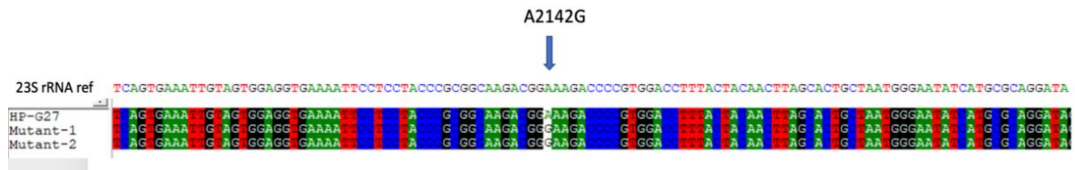

Figure 9. Multiple sequence alignment of 23S rRNA gene sequences of *H. pylori* G27, mutant-1 and mutant-2 compared to a reference gene.

5 DISCUSSION

The discovery of *H. pylori* infection and its role in various diseases, including gastric cancer, has completely changed the handle of the therapy for patients with this condition. Currently, the only available effective therapy for *H. pylori* infection is antibiotics. On the other hand, not many antibiotics can act against this bacterium that lives in acidic stomach conditions. According to guidelines, standard triple therapy, including a proton pump inhibitor, clarithromycin, and amoxicillin or metronidazole, has been used as first-line treatment for eradicating *H. pylori* in the region where clarithromycin resistance rates lower than 15%. The success of the treatment relies on factors like smoking, patient observance, and antibiotic resistance. Clarithromycin resistance was reported as the most critical factor in treatment failure (155, 192).

Clarithromycin is a bacteriostatic antibiotic that binds reversibly to the peptidyl transferase loop of domain V of the 23S rRNA molecule. As a result of this binding, protein elongation is inhibited, effectively preventing the synthesis of proteins in bacteria (140). In most Gram-negative bacteria, the resistance mechanism of clarithromycin or other macrolides was associated with modification of the target region (nucleotide mutations and methylation of the 23S rRNA) or via overexpression of the efflux pumps (193). On the other hand, the resistance to clarithromycin and other macrolides was reported depending on the various point mutations in the peptidyltransferase region of domain V of 23S rRNA in *H. pylori*. The most common mutation is the transformation of A to G at positions 2142 and 2143. In recent studies, other 23S rRNA gene mutations have also been reported that might be associated with clarithromycin resistance (194-196).

In the mutant-1 strain, six single nucleotide mutations at different positions in 23S rRNA were detected by next-generation sequencing. One of these mutations is A2142G which is frequently reported as associated with high clarithromycin MIC in the literature (197,198). Sanger sequencing was performed for the clarithromycin's target region to confirm the known mutation in mutant strains. Other mutations (T1476A, T1490A, T1494A, T1495A, G1472T) were detected out of the peptidyl

transferase region by whole genome sequencing. To our knowledge, these mutations were not reported before in the literature. Ultimately all these five candidate mutations also should be confirmed by the Sanger sequencing method. Furthermore, the relationship between clinical clarithromycin resistance and these new mutations should be studied using clinical strains or samples in future studies.

Clarithromycin-resistant strains with no mutation in their 23S rRNA are also reported indicating that unknown genes outside 23SrRNA are probably included in clarithromycin resistance in *H. pylori*. Binh *et al.* discovered two novel gene mutations (*infB* and *rpl22*) related to clarithromycin resistance in *H. pylori* that have synergic effects with 23S rRNA resulting in higher MICs (155). In this thesis study, these mutations were not observed.

A gene mutation as a novel candidate mechanism was observed in the clarithromycin-resistant mutant strain. This gene encodes the sulfite exporter TauE/SafE family protein. The mutation in this gen caused the alteration in amino acid structure from Leucine to Proline. Weinitschke *et al.,* reported that TauE/SafE proteins act as sulfite/organosulfonate exporters in the metabolism of C2 sulfonates (199). Furthermore, Rakitin et al. demonstrated that the two copies of genes that encode TauE/SafE family sulfite exporter were found in environmental isolates, not in clinical isolates of *Acinetobacter lwoffii* in their study, which compared the genomes of clinical and permafrost strains of *A. lwoffii*. Depending on the knowledge that organosulfonates are widespread in nature; they probably considered the utilization of organosulfonates by permafrost strains (200). To the best of our knowledge, the relationship between sulfite exporter TauE/SafE family proteins and antibiotic resistance has not been reported for any bacteria.

Based on this knowledge, if clarithromycin is found in the anionic form, it can be considered that as an anion transporter, the sulfite exporter TauE/SafE family protein might play a role as a drug efflux pump. Therefore, mutation in this mechanism can cause drug resistance. On the other hand, in the studies, organic anion transporter family members 1B1 and 1B3 were indicated as a target of clarithromycin in

mammalian cells. It was reported that clarithromycin is an inhibitor for these two proteins and causes interaction with the substrate drugs. (201). Therefore, It can also be predicted that inhibition of the sulfite exporter TauE/SafE family protein by a high dose of clarithromycin may cause cross-resistance for the substrate anionic antibiotics such as a fluoroquinolone, tetracycline, ampicillin, rifampicin that used in the treatment of *H. pylori* infection. Future works for performing the antibiotic susceptibility test for anionic antibiotics towards *H. pylori* G27 and its mutant are needed to confirm this hypothesis.

In this thesis study, the possible cross-resistance between clarithromycin and josamycin was also evaluated. A macrolide of low cost, josamycin is reported to be less effective than clarithromycin and has been suggested as a possible alternative but has not been tested in cases of drug resistance (202). There is very little research on josamycin activities in *H. pylori*. Baryshnikova *et al.* investigated the possibility of using josamycin instead of clarithromycin. Their study aimed to estimate the efficacy and safety of triple-modified eradication therapy with josamycin compared to the standard eradication therapy in patients infected with *H. pylori*. According to their results, modified triple eradication therapy with josamycin shows better efficacy and safety than standard triple eradication therapy. They showed that it could be recommended to use in clinical practice in case confirm these results with further trials (15). To the best of our knowledge, this thesis study is the first that evaluates the in vitro activity of josamycin on mutant strains at different MICs. Development of the resistance to josamycin was observed simultaneously with clarithromycin resistance in the achieved mutant strain in the study. Based on these results, we can predict that the usage of josamycin will not be practical in clarithromycin-resistant *H. pylori* infections. However, future studies should be performed that include clinical strains to confirm this prediction.

In this thesis study, during the bioinformatic analysis of the sequence results, it was observed that there were some breakings in the rRNA, and surprisingly, these breakings occurred in the same regions. These were predicted as hypothetically methylated sites and estimated caused bacterial resistance to antibiotics by decreasing

clarithromycin affinity and resulting in changing ribosome structure. In the literature, the clarithromycin and other macrolide resistance in Gram-positive and Gram-negative bacteria were associated with post-transcriptional methylation of the 23S rRNA (140,193).

However, to our knowledge, no study presents the relation between RNA methylation and clarithromycin resistance in *H. pylori*. Therefore, in case proving the putative finding in the thesis about methylation and clarithromycin resistance in *H. pylori*, it will be the first report in the literature. It is necessary to conduct further studies to determine whether the proof regions are differentially methylated to confirm this claim.

6 CONCLUSION

In conclusion, novel candidate resistance mechanisms for clarithromycin in *H. pylori* were suggested in this study. The new mutations detected in the 23S rRNA region that has not been reported before can be presented as targets for molecular diagnosis of clarithromycin resistance after validation studies. The other novel finding in the thesis is a new mutation in a gene that encodes sulfite exporter TauE/SafE family protein indicating new targets for future studies related to clarithromycin resistance or cross-resistance with other antibiotics. Furthermore, this study is crucial because it is the first to evaluate the in vitro activity of josamycin on clarithromycin resistance mutants. Since the cross-resistance to josamycin was observed in this study, it is possible to say this drug cannot be suitable as an alternative to clarithromycin. Still, advanced studies, including clinical strains and in-vivo studies, are also needed for confirmed this claim. Finally, hypothetically methylated regions in 23S rRNA were demonstrated in the mutant genome is important because it is the first time mentioned in this thesis study and can be a target for advanced studies.

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