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Molecular mechanisms of antibiotic resistance in *Helicobacter pylori*

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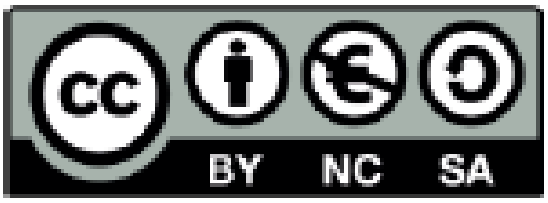

ABSTRACT

Molecular mechanisms of antibiotic resistance in *Helicobacter pylori*

Helicobacter pylori is a pathogen that has adapted to survive in the stomach, one of the harshest environments in the human organism. It is the aetiologic agent of several gastrointestinal system pathologies, such as peptic ulcer, gastritis and two types of cancer (gastric adenocarcinoma and MALT lymphoma, reason for which it is considered a class I carcinogen. Currently, there exist different drug combinations to treat the infection, but unfortunately, in recent years, the effectiveness of these drugs has significantly decreased, to the point of being totally lost in some cases. The main cause is the continuing emergence of resistances to these drugs. Nonetheless, the mechanisms that explain how *H. pylori* acquires these resistances are not fully understood. In this project, in addition to a bibliographic study, a genetic analysis of 52 strains of the pathogen from the Hospital Parc Taulí, was carried out, from which the main genes related to antibiotic resistance were analysed using genome sequences obtaind by Microbiology Unit of the Faculty of Pharmacy and Food Science of the Universitat de Barcelona. The results showed the existence of a wide variety of mutations in the different resistance genes, some of them not described in the studies published so far, which could lead to the antibiotic resistance of the strains studied. These results, together with findings from the literature review, prove that the development of resistance to antibiotics in *H. pylori* is a multifactorial process in which a large variety of mechanisms acts concurrently to confer multidrug-resistance.

Key words: *Helicobacter pylori*, molecular mechanisms, antibiotic resistance

RESUM

Mecanismes moleculars de resistència als antibiòtics en *Helicobacter pylori*

Helicobacter pylori és un patogen que s'ha adaptat per sobreviure a l'estómac, un dels ambients més hostils de l'organisme. És l'agent etiològic de diverses patologies del sistema gastrointestinal, com la úlcera pèptica, la gastritis i dos tipus de càncer (adenocarcinoma gàstric i limfoma MALT), motiu pel qual es considera un carcinogen de classe I. Actualment, existeixen diferents combinacions terapèutiques per a tractar la infecció, però, malauradament, en els darrers anys, l'efectivitat d'aquestes s'ha vist significativament reduïda, fins al punt d'haver-se perdut totalment en alguns casos. La principal causa és la continua aparició de resistències a aquests fàrmacs. No obstant, els mecanismes que expliquen com *H. pylori* adquireix aquestes resistències no es coneixen del tot. En aquest projecte, a part d'un estudi bibliogràfic s'ha fet un estudi genètic de 52 soques procedents de l'Hospital Parc Taulí, de les quals s'han analitzat els principals gens relacionats amb les resistències als antibiòtics utilitzant les seqüències genòmiques obtingudes per la Unitat de Microbiologia de la Facultat de Farmàcia i Ciències de l'Alimentació de la Universitat de Barcelona. Els resultats van mostrar l'existència d'una gran varietat de mutacions en els diferents gens de resistència, algunes no descrites als estudis publicats fins ara, que podrien donar lloc a la resistència de les soques estudiades. Aquests resultats, juntament amb els obtinguts de la cerca bibliogràfica van demostrar que el desenvolupament de les resistències als antibiòtics en *H. pylori* és un procés multifactorial en el que una gran varietat de mecanismes actuen conjuntament per atorgar multiresistència a *H. pylori*.

Paraules clau: *Helicobacter pylori*, mecanismes moleculars, resistència als antibiòtics

INTEGRATION OF THE DIFFERENT FIELDS IN THE PROJECT

The principal scope in which this project is framed is **Microbiology**, as it focuses on the study of *Helicobacter pylori*, a pathogenic microorganism responsible for producing multiple diseases in humans, and particularly on how, through multiple molecular mechanisms, it is capable of acquiring resistance to antibiotics.

In relation to the secondary areas, this project is integrated with the areas of **Biochemistry and Molecular Biology** and **Pharmacology and Therapeutics**. In relation to the former, since the appearance of molecular techniques at the turn of the 20th century, and especially with the development of whole genome sequencing techniques (WGS), the detection of resistance has become easier to carry out and more accessible, allowing more research teams to provide their knowledge on the subject. In this project, seven resistance-associated genes retrieved from the whole genomes of 52 *H. pylori* strains sequenced using WGS techniques were analysed in order to detect mutations potentially involved in *H. pylori* resistance to antibiotics.

Finally, because of the impact of antimicrobial resistance on the increasing incidence of eradication therapies failure, this work is also integrated in the Pharmacology and Therapeutics scope. The further study of known resistance mechanisms and the discovery of new ones will contribute to the optimisation of currently available treatments and will represent a step forward in the identification of new pharmacological targets and novel mechanisms of action that could be potentially applicable in the design and developing of new antibiotics.

SUSTAINABLE DEVELOPMENT GOALS (SDG)

This Final Degree Project is addressed to all those people who currently suffer or are at risk of suffering from diseases derived from *H. pylori* infection. Although this bacterium is found all over the world, its prevalence is much higher in developing countries, where infection rates can reach up to 90%, while in industrialised countries it is approximately 50%. The World Gastroenterology Organisation (WGO) stated in its 2021 report that low socio-economic status and poor healthcare conditions are the major determinants of *H. pylori* infection. These are particularly critical during the first ten years of life, when most people are first exposed to the pathogen. While 90% of cases of *H. pylori* infection are completely asymptomatic, eventually complications of various kinds can arise, such as peptic ulcer, gastritis or, in the most severe cases, malignant pathologies such as gastric adenocarcinoma or MALT lymphoma (mucosa-associated lymphoid tissue lymphoma). For this reason, it is essential to establish the earliest possible diagnosis of the disease in order to establish the best treatment. Unfortunately, low socio-economic status not only influences the prevalence of infection, but also prevents most vulnerable people from accessing diagnostic tests and available treatments. Different combinations of antibiotics and inhibitors of acid secretion in the stomach, such as proton pump inhibitors or bismuth salts, are currently available for the treatment of *H. pylori* infection. However, in recent years, most of these combinations have become less effective or even ineffective against the infection. This alarming situation is due to the continuing emergence of antibiotic resistance.

Antibiotic resistance occurs naturally over time, but can be accelerated by factors such as inappropriate use of antibiotics, lack of access to health care, and the presence of antibiotics in soil, crops and water. The US Centers for Disease Control and Prevention (CDC) describes antibiotic resistance as one of the greatest public health threats of our time. As a consequence of this alarming reality, in 2017 the World Health Organisation published a list of three categories of bacteria for which the development of new antibiotics was urgently needed. Category 2 of the list, which comprises high-priority microorganisms, includes well-known pathogens such as methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *Enterococcus faecium* (VRE) as well as clarithromycin-resistant *Helicobacter pylori*. This paper aims to contribute to the increase of knowledge about the molecular mechanisms of antibiotic resistance in this pathogen. For this purpose, the different mechanisms related to the resistance of *H. pylori* to the different antibiotics used in the treatment of the infection and the genes and proteins involved in it are discussed. In addition, the results of the molecular detection of resistance mutations of 52 *H. pylori* strains along seven genes associated with resistance to different antibiotics are presented.

In accordance with the above, it is considered that this research project is mainly covered by **Goal 3 "Health and well-being"**, specifically goals 3. 8: "achieve universal health coverage, access to quality essential health services and access to safe, effective, affordable and quality medicines and vaccines for all", and 3d: "strengthen the capacity of all countries, particularly developing countries, in early warning, risk reduction and risk management for national and global health".

Parallely, this project has an impact on **Goal 1 "End poverty"**, specifically on target 1.2: "reduce by at least half the proportion of men, women and children of all ages living in poverty in all its dimensions according to national definitions", and on **Goal 6 "Clean water and sanitation"**, specifically on target 6.3: "By 2030, achieve access to adequate and equitable sanitation and hygiene for all ad end open defecation, paying special attention to the needs of women and girls, and those in vulnerable situations. Lastly, this research has a potential impact on **Goal 9 "Industry, innovation and infrastructure"** because its conclusions can contribute to a deeper comprehension of *H. pylori* antibiotic resistance mechanisms which can be applied in the design and development of new antibiotics to treat multiresistant *H. pylori* infections. Sepecifically, this project is framed under target 9.5: "Enhance scientific research, upgrade the technological capabilities of industrial sectors in all countries, in particular developing countries, including, by 2030, encouraging innovation and substantially increasing the number of research and development workers per 1 milion people and public and private research and development spending.

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1. INTRODUCTION

1.1. History and discovery

On 11 June 1979, during the analysis of a routine gastric biopsy showing severe chronic active gastritis, Dr. Robin Warren (North Adelaide, Australia, 1937) first observed a previously unknown microorganism, which would be named later as *Helicobacter pylori*. Through the histological study of the sample, the Australian pathologist observed that the cell nuclei were misaligned, the mucus layer was significantly reduced and he noticed the presence of intra-epithelial polymorphonuclear leukocytes. Using Warthin-Starry silver stain and examining the sample at 1000x magnification, he observed small spiral-shaped bacilli in close proximity to the epithelial surface. This discovery collided head-on with the dogma that until then had been considered unquestionable: the human stomach was not a sterile environment **(1)**. Warren continued his research alone until 1981 when he was joined by young researcher Barry Marshall, who needed to undertake a research project to complete a clinical fellowship programme at the Royal Australian College of Physicians. The success of both researchers, who have become colleagues, came in 1982 when they succeeded in obtaining cultures of the helical-shaped bacterium after numerous failed attempts using the technique described by Skirrow in 1977 for the isolation of bacteria belonging to the genus *Campylobacter*. After letting the cultures incubate for seven days, during Easter Holiday, they obtained for the first time a culture of the unknown bacterium. As a result of preliminary investigations, it was assigned to the genus *Campylobacter*, as it showed several characteristics of this genus, being named *Campylobacter pylori* **(1, 2).**

In 1984, two years after this discovery, Dr. Marshall was determined to prove Koch's postulates (Robert Koch, 1884). To do it, Marshall relied on the case of a patient with signs of dyspepsia, whose biopsy showed the presence of numerous *C. pylori*. After receiving a dual treatment of bismuth salts and metronidazole, the patient was healed. On these grounds, Dr. Marshall decided to ingest 30 mL of the culture obtained from the biopsy after having administered himself a 400 mg dose of cimetidine in order to inhibit the stomach hydrochloric acid production **(2)**. After a week, he developed symptoms of gastritis and the collected cultures showed the growth of *C. pylori*. For that reason, he started a 14-day therapy based on bismuth salts and metronidazole (the same treatment the patient received). The treatment was a success as the endoscopy performed one month later after its finalization showed no histological signs of gastritis and the taken bacteriological cultures were negative, indicating that the bacteria had disappeared **(2).**

Because of their findings, Drs. Warren and Marshall jointly received the Nobel Laureate in Physiology and Medicine in 2005.

The classification of this new bacterium in the genus *Campylobacter* was based on the fact that it shared different morphological, metabolic and biochemical characteristics with other members of this group, including the spiral shape and microaerophilia. Although many of these were shared within the genus, two of them marked the difference between *C. pylori* and all other members of the genus. These are the presence of multiple flagella on one pole of the cell and the high urease content **(2)**.

In 1989, Stewart *et al*. published a research in which, considering different parameters such as the absence of methylated menaquinone-6 (MNK-6) or the differences in susceptibility to antibiotics, they concluded that *C. pylori* could not belong to this genus. According to this statement, it was suggested that it might belong to the genus *Woinella*. However, this assumption was later rejected due to differences in ultrastructure, morphology, growth characteristics and enzymatic capabilities. In the end, the creation of a new genus, *Helicobacter*, was proposed and *Campylobacter pylori* was reclassified as *Helicobacter pylori* **(3)**.

1.2. Epidemiology and transmission

Helicobacter pylori is the aetiological agent of multiple pathologies affecting the gastrointestinal system. It has been associated with the development of gastritis, peptic ulcers, gastric adenocarcinoma and mucosa-associated tissue lymphoma (MALT), and is considered a risk factor for the development of these diseases. It is one of the most prevalent infectious agents worldwide, with a prevalence rate of 90% in developing countries and between 25-50% in developed ones **(4)**.

A meta-analysis conducted in 2018 by Zamani *et al* states that Southern Africa region have the highest infection rate in the world, 86.8% [83.3-90.3%, 95% CI) whereas the lowest infection rate is reported in North America, where the infection rate stands at 25.8% [20.7-36.3; 95% CI] **(5)**. Regarding country-specific data, the most affected countries are Nigeria (87.7%), Portugal (86.4%), Estonia (82.5%), Pakistan (81.0%) and Kazakhstan (79.5%). Conversely, the countries with the lowest rates of *H. pylori* infection are Switzerland (18.9%), Denmark (22.1%), New Zealand (24.0%), Australia (24.6%) and Sweden (26.2%) **(6)**. These data, in addition to those for other countries, are graphically represented in **Figure 1**.

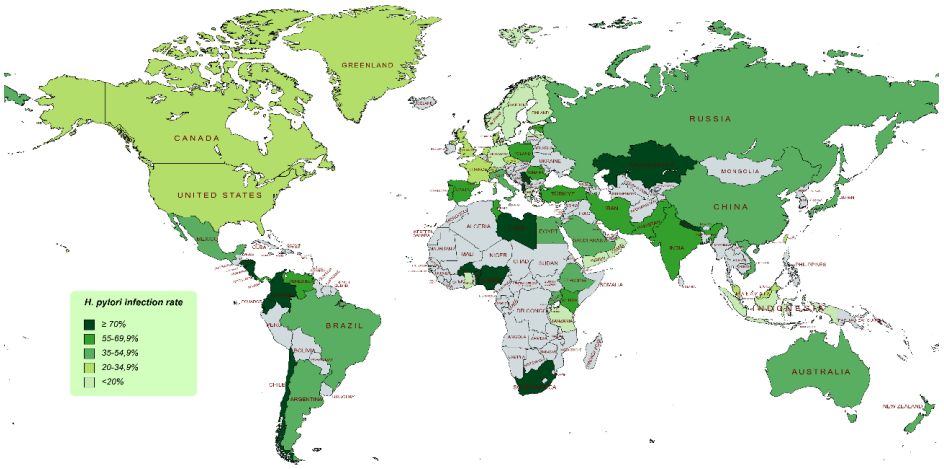

Figure 1. *Helicobacter pylori* infection rates in different countries around the world (obtained and modified from reference **(6)**).

The infection is mainly acquired during childhood, usually in the first 10 years of life. No consensus has yet been reached on the pathogen main mechanism of transmission but several epidemiological studies suggest the faecal-oral route as one of the most feasible answers to this unknown. Upper prevalence rates in developing regions reflect the fact that a low socioeconomic status and/or poor healthcare conditions during infancy are two determining factors contributing to the high prevalence of *H. pylori* in those regions **(7)**. In relation to the association of *H. pylori* infection with the aforementioned diseases, approximately 10-15% of the infected population will develop peptic ulcer, while the risk linked to the development of gastric adenocarcinoma or MALT lymphoma in those infected by the pathogen stands at 2–3% and 0.01%, respectively **(8)**. Due to its association with the potential development of neoplastic pathologies, the International Agency for Research on Cancer (IARC), catalogued *H. pylori* as a class I carcinogen in 1994, being the only bacterium belonging to this category **(9)**.

1.3. Diagnosis of the infection

For the diagnosis of *H. pylori* infection, various methods are currently available. These are classified into two groups, invasive methods, which are those requiring endoscopy or biopsy, and non-invasive methods **(10)**. All methods have both advantages and drawbacks related to their applicability. Invasive methods include histopathology, the Rapid Urease Test (RUT), culture, and the Polymerase Chain Reaction (PCR), which are conducted on a biopsy specimen. Non-invasive diagnostic methods, on the other hand, include the detection of antibodies from a patient's serum or urine sample, the Urea Breath Test (UBT) and the Stool Antigen Test (SAT) **(10, 11).**

According to the Position Paper of the Catalan Society of Digestology on the management of *H. pylori* infection, the UBT constitutes the preferred method for the diagnosis of *H. pylori* infection both before and after treatment. The same document considers SAT as an alternative to UBT in the pre-treatment diagnosis of the infection **(12)**. Nevertheless, none of the diagnostic methods is considered a gold standard and at least two matching tests are required to either confirm or reject the diagnosis **(11)**. The different diagnostic methods currently used have been listed in **Table 1**.

Table 1. Method used for the diagnosis of *Helicobacter pylori* infection, classified according whether they are invasive or non-invasive. **(10)**.

Method group	Diagnostic Method	Sensitivity	Specificity
Non-Invasive	Urea Breath Test (UBT)	95,9%	95,7%
	Stool Antigen Test (SAT)	94,0%	97,0%
	Serology	76-84%	79-90%
Invasive	Rapid Urease Test (RUT)	>90%	>90%
	Histopathology	80-95%	99-100%
	Bacteriological Culture	70-80%	100%
Invasive/Non-invasive	Polimerase Chain Reaction[a] (PCR)	>95%	>95%

[a]PCR can be classified as invasive or non invasive diagnostic method depending on the sample from which amplification is performed.

Considering both the sensitivity and specificity values of the different diagnostic methods, false negatives and false positives are unusual. However, even though being relatively seldom, these actually occur. False negatives may occur because none of the aforementioned tests has a sensitivity of 100%, being reduced if the patient has taken antibiotics over the previous 4 weeks, or proton pump inhibitors in the previous 2 weeks, or in case of gastrointestinal bleeding. False positives can arise because of the presence in the sample of other urease-producing bacteria like *Proteus mirabilis*, *Citrobacter freundii*, *Klebsiella pneumoniae*, *Enterobacter cloacae* and *Staphylococcus aureus* **(10, 11)**

1.4. Phenotypic and cultural characteristics of *Helicobacter pylori*

Helicobacter pylori is a gram-negative, spiral-shaped bacillus with a size of 0,5µm x 3µm. It belongs to the family *Helicobacteriaceae*, order *Campylobacterales*, class *Epsilonproteobacteria*, which is included in the *Pseudomonadota* phylum **(13)**. It is characterised by having 4 to 7 lophotrophic flagella, which are essential for its motility. These are coated with a lipidic sheath structurally similar to the outer membrane. The purpose of this sheath is primarily protective, as its function is to prevent degradation of the flagella by the acidic environment of the stomach **(14)**. *H. pylori* is mainly known for its typical spiral flagellated bacillary form. However, when under adverse condition for its development and growth (extreme temperatures, nutrient deficiency, increased oxygen pressure or presence of antibiotics), it is able to modify its morphology and adopt a coccoid form **(Figure 2) (14)**. This, unlike the bacillary form, is viable but non-culturable and is therefore known as CVNCF (Coccoid Viable Non-Culturable Form) **(14)**. This alternative form is generally resistant to antibiotics and can survive for up to one year under unfavourable conditions.

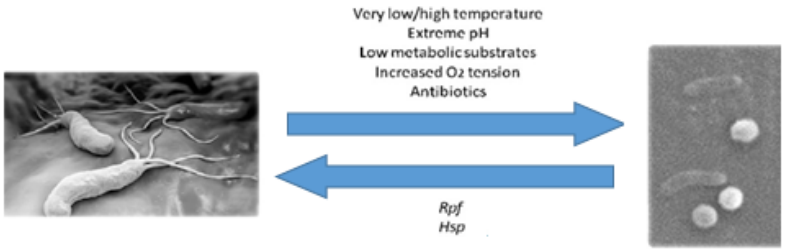

Figure 2. Transition from spiral-shaped viable form of *H. pylori* to viable but non-culturable (VBNC) coccoid forms (scanning electron microscope) and factors affecting this process. *Rpf* = resuscitation-promoting factor; *Hsp* = heat shock protein **(15)**.

The transition from the bacillary to the coccoid form is not direct, occurring via two intermediate forms called V and U. The coccoid form is not culturable axenically and it must be identified by electron microscopy and molecular techniques **(15)**. The survivability of *H. pylori* in this coccoid form, as well as the modification of its metabolic and morphological characteristics, may be a feasible explanation for therapeutic failure during treatment of the infection, when this is not due to the presence of antibiotic resistance **(14, 15)**

Growing *H. pylori* under laboratory conditions is a process that can be quite complex and for that reason it is considered a fastidious pathogen. For primary isolation in the laboratory, nutrient-rich media are required **(16)**. These include Columbia agar, which is a specific type of blood agar, Mueller-Hinton and Brucella Broth. The latter both require supplementation with blood or Fetal Bovine Serum (FBS) at a 5-10% concentration. *H. pylori* must be incubated in microaerophilic conditions (5–10% O_2, 5–10% CO_2, 80–90% N_2 and a 95% humidity) at a temperature between 35-39ºC, being 37ºC the optimal growth temperature. Although growth is commonly observed between the second and fifth day, in clinical practice cultures are incubated for 10 days before considered negative **(16)**.

One of the setback that can occur during isolation of *H. pylori* is the overgrowth of contaminants that can also be found in the biopsy. One solution to this problem is the addition of inhibitors to the culture medium that prevent the growth of these contaminants without affecting the viability of *H. pylori*. For this purpose it can be used the Dent supplement, which contains vancomycin, trimethoprim, cefsoludin and amphotericin B. *H. pylori* is a chemoorganotrophic microorganism with a respiratory metabolism. In terms of biochemistry, *H. pylori* tests negative for methyl red and Vogues-Proskauer and has three enzymes particularly useful for its identification: oxidase, catalase and urease, testing positive to all three **(17)**.

1.5. Pathogenicity: virulence factors

As mentioned above, *H. pylori* is closely linked to the development of multiple pathologies affecting the gastrointestinal system, mainly the stomach. The severity of these diseases is influence by various environmental and genetic factors, which are both host-related and pathogen related **(Figure 3) (18)**. *H. pylori* virulence factors are not only related to the induction of inflammatory responses, but have been shown to control and regulate these responses, allowing chronic inflammation **(19)**. These include enzymes, proteins, multiple antigens and even subcellular structures.

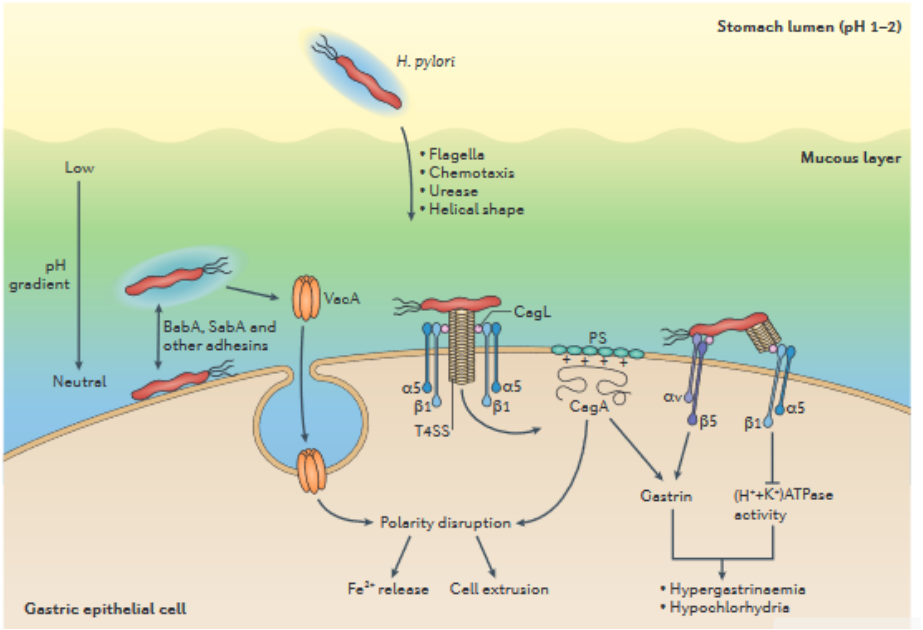

Figure 3. Main virulence factors of *Helicobacter pylori* **(20)**

1.5.1. Flagella

The lophotrophic flagella of *H. pylori* are a virulence factor whose presence is essential for colonisation to take place, as it has been shown that different mutant strains of the pathogen lacking these structures are unable to stablish infection. However, it has been demonstrated that the presence of flagella as the sole pathogenicity factor is not sufficient for colonisation and their synergic action together with others is required to stablish a persistent infection **(Figure 3) (21)**. A study published in 2014 showed that *csrA* mutant strains have decreased motility and adhesion compared to wild-type strains due to defective flagella formation caused by CsrA protein deficiency **(22)**.

1.5.2. Urease

In order to establish colonisation of the gastric epithelium and subsequent infection, apart from the ability to move, *H. pylori* requires neutralisation of the hydrochloric acid (HCl) present in the gastric juice. For this purpose, it has urease at its disposal. Urease catalyses the hydrolysis of urea into ammonia and carbamate, which is subsequently broken down into carbonic acid and a second ammonia molecule that promotes the alkalinisation of the medium surrounding the pathogen, allowing it to survive **(Figure 3) (18, 19)**. This reaction requires the use of nickel (Ni^{2+}) as a cofactor, which is taken from the host blood by the outer membrane proteins FecA3 and FrpB4 **(14)**. Although this enzyme is particularly useful for identification and is the basis for some diagnostic methods, as discussed above, it is not unique to *H. pylori* and can be found in other bacteria. Like flagella, urease plays an essential role in the colonisation of the gastric epithelium. There exists a relationship between urease activity and the morphological form of *H. pylori*, with urease activity being higher in the bacillary form compared to the coccoid form **(19)**.

1.5.3. Ahesion factors

The adhesion of *H. pylori* to the gastric epithelium represents an essential stage of the infection's pathogenesis. This process has two main functions: to protect the pathogen from the body's natural elimination mechanisms such as gastric emptying or peristaltic movements and to serve as a route for nutrient uptake and inoculation of its toxins into the host cell **(14)**. Within the proteins involved in the adhesion of the bacterium to the gastric mucosa are BabA/B/C, SabA and AlpA/B, which belong to the OMP (Outer Membrane Protein) family, and OipA, HopZ and HopQ, belonging to the HOP (Outer Membrane Porins) family **(Figure 3) (14, 18, 19).**

1.5.4. Cytotoxins

1.5.4.1. Citotoxin-associated gene A

The citotoxin-associated gene A (CagA) is encoded at one end of the *cagPAI* pathogenicity island, a locus approximately 40 kB in size, which codes for 32 genes. Among these are the gene coding the CagA toxin and genes coding for components of the type IV secretion system (T4SS) **(Figure 3) (21)**. The *cagPAI* is found in the most virulent strains of *H. pylori* but is absent in less virulent ones. The different strains can be classified accordingly as *cagA+* or *cagA-* **(18)**. CagA is a 120-140 kDa bacterial oncoprotein with a high immunogenic capacity **(21)**. When *H. pylori* is attached to the host cell, it injects, via the T4SS, the CagA protein into the cytoplasm of the cell. Once inside, it is phosphorylated at tyrosine residues by Src and Abl family kinases in the EPIYA domains **(23)**. These domains are located at the C-terminus of the protein and consist of the amino acid sequence Glu-Pro-Ile-Tyr-Ala **(23)**. EPIYA motifs can be classified as EPIYA-A, EPIYA-B, EPIYA-C and EPIYA-D. The repetition of these sequences is linked to the virulence level. *H. pylori* strains with a higher number of EPIIYA-C motifs or the presence of EPIYA-D have been associated with an increased risk of gastric cancer **(18)**.

Once inside the cell and when phosphorylated, it promotes the expression of pro-inflammatory cytokines and chemokines and alters the regulation of cell signaling pathways, both mitotic such as PI3K-Akt or β-catenin-Wnt pathways, and apoptosis by interfering with tumour suppressor genes such as p53 or RUNX3. All these pathways and genes are involved in the control of cell shape, adhesion and transformations, which are all significantly altered in neoplasic diseases **(21, 24)**.

1.5.4.2. Vacuolating cytotoxin A

Vacuolating cytotoxin A (VacA) is initially synthesised as a 140-kDa precursor, which, after proteolytic maturation, becomes the mature and active form with a mass of 88 kDa. When *H. pylori* is attached to the gastric cell, it secretes VacA via a V-type secretion system, remaining in a soluble form. VacA then binds to its membrane receptors that include RPTPα, RPTPβ, LRP-1, sphingomyelin and CD-18. The latter is a β-integrin found on the membrane of T lymphocytes **(15)**. The structure of the mature protein consist of two subunits, p33 and p55. The former is located at the N-terminal end while the latter corresponds to the C-terminus. VacA activity in the host cell contributes to multiple functional and structural alterations **(Figure 3)**. In the mitochondria, VacA toxin causes a decrease in membrane potential, induces the activation of BAX and BAC, two pro-apoptotic proteins of the Bcl2 family, and facilitates the release of mitochondrial cytochrome C. This triggers mitochondrial fragmentation and ultimately cell apoptosis **(15, 25)**. Other observed effects of VacA include interference with some immune mechanisms such as phagocytosis or antigen presentation **(15, 21)**. VacA is encoded in the homonymous gene *vacA*. This gene has three main polymorphic regions, *signal* (*s*), *intermediate* (*i*) and *mid* (*m*), with each region having two main alleles (1 and 2) **(26)**. In contrast to CagA, all *H. pylori* strains produce the VacA toxin, but its activity is not equal. The variation of the amount of vacuolating activity in each strain is precisely due to allelic variability in the *vacA* gene. Considering the possible allelic combinations, an association between genotype and VacA activity has been determined. VacA activity is higher in strains with de *s1i1m1* genotype whereas it is lower, or even absent, in those strains with the *s2i2m2* genotype **(26)**. The structure of both, the gene and the toxin are shown in **Figure 4.**

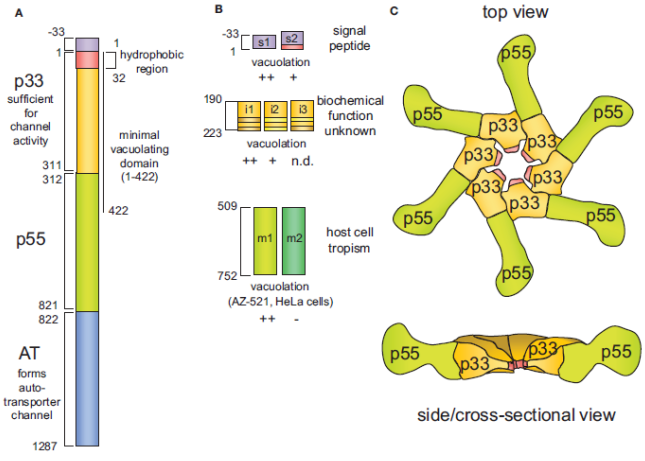

Figure 4. *Helicobacter pylori vacA* gene and VacA cytotoxin structures **(27)**

1.5.5. Other virulence factors

In addition to the virulence factors mentioned above, others have been described. These include HrgA, GGT, HP-NAP, etc. The restriction endonuclease-replacing gene A (*hrgA*) is considered an overriding determinant in the pathogenesis of *H. pylori*-associated gastric diseases such as adenocarcinoma **(21)**. GGT (γ-glutamyl transpeptidase) catalyses the transpeptidation and hydrolysis of the γ-glutamyl group and glutathione **(18)**. The enzyme has been linked to increased levels of IL-8 and oxide peroxide and

to the alteration of different biological processes leading to cell cycle dysregulation **(19, 21).** The neutrophil-activating protein (HP-NAP) enhances the activation of neutrophils and mast cells and promotes the secretion of multiple pro-inflammatory factors (IL-8, MIP1α, MIP1β, TNFα, IL-6 etc). It also facilitates the adhesion of neutrophils to gastric epithelium and induces alterations in cell tight junctions and in basal membranes. It also facilitates growth of *H. pylori* **(19, 21)**.

1.6. Treatment

In recent decades, several consensus documents and clinical practice guidelines have been published on the management of antibiotic treatment of *H. pylori* infection. Some examples are the Maastricht VI/Florence consensus report, published in 2022, or, more specifically in Spain, the clinical practice guideline based on the V Spanish Consensus conference on the treatment of *Helicobacter pylori* infection, published in 2021. Both documents state that, to be considered effective, a treatment must be able to eliminate the infection in at least 90% of treated patients **(28, 29)**. At present, there is a wide variety of therapeutic approaches available to treat *H. pylori* infection. The choice of first-line treatment depends mainly on the clinical situation of the patient, as well as local resistance rates to the different antibacterial agents. In Spain, classical triple therapy (PPI+CLR+AMX) is not recommended unless the CLR resistance rate is below 15%, as with higher rates, the efficacy of treatment drops below acceptable limits **(28, 29)**. Therefore, it has been progressively replaced in the last few years by other therapies. These are described in **Table 2.**

Table 2. Recommended *H. pylori* eradication treatments.

Concomitant quadruple therapy		
PPI	s.d / 12 h	14 days
AMX	1 g / 12 h	
CLR	500 mg / 12 h	
MTZ	500 mg / 12 h	
Quadruple therapy with bismuth (Pylera®)		
PPI	s.d / 12 h	10 days
Pylera®	3 capsules / 6 h	
Quadruple therapy with Levofloxacin		
PPI	s.d/12h	14 days
AMX	1 g / 12 h	
LEV	500 mg / 24 h	
BSC	240 mg / 12 h	
Therapy with rifabutin (with/without bismuth subcitrate)		
PPI	s.d / 12h	10-12 days
AMX	1 g / 12 h	
RFB	150 mg / 12 h	
BSC(±)	240 mg / 12 h	

PPI=proton pump inhibitor, AMX=amoxicillin, CLR=clarithromycin, MTZ=metronidazole, BSC=bismuth subcitrate, RFB=rifabutine. Pylera® is a commercial drug that includes BSC, MTZ and TCL Hcl in doses of 140 mg, 125 mg and 125 mg respectively. s.d=standard dosage (modified from **(28)**)

1.7. Antibiotic resistance

Since the turn of the twenty-first century, *H. pylori* eradication rates and, consequently, treatment effectiveness have been steadily declining. The decrease in the effectiveness or even treatment failure is due to the continuous emergence of resistance against one or multiple antibiotics used in the different dosing regimens **(30)**.

1.7.1. Prevalence of antibiotic resistance

1.7.1.1. Amoxicillin

Amoxicillin (AMX) is the classic example of a β-lactam antibiotic and is the key drug of most first-line therapies against *H. pylori*. Compared to other antibiotics such as clarithromycin (CLR) or rifabutin (RFB), the resistance rate to AMX is relatively low. In fact, it is the antibiotic with the lowest associated resistance rate. Even so, it has been increasing in recent years. In 2006, the resistance rate was between 0.8-1.4% and by 2021, it had reached 10% **(31, 32)**.

1.7.1.2. Metronidazole

Metronidazole (MTZ) is the antibiotic used against *H. pylori* that currently has the highest resistance rates. In 2018, the resistance rate of *H. pylori* to MTZ in European countries was 38.9% **(33)**. Comparatively, prevalence values for *H. pylori* resistance to MTZ in other regions were higher. These include Africa (91%), Southeast Asia (59%) and the Western Pacific (55%) **(34)**. The overall prevalence of *H. pylori* MTZ resistance is shown in **Figure 5**.

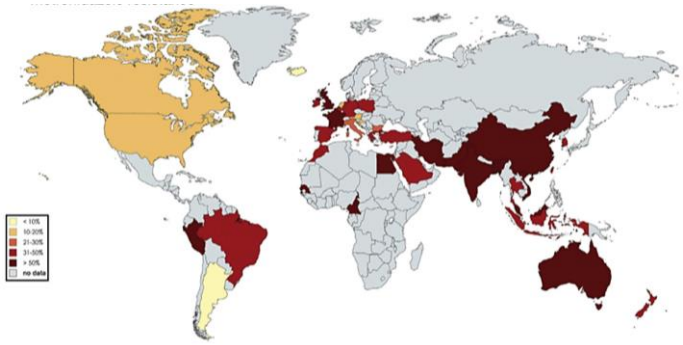

Figure 5. Pooled prevalence of resistance to MTZ during the period 2006-2016. Obtained from **(34)**

1.7.1.3. Clarithromycin

The resistance rate of *H. pylori* to CLR, a macrolide antibiotic, has increased significantly in most regions of the world **(35)**. In 2009, the rate of *H. pylori* resistance to clarithromycin in southern Europe countries was 21.5% **(36)**. In northern Europe countries the rate was lower (7.7%) **(36)**. By 2018, the average prevalence of CLR resistance in Europe stood at 32% **(34)**. This trend has been described in other regions of the world. In the United States, the *H. pylori* resistance rate to CLR increased from 13% in 2002 to 32.3% in 2016 **(37, 38)**. Similarly, the rate of *H. pylori* resistance to CLR in China was 55.03% in 2022, compared to 14.8% in 2000 **(39, 40)**. The overall prevalence of *H. pylori* resistance to clarithromycin is represented in **Figure 6.**

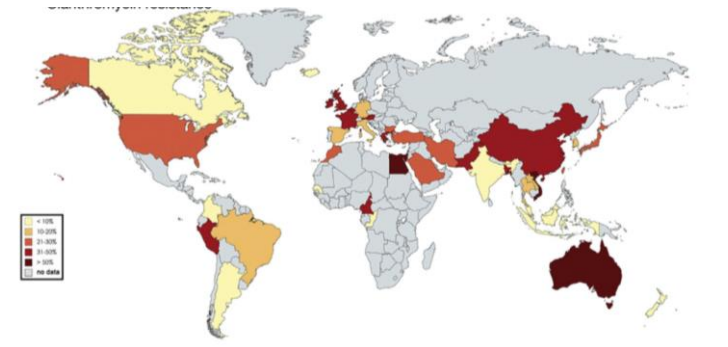

Figure 6. Pooled prevalence of resistance to CLR during the period 2006-2016. Obtained from **(34)**

Against this backdrop, in 2017 the World Health Organisation included clarithromycin-resistant *H. pylori* on its priority list for research and development of new antibiotics, with a high priority level (Level 2). Other resistant pathogens such as methicillin-resistant *Staphylococcus aureus* (MRSA) or vancomycin-resistant *Enterococcus faecium* (VRE) are also on the same level of the list **(41)**. The complete list can be found in **Annex 1**.

1.7.1.4. Levofloxacin

Levofloxacin (LEV) resistance is associated with the widespread use of fluoroquinolone antibiotics **(42)**. The rate of *H. pylori* resistance to this antibiotic varies significantly by region. In Europe, the resistance rate is 14-16% **(34, 36)** while in other regions of the world, such as Asia or the Western Pacific it reaches 30% and 24% respectively **(34)**. The global prevalence of *H. pylori* resistance to LEV is illustrated in **Figure 7.**

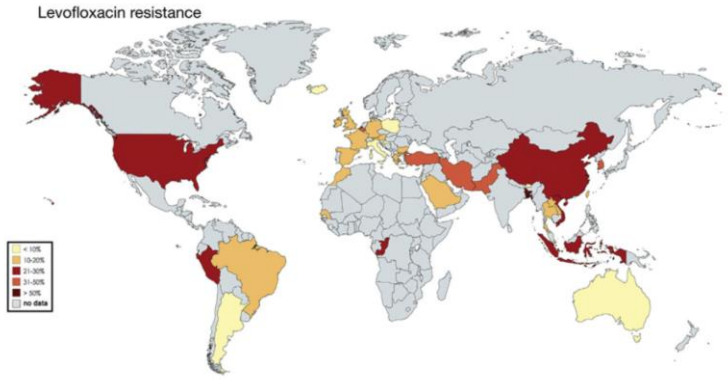

Figure 7. Pooled prevalence of resistance to LEV during the period 2006-2016. Obtained from **(34)**

1.7.1.5. Tetracycline

Currently, tetracycline (TET) is mainly used as a second or third line treatment when AMX, CLR or MTZ have proven to be ineffective **(43)**. Thus, resistance rates are generally low (<5%) **(44)**. However, in developing countries –mainly for economic reasons– the antibiotic is still used extensively **(43)**. This leads to higher resistance prevalence in some regions such as Africa, where the resistance rate to TET is estimated to be 48,7% **(45)**.

1.7.1.6. Rifabutin

Rifabutin (RFB) is an example of rifamycins. It is mainly used in resistant infections as a second-line treatment **(43)**. Resistance rates to this antibiotic are generally low in the major part of the globe (<5%) **(44)**. The main drawbacks of rifabutin-based therapies are the associated risk of reversible myelotoxicity and the potential for increasing resistance rates of *Mycobacterium tuberculosis* to this group of antibiotics **(46)**.

1.7.2. Helicobacter pylori resistance mechanisms: general concepts and resistance patterns

H. pylori antibiotic resistance can be explained through different mechanisms. Such mechanisms can be classified into three main groups: genetic changes, physiological alterations and cellular adaptations **(47)**. In addition, there are other mechanisms, such as enzymatic inactivation, can also explain some cases of resistance **(7)**. **Figure 8** shows a general scheme of the principal resistance mechanisms described in *H. pylori*.

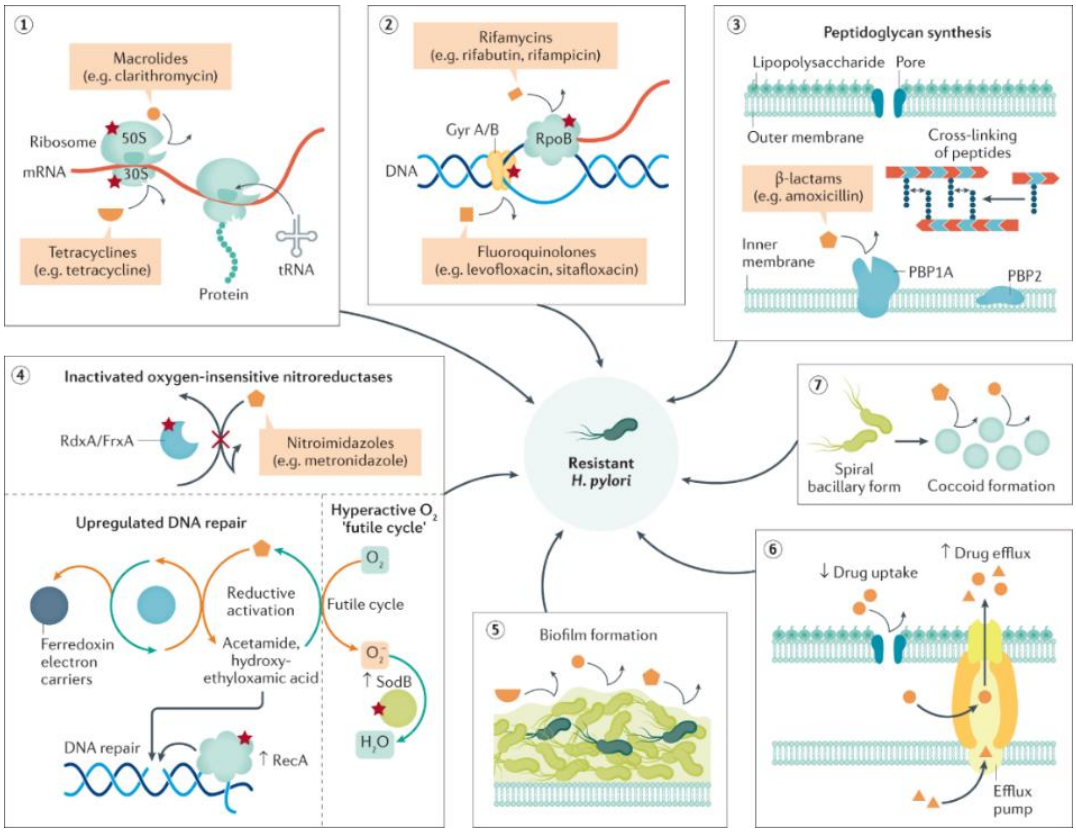

Figure 8. Main resistance mechanisms described in *H. pylori*. Obtained from **(47)**

Three resistance patterns have been described for *H. pylori*: single-drug resistance (SDR), multidrug resistance (MDR) and heteroresistance (HR). The SDR pattern refers to the resistance of *H. pylori* to each antibiotic family independently. In most cases, this family-related resistance is due to genetic alterations that cause both alterations in the antibiotic targets or in their activation process, as in the case of metronidazole. These alterations are mostly point mutations, mainly located in the bacterial chromosome **(47)**. Multidrug resistance (MDR) is described as the resistance of one strain of *H. pylori* to three or more antibiotics from different families **(44)**. In general, a strain can become multi-resistant if multiple mutations that induce resistance to each antibiotic individually occur at the same time **(47)**. However, other mechanisms have been described as facilitating the emergence of this resistance pattern. These include overexpression of efflux proteins, biofilm formation or the coccoid morphology exhibited by *H. pylori* **(44, 47)**. In most cases, all these mechanisms exert a synergistic effect on the development of resistance. Finally, there is the heteroresistance (HR) pattern, which is defined as the presence of one or more subpopulations with increased levels of resistance compared to the main population **(47)**.

2. OBJECTIVES

The main objective of this research is to analyze and to study the different molecular mechanisms of antibiotic resistance in *Helicobacter pylori*. In order to achieve this goal, secondary aims, listed below, have been defined:

- To carry out a literature review on the different mechanisms of antibiotic resistance described in *H. pylori*, especially in molecular terms: genetic alterations, physiological changes and cellular adaptations
- Learn how to correctly handle different databases and specific sequence analysis programmes
- To analyse the sequences of 7 resistance genes from 52 *H. pylori* strains to detect resistance-associated mutations
- Try to predict the correlation between detected mutations and antibiotic resistance in the 52 strains included in this study

3. MATERIAL AND METHODS

3.1. Bibliographic research

A bibliographic search was conducted to acquire the most updated knowledge available regarding the *H. pylori* resistance to antibiotics, mainly on the molecular mechanisms involved. This search was carried out by accessing multiples bibliographic databases such as PubMed (Medline), Scopus, Springer Link or SciELO. Specialized books on the subject and other specialized databases such as GenBank, Uniprot or Protein Data Bank (PBD) were also consulted. In a preliminary search, the concepts "*Helicobacter pylori*" and "antibiotic resistance" were employed to find boarder information on the subject. Subsequent searches were made using more specific terms to narrow down the results obtained. Keywords such as "*Helicobacter pylori*", "antibiotic resistance [AND] molecular mechanisms", "*H. pylori* [AND] treatment", among others, were used for this second query. The language employed in the consulted articles were limited to Spanish and English. All the bibliography has been compiled using Mendeley reference manager and the citations have been written following the Vancouver style criteria.

3.2. Bioinformatics study of antibiotic resistance in *Helicobacter pylori*

3.2.1. Helicobacter pylori strains

For this project, 52 *H. pylori* strains belonging to the *H. pylori* collection of the Digestive Diseases Service of the Hospital Parc Taulí (Sabadell, Barcelona) have been selected. More specifically, the genomes obtained by the Microbiology Section of the Faculty of Pharmacy and Food Sciences of the Universitat de Barcelona have been used (BioProject Accession PRJNA449871). **Annex 2** shows a table with the 52 strains selected for the study, as well as their genetic label (GenBank Accession Number) and the locus tag of all the genes under study.

3.2.2. Sequence collection

For each of the 52 *H. pylori* strains, seven genes were analyzed: *pbp1*, 16S rRNA, 23S rRNA, *rdxA*, *frxA*, *gyrA* and *rpoB*, which are related to *H. pylori* resistance to AMX, TET, CLR, MTZ, LVX and RFB respectively. The sequences of the genes were retrieved from the publicly accessible GenBank database of the NCBI (National Center for Biotechnology Information). To search for the sequences needed, the "Nucleotide" option was selected and the name of the species was indicated together with the strain and gene required.

3.2.3. Analysis of resistance associated-genes

To conduct the analysis of the seven resistance genes mentioned above, the MEGA XI programme (Molecular Evolutionary Genetics Analysis, Pennsylvania State University, USA) was used. The sequences obtained and saved in fasta (.fas) format files were uploaded to the programme and aligned. The alignment was performed using the "Align by ClustalW" option. Once the sequences were aligned, the visualisation was optimised using the "Toggle Conserved Sites" tool. Using this option, the program only highlights the nucleotide positions that differ between the strains. Once all the necessary adjustments were made, the analysis was carried out. In this case, specific positions known to be associated with resistance were analysed in order to identify mutations or other alterations in the sequences that could indicate a potential resistance. In order to perform a rigorous analysis of the results, in all alignments the sequences of the strains included in the study were compared with those corresponding to the *H. pylori* ATCC 26696 reference strain (GenBank Accession Number AE000511.1).

4. RESULTS AND DISCUSSION

During the course of this research, a bibliographic search was carried out to understand the different molecular mechanisms by which *Helicobacter pylori* can become resistant to the antibiotics used in the treatment of the infection. A genetic study of 52 *H. pylori* strains was conducted to complement the information obtained. For each strain, seven genes have been analysed: *pbp1*, *16S*, *23S*, *rdxA*, *frxA*, *gyrA* and *rpoB*, which have been associated with *H. pylori* resistance to AMX, TET, CLR, MTZ, LEV and RFB, respectively (both genes, *rdxA* and *frxA* are related to MTZ resistance). The first part of this section presents the results derived from the bibliographic research. The second part describes the results obtained from the genetic analysis of the selected *H. pylori* strains. Throughout this part, the discussion of the results is also developed, with the aim to provide and answer to the objectives pursued in this research.

4.1. Molecular mechanisms of resistance

4.1.1. Genetic alterations

Structural alterations in the genetic sequence of different genes encoded in the chromosome represent the major cause of antibiotic resistance in *H. pylori* **(48)**. These mutations lead to modifications of the pharmacological targets of antibiotics or prevent their activation **(47)**. In both cases, there is a disruption of antibacterial activity and a loss of therapeutic effectiveness.

4.1.1.1. Amoxicillin resistance

The main mechanism of amoxicillin resistance in *H. pylori* involves point mutations in the genes encoding penicillin-binding proteins (PBPs) **(32, 47, 48, 49)**. PBPs are a group of enzymatically active proteins located in the plasmatic membrane of bacteria **(48)**. They act in the last two reactions of peptidoglycan (PG) biosynthesis: the polymerisation of PG structural units and transpeptidation. PG is a macropolymer constituted by N-acetyl glucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc) units linked together by peptides to form a mesh-like structure that surrounds and stabilises the cell **(50)**.

Using digoxigenin-labelled ampicillin (DIG-ampicillin), Harris *et al.* (2000) identified eight PBPs of *H. pylori* along with a ninth protein that they listed as a possible PBP **(48)**. These are currently the nine known PBPs of *H. pylori* and are referred to as PBP1 to PBP9. Of these, there are three high-molecular weight PBPs (PBP1 to PBP3) while the remaining are low-molecular weight PBPs (PBP4 to PBP9) **(7)**. There is the so-called amoxicillin tolerance (Amx^T), an unstable resistance phenotype that has been hypothesised to be associated with an deficiency of PBP4 **(31, 47)**.

However, only high-molecular weight ones have been associated with stable resistance to amoxicillin (and to β-lactams in general) **(48)**. Among the three high molecular weight PBPs described for *H. pylori*, the one mainly associated with resistance is PBP1, encoded in the *pbp1* gene **(43, 47)**. PBP1 is biochemically characterised as a bifunctional enzyme with glycosil transferase and acyl-transpeptidase activity **(31)**. Attaran *et al.* (2021) determined by pairwise alignment of the sequences of *Streptococcus pneumoniae* PBP1 with that of *H. pylori* that Ser368 is the catalytic residue in *H. pylori* **(32)**. When AMX binds to PBP1, Ser368 acts as a nucleophile and attacks the β-lactam ring, forming and acyl-enzyme complex that inactivates the protein and prevents entry of the nascent peptidoglycan molecule **(32, 51)**. Once formed, the Ser368-AMX complex interacts non-covalently with other amino acid residues located at the binding site of PBP1 **(32)**. The structure of *H. pylori* PBP1 and the non-covalent interactions are shown in **Figure 9.**

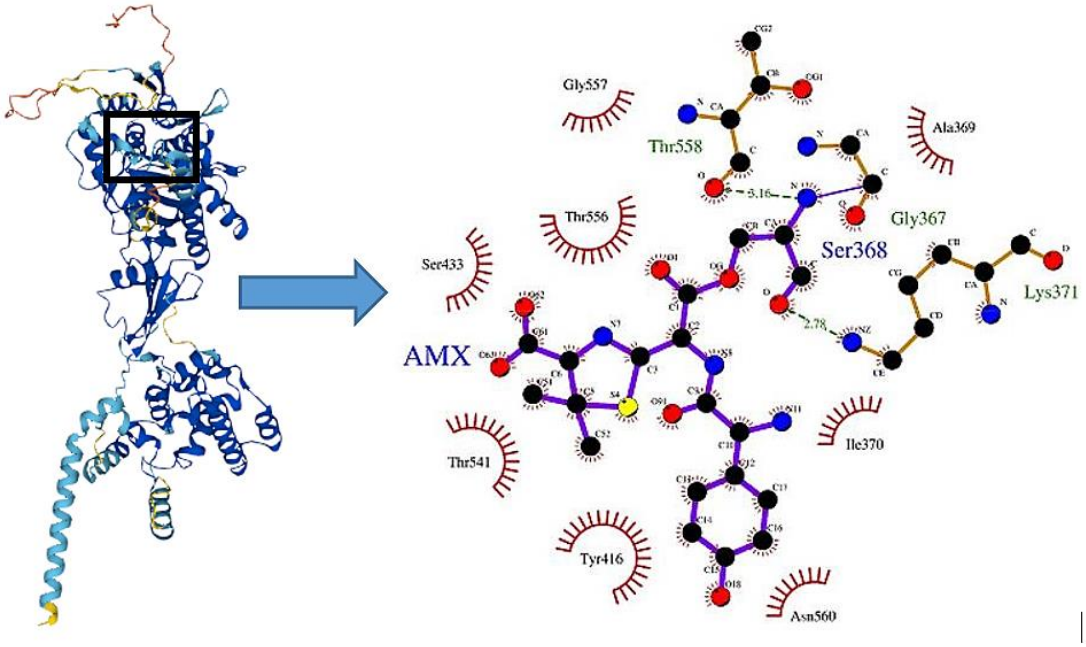

Figure 9. Left; 3D structure of *H. pylori* PBP1 (Uniprot ID Q86103). Right; Covalent interaction between amoxicillin (AMX) and the Ser368 residue of PBP1. Red arcs (hydrophobic interactions) and green dotted lines (H bonds) represent amino acid residues involved in non-covalent interactions with the Ser368-AMX complex. Figure taken and modified from **(32)**.

AMX resistance in *H. pylori* is related to point mutations in the C-terminal region where highly conserved amino acid sequences called penicillin-binding motifs are located. These are $STGK_{338-341}$, $SAIK_{368-371}$, $SKN_{402-404}$, $SLN_{433-435}$, $KTG_{555-557}$ and $SNN_{559-561}$ and they correspond to the sites where AMX must bind to exert its bactericidal effect **(7)**. Consequently, amino acid modifications in or near these regions due to point mutations induce the development of resistance **(7, 52, 53)**. Genetic alterations in the other two high molecular weight PBPs (PBP2 and PBP3) have also been linked to *H. pylori* resistance to β-lactam antibiotics, although in this case they appear to facilitate and enhance resistance induced by mutations in the *pbp1* gene **(47)**. Accordingly, resistance is higher in those strains with alterations in all three proteins compared to those with only the gene coding for PBP1 affected, suggesting that they act synergistically. Some authors such as Ansari *et al.* (2022) **(7)** or Rimbara *et al.* (2008) **(49)** have reported mutations in these proteins and their possible relationship with AMX resistance. However, knowledge about the actual role of these mutations in the development of *H. pylori* resistance to AMX is still scarce and more studies are needed.

4.1.1.2. Tetracycline resistance

Tetracycline (TET) is a broad-spectrum antibiotic that has been shown to be active even against some protozoal parasites **(54)**. Stability at acidic pH and the fact that it reaches high concentrations in the gastric juice and mucosa make TET a potentially useful alternative for the treatment of *H. pylori* infection when first-line options (AMX, CLR and MTZ) are not effective **(43, 47)**. It belongs to the polyketide group. Its structure consist of a hydonaphthacene core to which different hydrophilic groups are attached. These are mainly located on one side of the molecule **(55)**.

TET is actively uptaken by the bacterium by specific transport systems through an energy-dependent process and accumulates in the cytoplasm **(55, 56).** Once in the cytoplasm, is directed to the ribosome where it reversibly binds to the pocket enveloping the 16S rRNA molecule in the small subunit (30S) **(47)**. As a result, the binding of the aminoacyl-tRNA to the acceptor site (A site) on the 30S subunit is blocked and the subsequent binding of release factors 1 and 2 (RF-1 and RF-2) during the termination phase is prevented **(7, 43, 47)**. This results in a blockage of protein biosynthesis, thereby inhibiting bacterial growth. Regarding the binding of TET to the 30S subunit of the ribosome, studies published by Brodersen *et al.* (2000) and Pioletti *et al.* (2001) determined the existence of one primary TET binding site and more than five secondary binding sites **(57, 58)**.

The 16S rRNA is encoded in the *rrnA* and *rrnB* genes. Both genes are involved in the resistance of *H. pylori* to tetracycline **(59).** The main cause of resistance is the change of the 926AGA928 triplet into 926TTC928 triplet, which has been reported in several studies **(7, 43, 54, 59).** The 16S rRNA genes of *H. pylori* strains 26695 (TET^S) and 181 (TET^R) are schematized in **Figure 10.**

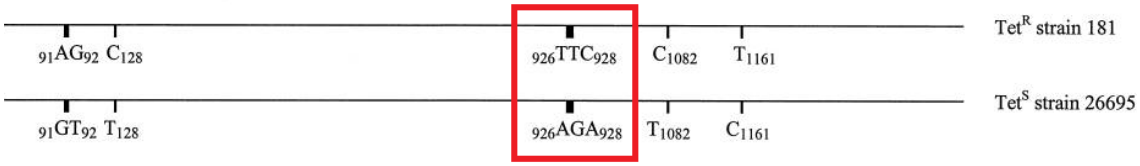

Figure 10. Schematic representation of the 16S rRNA genes of strains 181 (TET^R) and 26695 (TET^S). The triplet 926AGA928 is outlined in red. Taken and modified from **(59)**

The magnitude of *H. pylori* resistance to tetracycline is related to the number of mutations present in the 926AGA928 triplet. Thus, *H. pylori* strains with the triple mutation 926TTC928 have a high resistance grade while strains with only one or two mutated nucleotides in the triplet have been linked to low level resistance **(59, 60).** It is noteworthy that the probability of the triple mutations occurring is small, being a plausible explanation for the low rates of *H. pylori* resistance to this antibiotic. The effect of this mutation results in decreased binding of TET to its site of action. In the study published by Nonaka *et al.* (2005), it was found that in strains whose ribosomes had the triple mutations in the 926AGA928 triplet, tetracycline binding to them was 2,5-fold lower compared to strains without this mutation **(61)**.

4.1.1.3. Clarithromycin resistance

Clarithromycin (CLR) is the classical example of macrolide antibiotic. Macrolides are characterised by a macrocyclic lactone structure with a ring of 12 or more members derived from a polyketide **(62)**. Specifically, CLR belongs to the 2nd generation macrolide class and its structure consists of a 14-membered macrocyclic lactone ring **(43)**. It is a semi-synthetic macrolide structurally derived from erythromycin A, which is synthesized by *Saccharopolyspora erithraea* **(62)**. The structures of both macrolides are shown in **Figure 11**.

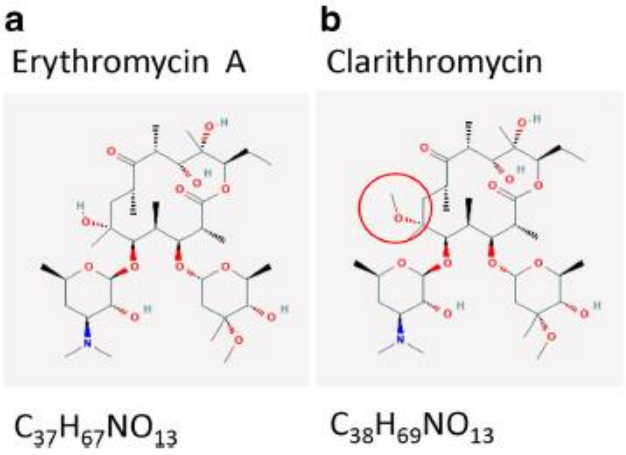

Figure 11. Chemical structures if erythromycin A produced by *Saccharopolyspora erithraea* (A) and CLR (B). Retrieved from **(62)**

CLR is part of most first-line therapies for the treatment of *H. pylori* infection. Its choice over other macrolides is mainly due to two pharmacokinetic advantatges: its stability in acidic media environment and improved absorption through the gastric mucosa **(47)**. It has a bacteriostatic effect whose mechanism of action involves reversible binding to the peptidyl-transferase loop of the V domain of 23S rRNA molecules of the 50S subunit of the bacterial ribosome **(47)**. The binding of CLR to its site of action also prevents the entrance into the 50S subunit of the Nascent Exit Peptide Tunnel (NPET) **(63)**.

The main cause of CLR resistance in *H. pylori* involves point mutations in the 23S rRNA. This is encoded in the *rrl* gene **(64)**. *H. pylori* harbours two copies of the *rrl* gene **(7)**. Most resistant strains show mutations in both copies. However, strains with only one muted copy of the gene (heterozygous phenotype) have also developed resistance **(62)**.

Binding of macrolides –including CLR– takes place in the macrolide-binding pocket (MBP), a high affinity pocket formed by some 23S rRNA nucleotides. The major nucleotides constituting this binding pocket are A2142 and A2143. In sensitive *H. pylori* strains, the interaction between CLR and the MBP occurs via hydrogen bonds and hydrophobic interactions. The former are established with residues A2142 and A2587, while the latter are formed with residues A2143, A2144, G2145, A2146, C2526 and C2527 **(63).** The alteration of some of these nucleotides by point mutations constitutes the main cause of CLR resistance in *H.pylori*.

In relation to 23S rRNA-associated resistance mutations, multiple studies agree that the most clinically relevant mutations occur in the nucleotides at positions 2142 and 2143 and involve the substitution of adenine (A) for a guanine (G) at both positions (A2142G and A2143G) **(7, 47, 62)**. The A2142C substitution is also considered as being clinically significant, although it is less common than the other two **(62)**. These three mutations (A2142G, A2143G and A2142C) account for nearly 90% of resistance cases in western countries **(62, 64)**. Other resistance-related point mutations include: G1939A, A1957C, T1958G, G1964T, A2115G, T2117C, G2141A, A2144G, A2144T, C2147G, T2182C, G2212A, T2215C, G2223A, G2224A, T2288C, T2711C, C2759T and C2772T **(7, 43, 62).** Additionally, C2647A and T21717C substitutions have been associated with low levels of resistance while T2182C has been described in both resistant and susceptible *H. pylori* strains **(62)**.

Resistance of *H. pylori* to CLR, however, is not only due to mutations in the gene coding for 23S rRNA. Mutations in the *infB* and *rpl22* genes, which encode for the initiation factor IF-2 and ribosomal protein L22 respectively, have been linked to CLR resistance **(43)**. Alterations in these two genes exert a synergistic effect with those present in 23S rRNA gene, as strains with mutations in both genes (*infB* or *rpl22* + 23S rRNA) exhibit a higher MIC values compared to those with mutated *infB* or *rpl22* but unaltered 23S rRNA **(62)**.

4.1.1.4. Metronidazole resistance

Metronidazole (MTZ) is a synthetic derivative of nitroimidazole **(7)**. After its administration, is actively released into the gastric juice, where its activity is slightly affected by the low gastric pH. Despite this, the acidic environment does not significantly decreases the activity and effectiveness of the drug **(47)**.

As a prodrug, it requires a prior bioactivation process to exert its bactericidal effect **(47)**. Through passive diffusion systems, MTZ penetrates into the cytoplasm. The inward flow is facilitated by the decrease in drug concentration resulting from metabolic reactions **(65)**. In the bacterial cytoplasm, the nitro group ($-NO_2$) of the imidazole ring is reduced by nitroreductases of bacterial anaerobic metabolism, generating biological active metabolites **(55)**. During this process, nitroanionic radicals are generated, along with nitrosoderivatives and hydroxylamine, which are responsible for cytotoxic activity by inducing the destruction of the helical structure of DNA **(66).** In *H. pylori*, the main protein involved in the reduction of metronidazole into its active form is the oxygen-insensitive NAD(P)H nitroreductase (RdxA) **(47)**. Nevertheless, other proteins such as NADPH-flavin oxidoreductase (FrxA), ferrodoxin (FdxA), flavodoxin (FldA) or various ferrodoxin-like proteins (FdxB, OorDABC, PorCDAB) have also been found to be involved in the MTZ bioactivation pathway **(7, 65)**

Although the mechanism underlying *H. pylori* resistance to MTZ has not been described in as much detail as the other antibiotics, inactivation of the enzymes involved in the bioactivation process is considered to be the main cause, constituting an indirect resistance mechanism **(43, 65)**. Of all the proteins involved in MTZ reduction, conventional molecular techniques have identified mutations in the *rdxA* and *frxA* genes, which encodes the RdxA and FrxA reductases respectively, as the major factors inducing resistance **(65, 67)**.

In their study, Martínez-Júlvez *et al*. (2012) structurally characterised the RdxA nitroreductase from *H. pylori*. It consists of two homodimers, each containing two flavo-mononucleotide cofactors (FMN). Each monomer contains 210 amino acids folded into a α+β motif, consisting in 5 α-helices and 5 β-sheets **(68)**. The molecular structure of RdxA shown in the study is available in the Protein Data Bank (PBD code: 3QDL).

Saracino *et al.* (2021) described that R16C, R16H, A67V, A68E, K64N, R90S or P106S substitutions have only been found in MTZ-resistant *H. pylori* strains. Other mutations, including those at positions 62, 96 and 162, have been found in both susceptible and resistant strains, meaning that they are not related to resistance **(67).** Other point mutations in *rdxA* have been described in other studies as being linked to the development of resistance. These include V57S, H99R, S45N, S46F, Y47C, N48Y, I160V, G162R and G163D **(69, 70)**.

The FrxA nitroreductase is structurally related to RdxA and has similar interactions with FMN cofactors **(68)**. However, there is still no consensus on the actual role of alterations in this gene on the MTZ resistance emergence. While it is true that some mutations such as R58C, A85V, I177M and E169K have been described as being associated with resistance **(69, 70),** there are discrepancies between authors on this regard. There are authors, such as Kwon *et al.* (2000), who claim that the effects of *frxA* inactivation are equivalent to those produced by *rdxA* inactivation. In contrast, other authors such as Jeong *et al.* (2000) or Ansari *et al.* (2022) defend the hypothesis that *frxA* inactivation only has an effect on those *H. pylori* strains with mutated *rdxA*, suggesting that inactivation of *frxA* alone does not induce resistance in strains with unaltered *rdxA*. **(7, 71, 72).**

However, despite the large number of articles published on the genetic resistance of *H. pylori* to MTZ, the distribution of mutations detected in *rdxA* and *frxA* is haphazard and irregular **(73)**. Furthermore, as described in several studies, genetic alterations in these genes are not limited to point mutations, but also include frameshift mutations (deletions and insertions) and premature STOP codons leading to truncated proteins **(7, 47, 67, 74)**. These factors hinder the correlation between genetic alterations and phenotypic MTZ resistance.

In recent years, with the development of WGS techniques, other genes that may be related to *H. pylori* MTZ resistance have been detected. These include the *recA* (RecA protein for the repair and maintenance of DNA), *sodB* (superoxide dismutase B), *fur* (ferric uptake regulator protein), *mdaB* (NADPH quinone oxidoreductase), *ribF* (riboflavin biosynthesis protein), *omp11* (outer membrane protein 11) and *rpsU* (30S ribosomal protein S1) genes **(67, 74)**. However, the role of these genes in MTZ resistance remains unexplored. The underlying mechanism is still unknown in some cases, as in the case of the *rpsU* gene **(7)**. Other mechanisms, such as overexpression of SodB mediated by mutations in the *fur* regulatory gene have been suggested as a potential mechanism for inducing MTZ resistance regardless genetic alterations in RdxA **(75)**. However, more studies are required to elucidate the detailed molecular mechanisms driving MTZ in *H. pylori*.

4.1.1.5. Levofloxacin resistance

Levofloxacin (LEV) is a third-generation fluoroquinolone that has been shown to exhibit strong activity against fastidious pathogens like *H. pylori* **(55)**. Quinolones act by inhibiting two bacterial enzymes: DNA gyrase and topoisomerase IV (topoIV). In general, DNA gyrase is the antibiotic target in gram-negative bacteria while in gram-positive bacteria it is topoIV **(7)**. TopoIV is encoded in the *parC* and *parE* genes, neither of which exists in the *H. pylori* genome **(7, 43)**. These genes have homology with the GyrA and GyrB subunits of DNA gyrase, respectively **(65)**. Thus, the *H. pylori* resistance to LEV is essentially mediated by mutations in one or both of the DNA gyrase-encoding genes (*gyrA* and gyrB) **(47)**.

DNA gyrase is an 826 amino acid protein belonging to the type II topoisomerases class. Its main function is the negative supercoiling of the DNA double strand during the initial phase of DNA replication **(65)**. This enzyme displays a tetrameric structure consisting of two A subunits (encoded in *gyrA*) and two B subunits (encoded in *gyrB*) **(76)**. Resistance of *H. pylori* to LEV is caused by mutations in the quinolone-resistance-determining region (QRDR), a hipermutable region located in the GyrA subunit of DNA gyrase **(7, 77)**. In sensitive strains, which do not harbour any mutations in the QRDR, the interaction of LEV with GyrA is established by five hydrogen bonds (Y90, S115, I116, G118 and D119) and six hydrophobic interactions (G35, N87, D91, V94, G114 and D117) **(77)**. The interactions between levofloxacin and *gyrA* are shown in **Figure 12.**

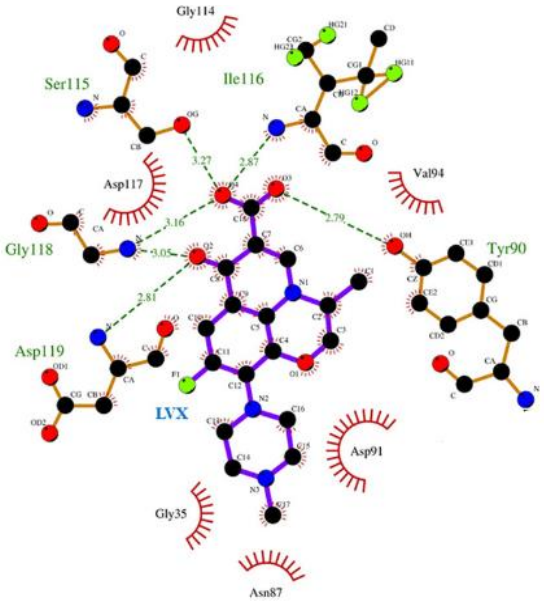

Figura 12. Representations of the molecular interactions between levofloxacin and GyrA. Red arcs represent hydrophobic interactions and green dotted lines represent hydrogen bonds. The chemical structure of levofloxacin is shown in purple. Retrieved from **(77)**

Several studies agreed that point mutations in *gyrA* gene codons 87 and 91 are the most prevalent **(7, 78-80)**. Codons 87 and 91 code for asparagine (Asn, N) and aspartic acid (Asp, D), respectively. At these positions, the most frequently described mutations in *H. pylori* isolates are N87K and D91N **(65, 78).** In addition, other common mutations have been described at these same. These include N87T, N87Y, N87I, D91Y, D91G and D91H **(7, 81).**

In a study published in 2011, Lee *et al*. observed that in the case of two strains with the N87K mutations, its presence triggered therapeutic failure. However, in three strains carrying the D91N substitutions, no cases of eradication treatment failure were observed **(82)**. The authors suggested that N87K substitution conferred a greater LEV resistance than the D91N, concluding that the former might be a major determinant of quinolone therapy failure **(82)**. Another study published the following year found similar results. In this study, it was observed that by exchanging the D91N substitution for the N87K in resistant strains the MIC of LEV increased (from 0.125 mg/L to 8 mg/L), concluding that the N87K substitution conferred a higher resistance to LVX compared to substitution in position 91 **(80)**. Despite being the most commonly reported and the most clinically significant in relation to LEV resistance, these are not the only reported mutations. Other mutations reported less frequently include D34N/Y, S63P, A88P, D99V, A129T, R130K, R140K, D161N, V172I, P188S and D192N **(7)**.

Besides, a five amino acid insertion (QDNSV) at the N-terminus of GyrA has also been identified and found exclusively in *H. pylori* LEV-resistant strains. This alteration alters the conformation of the protein, affecting levofloxacin binding **(67)**.

The *gyrB* gene has been the object of fewer studies compared to *gyrA*. Consequently, a consensus on the possible role of point mutations in this gene and its relevance towards *H. pylori* resistance remains awaited. The QRDR of GyrB is located between residues E415 and S454 **(47)**. Although some mutations in gyrB, such as the one reported by Rimbara *et al.* (2012) located at position 463 (E463K) has been linked to resistance development **(80)**, others located within and near the QRDR (D435N, V437L, R484K and R579C) do not correlate as strongly with phenotypic resistance **(67).** In the studies conducted by Wang *et al.* (2018) and Farzi *et al.* (2019), D481E and R484K substitutions were described in both susceptible and resistant strains **(70, 83)**. It appears, therefore, that mutations in the *gyrB* gene do not induce resistance on their own. However, the existence of a synergistic effect between point mutations in *gyrA* and *gyrB* needs to be evaluated in forthcoming investigations.

4.1.1.6. Rifabutin resistance

Rifamycins are a group of bactericidal agents used in the treatment of various bacterial diseases. Their mechanism of action consists on the inhibition of the initial stages of mRNA and tRNA biosynthesis during transcription by blocking the DNA-dependent RNA polymerase β-subunit, which is encoded in the *rpoB* gene **(55).** The classic member of this group of antibiotics is rifampicin (RIF), a semi-synthetic derivative of rifamycin B, used in the treatment of tuberculosis, a disease caused by *Mycobacterium tuberculosis*. However, RIF is not used in the treatment of *H. pylori* infection. In this case, rifabutin, another drug of the same family is used. The use of rifabutin has been increasing in recent years, mainly due to the exponential increase in resistance rates against first-line drugs (AMX, CLR and MTZ). The pharmacological activity of rifabutin is not affected by stomach low pH, which constitutes an advantatge in its usage against *H. pylori* **(67)**. Rifampicin has been routinely used in screening for *H. pylori* resistance to rifabutin. The reason for this has been that cross-resistance between rifamycins was assumed. Nevertheless, the study published by Yang *et al*. (2022) concluded that there was a lack of cross-resistance between rifamycins and that the use of rifampicin in rifabutin resistance screening was therefore inappropriate **(84)**.

H. pylori resistance to rifabutin has been linked to point mutations in a specific region of the *rpoB* gene called rifampicin-resistance determining region (RRDR) **(67).** These mutations are mainly located in codons 525-545, 547 and 586 of this region (positions numbers corresponding to *H. pylori* ATCC 26695

genome) **(85).** Within this region, mutations at codons 525, 530 and 540 play a critical role in rifabutin resistance. The most frequently described mutations at these positions are L525I/P, D530N/G/E/V, H540N and L547F **(85, 86)**. Hays *et al.* (2018), using a directed-mutagenesis assay triplet 1639-1641 (CTC, Lys in ATC 26695), discovered the substitution L547F. The authors concluded that this substitution led to the development of rifabutin resistance by *H. pylori* **(86).**

4.1.2. Enzymatic inactivation

In addition to alterations in the pharmacological targets of the different antibiotics used in *H. pylori* infection treatment, enzymatic drug inactivation has been described as another common mechanism by which bacteria acquire antibiotic resistance.

In gram-negative bacteria, resistance to β-lactam antibiotics is mainly mediated by the production of β-lactamases **(7, 31, 47)**. These enzymes can be found encoded in the bacterial chromosome or in plasmids and act by hydrolysing the β-lactam ring inducing inactivation of the antibiotic and loss of its antibacterial function **(31).** Tseng *et al.* (2009) first described the role of β-lactamases in the development of resistance in *H. pylori*. The authors detected the *blaTEM1* gene in the genome of *H. pylori* strain 3778 (GenBank Accession number: EU726527), which was highly resistant to AMX, with a MIC ≥ 256 mg/L **(87)**. However, several studies have reported that the production of β-lactamases do not constitute the main mechanism of AMX resistance in *H. pylori* **(31, 32, 47, 48, 49)**. Still, the use of β-lactamase inhibitors such as clavulanic acid (CA) together with AMX has proven to be useful, as an increased eradication rate has been observed after co-administration with AMX **(88, 89**). This suggest that, although uncommon, the production of β-lactamases is a resistance mechanism in *H. pylori* that needs to be accounted for and provides a rationale for the possible use of CA in new *H. pylori* therapies.

Enzymatic inactivation as a resistance mechanism has also been reported for TET. Several studies establish that TetX, an NADPH-dependent oxidoreductase, in presence of NADPH and molecular oxygen, induces antibiotic degradation by catalysing the generation of inactive metabolites **(59, 89).** Regarding the other antibiotics used against *H. pylori* infection, no data have been found suggesting enzyme inactivation as a resistance mechanism.

4.1.3. Alterations in Outer Membrane Proteins: decreased drug uptake and efflux pumps overexpression

In the bacterial response to the pharmacological action of antibiotics, one of the most determining factors is the balance of drug concentrations outside and inside the cell. This equilibrium can be modified if the systems that maintain it are altered. Thus, a decrease in the drug uptake or an increase in the efflux leads to a reduction in the antibiotic concentration inside the bacterium. If the resulting concentration of the antibiotic is lower than the minimum effective concentration, the antibiotic has no effect and it is when resistance appears.

Outer Membrane Proteins (OMPs) are involved in both the uptake and expulsion of antibiotics by the bacterium. Therefore, the alteration of these proteins represents an important mechanism of antibiotic resistance. Most studies report that the *H. pylori* genome encodes about 32 OMPs **(43, 90, 91)**. Other studies, such as that of Sharndama *et al*. (2022) however, describe that *H. pylori* expresses around 64 OMPs **(14)**. These OMPs are classified in five groups: adhesins, porins, iron transporters, efflux proteins and proteins whose function remains unknown **(90)**.

In relation to the *H. pylori* AMX resistance, it has been described in several studies that genetic alterations in the *hopB* and *hopC* genes (encoding two porins) reduce the membrane permeability to the drug, resulting in a reduction of its concentration inside the cell, and consequently in the emergence of resistance **(7, 43, 47)**. On the other hand, in the study published by Godoy *et al*. (2006) it was observed that exposure to AMX caused a down regulation of the Omp32 porin **(92).**

For CLR, Smiley *et al*. published a study in 2013 where the proteomics of CLR-sensitive (ATCC 43504) and a CLR-resistant strain (ATCC 700684) were compared. In this study, it was observed how in the CLR-resistant strain, HopT (BabB), OMP31 and HofC proteins, an adhesin, a porin and a surface protein, respectively, were overexpressed. In contrast, the expression levels of iron-regulated membrane protein (FrpB1), urease B, elongation factor Tu (EF-Tu) and other OMPs were lower compared to the CLR-sensitive strain **(91)**. In this study, efflux proteins were found to be unaffected. These data support the idea that alteration of OMPs constitutes a novel mechanism of CLR resistance in *H. pylori*. However, the specific mechanisms involved are not entirely described and further studies are required. For MTZ, mutations in genes different from *rdxA* and *frxA*, such as *fur* and *omp11* were reported in different studies as an additional resistance mechanism to this antibiotic **(67, 69, 74)**.

Efflux proteins play a critical role in the development of antibiotic resistance **(14)**. These are located in the plasmatic membrane and in some cases, there are also anchored to the outer membrane of the bacterium. Their function involves recognizing and excreting substances that could have noxious effects to the bacterium **(7)**. Efflux proteins can recognize a wide variety of substances, including antibiotics **(7)**. Therefore, alteration of these proteins represents a key mechanism underlying MDR in *H. pylori*.

At the present, six families of bacterial efflux proteins are known. These are: ABC (ATP-binding cassette), MFS (Major Facilitator Superfamily), MATE (Multidrug and Toxin Extrusion family), SMR (Small Multidrug Resistance family), RND (Resistance-Nodulation-Division superfamily) and PACE (Proteobacterial Antimicrobial Compound Efflux family) **(93)**. With the exception of the last family (PACE), all families have been described in *H. pylori* **(90).** The ABC family functions thorugh energy obtained from ATP hydrolysis, while the rest operates through energy provided by transmembrane ion gradients, mainly proton gradients **(43, 94)**. Four of these families are found in both gram-positive and gram-negative bacteria. The RND family, on the other hand, is only found in gram-negative bacteria **(94)**.

The RND family efflux proteins are the most relevant in relation to antibiotic resistance. Unlike the other families, their molecular structure consist of three components: an IEP (inner membrane protein), a PEP (periplasmatic efflux protein) and an OEP (outer membrane efflux protein) **(90)**. This structure facilitates the direct expulsion of various antibiotics from the cytoplasm or periplasmic space to the outside **(94)**. Four RND efflux proteins associated with multidrug resistance have been identified in *H. pylori*. These are: HP0605/06/07 (HefABC), HP0969/970/971 (HefDEF), HP1327/28/29 (HefGHI) and HP1487/88/89 **(7).** The genetic structure of these four proteins is schematically depicted in **Figure 13**.

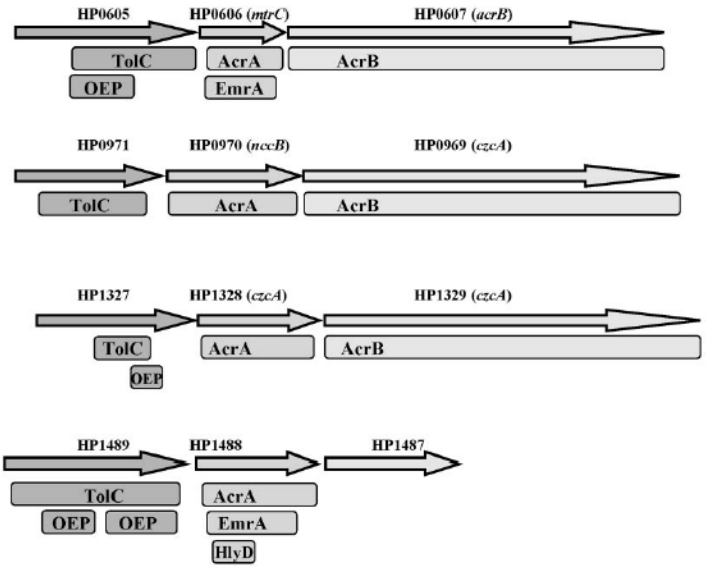

Figure 13*. Schematic representation of the genetic structure of the RND family efflux proteins described in* H. pylori*. Figure obtained from* ***(95)***

The best characterized efflux protein within the RND family is HefABC. One component of the structure of HefABC is the OEP HefA. It has been reported that the *hefA* gene, plays a key role in the pump activity **(43, 96)**. Thus, Liu *et al*. (2008) concluded that overexpression of *hefA* increased the efflux of different antibiotics, resulting in the emergence of multidrug resistant *H. pylori* strains. The antibiotics whose efflux is mediated by RND efflux pumps include AMX, TET, CLR and MTZ **(96)**. The participation of RND family efflux pumps in the resistance of *H. pylori* to CLR and MTZ could provide an explanation for the high prevalence of CLR+MTZ dual resistance in *H. pylori*. The prevalence of this dual resistance averaged 13% in the period 2013-2020, according to data from the European *H. pylori* Management Registry **(97).**

The available evidence on efflux pumps as resistance mechanisms has served as a bases for the use of efflux pump inhibitors (EPIs) as a therapeutic approach for the treatment of *H. pylori* infection. To evaluate the effect of these compounds on MDR *H. pylori* strains, Zhang *et al.* (2010) conducted an experiment using CCCP (carbonyl cyanide m-chlorophenyl hidrazone) **(98)**. CCCP prevents proper proton gradient and is therefore active against pumps that use this mechanism for obtaining energy **(94)**. Specifically, it has shown activity against efflux pumps of the MATE, RND and SMR families **(98)**. The authors observed that after CCCP treatment, MICs of five antibiotics (tetracycline, erythromycin, chloramphenicol, cefotaxime and ceftriaxone) decreased at least 4-fold. Also, they analyzed the expression profiles of the *hefA* gene and found that these were higher in MDR strains compared to sensitive strains. The results of this study backed up the role of efflux pumps in *H. pylori* MDR and particularly, the predominant role of RND pumps **(98)**. In the same study, the effect of reserpine was also evaluated. Reserpine is an alkaloid that inhibits efflux proteins of the MFS and ABC superfamilies. The authors observed that MICs of the tested antibiotics did not change after the treatment with reserpine, suggesting that these families were not involved in *H. pylori* MDR **(98)**.

Another efflux protein reported to be involved in *H. pylori* antibiotic resistance is TetA (HP1165) **(43)**. TetA belongs to the MFS family and has been shown to play a significant role in the TET resistance of *H. pylori* 26695 **(99)**. In the study, the authors noticed that inactivation of the protein due to mutations in the *hp1165* gene resulted in increased susceptibility of strains, which did not harbor mutations in the 16S rRNA encoding genes. Based on the results, the authors demonstrated that *hp1165* gene was involved in the TET resistance of *H. pylori* **(99)**. Moreover, alterations in the expression of the *hp1165* have also been described in strains with resistance against AMX, CLR and MTZ, proving that the TetA protein, similarly to *hefA* gene, is linked to MDR in *H. pylori* **(90)**.

Therefore, the alterations OMPs in *H. pylori* have been proven to constitute a relevant mechanism linked to the emergence of multidrug-resistant strains. This reinforces the need to continue investigating these proteins in order not only to improve and optimize the currently used treatments, but also to develop new potential therapeutic compounds such as novel EPIs.

4.1.4. Cellular adaptation: implications of biofilms and coccoid morphology in Helicobacter pylori multidrug-resistance

Over the last years, it has become evident that cellular adaptation contribute significantly to the emergence of antibiotic resistance in *H. pylori* **(47)**. Biofilm formation and the modification of cell morphology may not be considered resistance mechanisms per se, as their primary purpose is to ensure the survival of the pathogen under adverse conditions. However, these conditions include the presence of antibiotics in the environment. It has been observed that both biofilm formation and the adoption of coccoid form by the bacterium can substantially promote the emergence of multidrug resistant *H. pylori* strains. Therefore, cellular adaptation is a phenomenon that must be considered when assessing the causes of treatment failure.

Biofilms are bacterial communities in which cells are attached to each other and to the surface by a polymeric matrix consisting mainly of extracellular polymeric substances (EPS). These include polysaccharides, proteins and extracellular DNA, among others **(100).** Although the structure and formation of biofilms have been extensively studied, the mechanisms through which these communities are related to antibiotic resistance in *H. pylori* remain unclear. However, based on the data available from the published studies, several potential mechanisms have been proposed.

In the first place, EPS form a physical barrier that confers *H. pylori* a class of antibiotic tolerance. This tolerance is transient and not genetically heritable as it is due to the physiology of the biofilms and not to genetic alterations **(101)**. The barrier also prevents the action of the immune system and the entrance of antibiotics into the bacterial cell. In addition, the entry of antibiotics is further impeded by the barrier of electrical charges constituted by EPS, which generally carry negative charges that can affect the antibiotic transport mechanisms across the membrane **(100)**. On the other hand, biofilms constitute a large genetic reservoir where a continuous genetic exchange occurs through different mechanisms. This includes antibiotic resistance-associated genes **(101)**.

Another potential mechanism described in biofilms is related with efflux proteins. As mentioned above, overexpression of these proteins has been linked to MDR in *H. pylori*. In relation to biofilms, it has been shown in several studies that in biofilm-forming cells, the expression of these proteins is higher compared to planktonic cells, which are not capable of establishing biofilms. Yonezawa *et al*. (2019) observed that the genes *hp0605*, *hp0971*, *hp1327* and *hp1489*, which encode the OEPs of the four RND family efflux pumps, were overexpressed in biofilm forming strains **(102)**. In other studies, additional efflux proteins have been described. Ge *et al.* (2018) reported how the enzyme SpoT (bifunctional (p)ppGpp synthase/hydrolase), which synthesises two regulatory factors for biofilm formation, induced overexpression of the *hp1174* gene, which encodes for the gluP (glucose/galactose transporter), an MFS family efflux pump **(103)**. Other efflux proteins, such as Hp118, are also overexpressed in biofilms and are associated with the emergence of multi-resistant *H. pylori* strains **(100).**

Finally, alongside the biofilm formation, when under adverse conditions, *H. pylori* transforms into CVNCF (Coccoid Viable Non-Culturable Form) in order to survive **(14)**. Upon entering this dormant state, cell size decreases and metabolic activity is significantly reduced. Still, in this form, *H. pylori* maintains its virulence and it has been shown that these forms can increase the risk of cancer and the capability of the pathogen to evade the immune system **(104).** However, evidence on the mechanisms by which the modification of *H. pylori* morphology is involved in the development of antibiotic resistance is insufficient and further studies are needed.

4.2. Identification of resistance-associated mutations by genetic screening of 52 *H. pylori* strains

As described in the Material and Methods section, in order to complement the information obtained from the bibliographic search on genetic alterations causing resistance, a genetic study of 52 *H. pylori* strains was carried out.

4.2.1. pbp1: amoxicillin resistance

Amoxicillin resistance in *H. pylori* is related to point mutations in the C-terminal region of PBP1. There are mostly located in or near the so-called penicillin-binding motifs (*see section 4.1.1.1*). The *pbp1* gene sequences of the 52 strains were aligned and compared with the reference strain 26695. Mutations were detected in over half of the analysed gene positions. The mutations are summarised in **Table 3**.

Table 3. Results of mutation detection in the *pbp1* gene (AMX)

Nucleotides	1096-7-8	1105-6-7	1120-1-2	1204-5-6	1216-7-8	1240-1-2	1249-50-1	1267-8-9	399-1400-	1603-4-5	1627-8-9	1666-7-8	1684-5-6	1765-6-7	1771-2-3	1777-8-9	1795-6-7	1801-2-3
Triplet (Sensitive)	TTT (=F)	GCG (=A)	GTG (=V)	AGT (=S)	GAA (=E)	AGC (=S)	TCT (=S)	CTT (=L)	TCT (=S)	GAT (=D)	AGT (=S)	ACC (=T)	AAT (=N)	AGC (=S)	GGA (=G)	ACA (=T)	GCG (=A)	GTG (=V)
Amino acid position	366	369	374	402	406	414	417	423	467	535	543	556	562	589	591	593	599	601
Resistance	L	T	L	G	A	R, A	T	F	T	N	R	S	Y	G	K	A	G	G
ATCC 26696 Amx-S)	F	A	V	S	E	S	S	L	S	D	S	T	N	S	G	T	A	V
AAP164	TTT	GCA (=A)	GTG	AGT	GCG (=A)	AGC	ACT (=T)	CTT	TCT	AAT (=N)	CGT (=R)	ACC	TAT (=Y)	GGC (=G)	GGA	ACA	GCA (=A)	GTG
AFR58	TTT	GCG	GTG	AGT	GAA	AGC	TCT	CTT	TCT	AAT (=N)	AAT (=N)	ACC	AAT	AGC	GGA	GCA (=A)	GCA (=A)	GTG
APR133	TTT	GCG	GTG	AGT	GAA	AGC	TCT	CTT	TCT	AAT (=N)	CAT (=H)	ACC	AAT	AGC	GGA	ACA	GCG	GTG
B126	TTT	GCG	GTG	AGT	GCG (=A)	AGC	ACT (=T)	CTT	TCT	AAT (=N)	AGT	ACC	AAT	AGC	GGA	ACA	GCA (=A)	GTG
B247A	TTT	GCG	GTG	AGT	GCG (=A)	AGC	ACT (=T)	CTT	TCT	AAT (=N)	AGT	ACT (=T)	AAT	AGT (=S)	GGA	ACA	GCG	GTG
B274	TTT	GCG	GTG	AGT	GCG (=A)	AGC	TCT	CTT	TCT	AAT (=N)	AAT (=N)	ACC	AAT	AGT (=S)	GGA	ACA	GCG	GTG
B297	TTT	GCG	GTG	AGT	GAA	CCC	TCT	CTT	TCT	AAT (=N)	CGT (=R)	ACT (=T)	AAT	AGC	GGA	ACA	GCA (=A)	GTG
B314	TTT	GCG	GTG	GGT (=G)	ACG (=T)	AGC	ACT (=T)	CTT	TCT	AAT (=N)	AGT	ACC	AAT	GGC (=G)	GGA	ACA	GCA (=A)	GTG
B319	TTT	GCG	TTG (=L)	AGT	GAA	AGC	TCT	CTT	TCT	AAT (=N)	AGT	ACC	AAT	AGC	GGA	ACA	GCA (=A)	GTG
B335	TTT	GCA (=A)	GTG	AGT	GTG (=V)	AGC	ACT (=T)	CTT	TCT	AAT (=N)	AAT (=N)	ACC	AAT	AGC	GGA	GCA (=A)	GCG	GTG
B344	TTT	GCG	GTG	AGC (=S)	GAA	AGC	TCT	CTT	TCT	AAT (=N)	CGT (=R)	ACC	AAT	AGC	GGA	GCA (=A)	GCG	GTG
B345	TTT	GCG	GTG	AGT	GTG (=V)	AGC	ACT (=T)	CTT	TCT	AAT (=N)	AGT	ACT (=T)	AAT	GGC (=G)	GGA	ACA	GCG	GTG
B355	TTT	GCG	GTG	AGT	GCG (=A)	AGC	ACT (=T)	CTT	TCT	AAT (=N)	CGT (=R)	ACC	AAT	GGC (=G)	GGA	ACA	GCA (=A)	GTG
B360	TTT	GCG	GTG	AGT	GAA	AGC	TCT	CTT	TCT	AAT (=N)	CGT (=R)	ACT (=T)	AAT	AGC	GGA	ACA	GCG	GTG
B362	TTT	GCG	GTG	AGT	GCG (=A)	AGC	ACT (=T)	CTT	TCT	AAT (=N)	CAT (=H)	ACC	AAT	AGC	GGA	ACA	GCG	GTG
B366	TTT	GCG	GTG	AGT	GCG (=A)	AGC	TCT	CTT	TCT	AAT (=N)	CGT (=R)	ACC	AAT	AGC	GGA	ACA	GCG	GTG
B368	TTT	GCG	GTG	AGT	GAA	AGC	TCT	CTT	TCT	AAT (=N)	CGT (=R)	ACC	AAT	AGC	GGA	ACA	GCA (=A)	GCG (=A)
B373	TTT	GCG	GTG	AGT	ACG (=T)	AGC	ACT (=T)	CTT	TCT	GAT	AGT	ACC	AAT	GGC (=G)	GGA	ACA	GCA (=A)	GTG
B400	TTT	GCG	GTG	AGT	GCG (=A)	AGC	ACT (=T)	CTT	TCT	GAT	AGT	ACT (=T)	AAT	AGC	GGA	GCA (=A)	GCA (=A)	GTG
B444A	TTT	GCG	GTG	AGT	GAA	AGC	TCT	CTT	TCT	AAT (=N)	CGT (=R)	ACC	GGT (=G)	AGC	GGA	ACA	GCA (=A)	GTG
B448	TTT	GCG	GTG	AGT	GCG (=A)	AGC	ACT (=T)	CTT	TCT	AAT (=N)	AGT	ACC	AAT	AGC	GGA	ACA	GCA (=A)	GTG
B455	TTT	GCA (=A)	GTG	AGT	GAA	AGC	TCT	CTT	TCT	AAT (=N)	AGT	ACT (=T)	AAT	AGC	GGA	ACA	GCA (=A)	GTG
B464A	TTT	GCG	GTG	AGT	GCG (=A)	AGC	TCT	CTT	TCT	AAT (=N)	AGT	ACC	AAT	GGC (=G)	GGA	ACA	GCA (=A)	GTG
B491	TTT	ACG (=T)	GTG	AGT	GAA	AGC	ACT (=T)	CTT	TCT	AAT (=N)	AGT	ACC	TAT (=Y)	GGC (=G)	GGA	ACA	ACA (=T)	GTG
B497A	TTT	GCG	GTG	AGT	GAA	AGC	TCT	CTT	TCT	AAT (=N)	AGT	ACC	AAT	AGT (=S)	GGA	ACA	GCA (=A)	GTG
B508-S1	TTT	GCG	GTG	AGT	GAA	AGC	TCT	CTT	TCT	AAT (=N)	AGT	ACG (=T)	AAT	AGC	GGA	ACA	GCG	GTG
B508A-T2A	TTT	GCG	GTG	AGT	GAA	AGC	TCT	CTT	TCT	AAT (=N)	AGT	ACG (=T)	AAT	AGC	GGA	ACA	GCG	GTG
B508A-T4	TTT	GCG	GTG	AGT	GAA	AGC	TCT	CTT	TCT	AAT (=N)	AGT	ACG (=T)	AAT	AGC	GGA	ACA	GCG	GTG
B518	TTT	GCG	GTG	AGT	GCG (=A)	AGC	ACT (=T)	CTT	TCT	AAT (=N)	AGT	ACC	AAT	GGC (=G)	GGA	ACA	GCG	GTG
B528A	TTT	ACG (=T)	GTG	AGT	GCG (=A)	AGC	TCT	CTT	TCT	AAT (=N)	CGT (=R)	ACC	AAT	AGC	GGA	ACA	GCG	GTG
B529	TTT	GCG	GTG	AGT	GCG (=A)	AGC	TCT	CTT	TCT	AAT (=N)	CGT (=R)	ACC	AAT	AGC	GGA	ACA	ACG (=T)	GTG
B547F	TTT	GCG	GTG	AGT	GCG (=A)	AGC	TCT	CTT	TCT	AAT (=N)	CAT (=H)	ACC	AAT	AGC	GGA	ACA	GCA (=A)	GTG
B572A	TTT	GCG	GTG	AGT	GAA	AGC	TCT	CTT	TCT	AAT (=N)	AAT (=N)	ACC	AAT	AGC	GGA	ACA	GCG	GTG
B630	TTT	ACG (=T)	GTG	AGT	GCA (=A)	AGC	ACT (=T)	CTT	TCT	AAT (=N)	AGT	ACG (=T)	AAT	AGC	GGA	ACA	GCA (=A)	GCG (=A)
B657-A1	TTT	GCG	GTG	AGT	GCG (=A)	AGC	ACT (=T)	CTT	TCT	AAT (=N)	CGT (=R)	ACC	AAT	AGT (=S)	GGA	GCG (=A)	AGC (=S)	GTG
B657-A4	TTT	GCG	GTG	AGT	GAA	AGC	TCT	CTT	TCT	AAT (=N)	CGT (=R)	ACC	AAT	AGT (=S)	GGA	GCA (=A)	GCG	GTG
B657-C1	TTT	GCG	GTG	AGT	GAA	AGC	TCT	CTT	TCT	AAT (=N)	CGT (=R)	ACC	AAT	AGT (=S)	GGA	GCA (=A)	GCG	GTG
B659-A1	TTT	GCG	GTG	AGC (=S)	GCA (=A)	AGC	ACT (=T)	CTT	TCT	AAT (=N)	AGT	ACC	AAT	AGT (=S)	GGA	GCA (=A)	GCG	GTG
B659-C2	TTT	GCG	GTG	AGC (=S)	GCA (=A)	AGC	ACT (=T)	CTT	TCT	AAT (=N)	AGT	ACC	AAT	AGT (=S)	GGA	GCA (=A)	GCG	GTG
B661A	TTT	GCG	GTG	AGT	GAA	AGC	TCT	CTT	TCT	AAT (=N)	CAT (=H)	ACC	AAT	AGC	GGA	ACA	GCG	GTG
B679	TTT	GCG	GTG	AGT	GTG (=V)	AGC	ACT (=T)	CTT	TCT	AAT (=N)	AGT	ACC	AAT	AGC	GGA	ACA	GCG	GTG
B712A	TTT	GCG	GTG	AGT	GAA	AGC	TCT	CTT	TCT	AAT (=N)	CGT (=R)	ACC	AAT	AGC	GGA	ACA	GCA (=A)	GTG
BMG112	TTT	GCG	GTG	AGT	GAA	AGC	ACT (=T)	CTT	TCT	AAT (=N)	CGT (=R)	ACC	AAT	AGC	GGA	ACA	GCA (=A)	GTG
CRL122	TTT	GCG	GTG	AGT	GCG (=A)	AGC	ACT (=T)	CTT	TCT	AAT (=N)	CGT (=R)	ACT (=T)	AAT	AGC	GGA	ACA	GCG	GTG
CRM21	TTT	GCG	GTG	AGT	GCG (=A)	AGC	ACT (=T)	CTT	TCT	GAT	AGT	ACC	AAT	GGC (=G)	GGA	ACA	GCA (=A)	GTG
JDX15	TTT	GCG	GTG	AGT	GAA	AGC	ACT (=T)	CTT	TCT	AAT (=N)	CGT (=R)	ACC	AAT	AGT (=S)	GGA	ACA	GCG	GTG
JGF25	TTT	GCG	GTG	AGT	GCG (=A)	AGC	TCT	CTT	TCT	AAT (=N)	CAT (=H)	ACT (=T)	AAT	AGC	GGA	ACA	GCG	GTG
JMM43	TTT	GCG	GTG	AGT	GCG (=A)	AGC	TCT	CTT	TCT	AAT (=N)	AGT	ACC	GAT (=D)	AGC	GGA	ACA	GCG	GTG
JSS185-B120	TTT	GCG	GTG	AGT	GCA (=A)	AGC	ACT (=T)	CTT	TCT	AAT (=N)	CGT (=R)	ACC	AAT	AGC	GGA	ACA	GCG	GTG
MMV242	TTT	GCG	GTG	AGT	GCG (=A)	AGC	TCT	CTT	TCT	AAT (=N)	AGT	ACC	AAT	AGC	GGA	ACA	GCG	GTG
MSL190	TTT	GCG	GTG	AGT	GAA	AGC	TCT	CTT	TCT	AAT (=N)	AAT (=N)	ACC	AAT	AGC	GGA	ACA	GCG	GTG
VCT187	TTT	GCG	GTG	AGT	GAA	AGT (=S)	TCT	CTT	TCT	AAT (=N)	CGT (=R)	ACT (=T)	AAT	AGC	GGA	GCG (=A)	GCG	GTG

Mutations described in the references **(7, 53, 78, 105, 106, 107)**
Mutation not described in the references
Red Key mutations that confers resistance to the antibiotic

One of the mutations observed was N562Y, which was detected in strains AAP164 and B491. This mutation has been described in multiple studies such as Zebretto *et al.* (2017), Kwon *et al.* (2017) and Rimbara *et al.* (2007), where it was detected only in AMX-resistant strains **(53, 78, 105).** Position 562 is adjacent to one of the conserced penicillin-binding motifs ($SNN_{559-561}$), which would explain its role in resistance. This mutation, along with the T556S and T593A substitutions, were described as the mutations conferring the highest resistance to *H. pylori* **(43)**. The T593A mutation was detected in 10/52 strains tested. However, its role in resistance development is not entirely clear, as there exist some contradiction between the experts. In some studies, it was associated with the appearance of AMX resistance **(49, 105, 106)**, while in others, such as Kageyama *et al.* (2019), this mutation was observed in both sensitive and resistant strains, suggesting that it was not involved in the development of resistance **(107)**. On the other hand, the T556S mutations was not found in any of the analysed strains. Based in these results, it was concluded that strains AAP164 and A491 could be resistant to AMX due to the presence of N562Y mutation.

Other mutations were observed, such as A369T, S402G or E406A, which are located in or near other penicillin-binding motifs. Among these three, the most notable is the A369T substitution, which is part of the SAIK368-371 motif, where the Serine-368 (Ser368), which is the catalytic residue of PBP1, is located. In this case there is a replacement of an alanine (A) by a threonine (T), resulting in a change from a hydrophobic (non-polar) amino acid to a hydrophilic (polar) one. This prevents the establishment of one of the hydrophobic bonds with the Ser368-AMX complex required for the correct activity of the antibiotic **(Figure 9)**, which would justify the resistance of strains B491 (which also has the N562Y mutation), B528A and B630 to amoxicillin. Finally, other mutations not described in the literature were also found, so their association with *H. pylori* resistance to AMX could not be predicted. In all cases, however, phenotypic tests would be necessary to confirm the correlation between the detected mutations and *H. pylori* resistance to amoxicillin.

4.2.2. 16S rRNA: tetracycline resistance

The resistance of *H. pylori* to tetracycline (TET) is due to alterations in the 926-928 triplet of the *rrnA* and *rrnB* genes that code for 16S rRNA. After analysing the genetic sequences of the 52 strains included in this study, only 3 strains (B679, B464A and CRM21) showed any alteration in this triplet. All three strains only had a single mutation in the triplet, occurring in all three cases at nucleotide 926 (corresponding to an A in the genome of the reference strain 26695), which corresponds to the first nucleotide of the triplet (*see section 4.1.1.2*). Sequence alignment of some of the strains studied is shown in **Figure 14**.

Figure 14. Part of the alignment of the sequences of the gene coding for 16S rRNA from 23/52 strains included in the study. Codon 926-928 of the gene is indicated in red. The blue arrow points to position 926. Black arrows indicate the three strains (B464, B679 and CRM21) in which mutations were detected.

In strain B679, an M was observed at position 926, meaning that this position could correspond to either an A or a C. In the case that the real nucleotide was an A, the triplet would be AGA, so there would be no change with respect to the reference strain, indicating thereby the sensitivity of strain B679. In relation to the A926C mutation (if M=C), to confirm the role of this mutation in the development of resistance, Lawson *et al.* (2005) transformed strain 26695 with the 16S rRNA of the TET-resistant strain H3615 (MIC=1,5 mg/L). The transformed strain obtained showed a MIC equal to that of the donor strain (H3615) suggesting that this mutation may induce tetracycline resistance in *H. pylori* **(108)**. However, the fact that the nucleotide at position 926 in strain B679 could be either an A or a C prevented the strain from being genetically classified as either sensitive or resistant. In this case, it would have been optimal to have more time to sequence the gene again to see which nucleotide was actually located at position 926 of the gene.

The A926C mutation was also detected in strain B464A and could therefore be resistant to tetracycline. It would have been valuable to perform a phenotypic study of strain B464A to confirm whether the mutation found (A926C) induced resistance.

Finally, the A926G mutation was detected in strain CRM21. The influence of this mutation on *H. pylori* resistance to tetracycline remains unclear. Studies by Lawson *et al.* (2005) and Bachir *et al.* (2018) have described this mutation in strains with a low level of tetracycline resistance **(108, 109)**. This is in accordance with findings reported in the studies published by Gerrits *et al.* (2002) and Nishizawa *et al.* (2014) (*see section 4.1.1.2*).

However, in other studies, this mutation was detected in tetracycline-sensitive strains **(69, 85, 110)**. Because of the contradictory results shown in the literature, it was not possible to establish the resistance or sensitivity of strain CRM21 to tetracycline solely based on genotypic data. Therefore, a phenotypic study of this strain would be required to confirm the correlation between this mutation and the resistance to the antibiotic. The mutations detected in the 16S rRNA gene are shown in **Table A2**.

4.2.3. 23S rRNA: clarithromycin resistance

To study the clarithromycin resistance of the 52 *H. pylori* strains studied, positions 2146, 2147, 2186, 2199 and 2227 were analysed and compared with the sequence of the reference strain 26695. These positions are equivalent to positions 2143, 2144, 2183, 2196 and 2224 of the 23S rRNA gene of strain ATCC 27270, being this strain the one used as a reference in most of the published studies. Mutations were detected in 7 of the strains studied, all located at positions 2146 and 2147 (2143 and 2144 of strain ATCC 27270).

Both of these nucleotides are part of the macrolide-binding pocket (*see section 4.1.1.3*). Thus, mutations in these two positions may prevent the binding of CLR to the 23S rRNA, inducing the emergence of a potential resistance to CLR in *H. pylori*. Results of the sequence alignment of some of the strains studied are depicted in **Figure 15**.

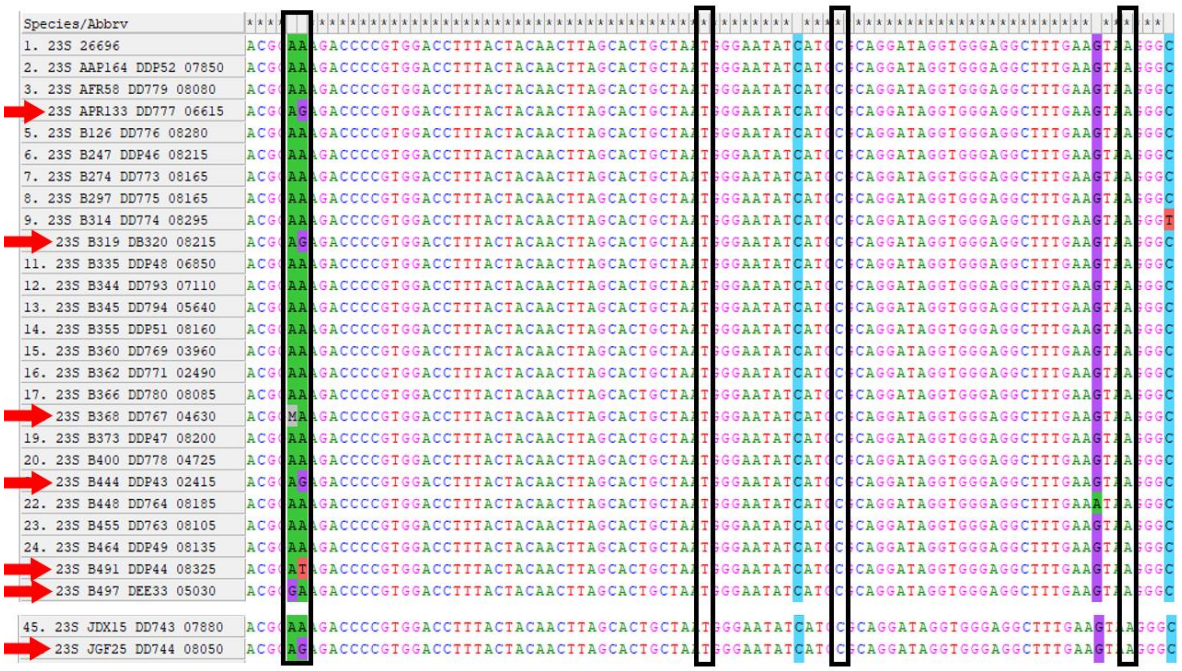

Figure 15. Part of the alignment of the sequences of the gene coding for 23S rRNA. Positions 2146, 2147, 2186, 2199 and 2227 of the gene are framed in black. Red arrows indicate the seven strains (APR133, B319, B368, B444, B491, B497 and JGF25) in which mutations in postions 2146 and 2147 were detected.

In two strains (B368 and B497), alterations were detected at position 2146. In strain B368 an M was observed, which indicated that this position could correspond to either an A or a C. In the first case (M=A), there would be no change compared to the reference strain. In case M was a C, mutation A2146C would be observed. On the other hand, the A2146G substitution was observed in the sequence corresponding to strain B497. The latter, together with the A2145G and A2145C mutations, constitute 90% of cases of clarithromycin-resistant *H. pylori* strains **(62, 64, 111)**. In the study by Lauener *et al.* (2019), the authors found a strong correlation between the presence of A2146C and A2146G mutations and *H. pylori* resistance to clarithromycin **(85)**. On this basis, it was determined that strain B497 carried a mutation that would confer resistance to clarithromycin. However, phenotypic tests should be performed to confirm it. For strain B368, a new sequencing of the gene would be required to confirm the nucleotide corresponding to position 2146 in order to obtain better results.

Regarding position 2147, alterations were observed in five strains. Four of them (APR133, B319, B444A and JGF25) carried the A2147G mutation and in one (B491) the A2147T mutation was observed. Mutations at this position have been the object of ongoing controversy and their role in *H. pylori* resistance to clarithromycin is not fully defined. Regarding the A2147T mutation located in strain B491, Kocazeybek *et al*. (2019) found that the presence of this mutation resulted in an increased probability of developing clarithromycin resistance of 36.92% **(112).** However, in the same study, the A2147T mutation was also detected in strains sensitive to the antibiotic. On the other hand, Francesco *et al*. (2014) detected the same mutation only in resistant strains with a mean MIC value of 134 mg/L **(113).** Therefore, the phenotype of strain B491 could not be predicted due to the controversy observed in the results of the published studies. The mutations detected in the 23S rRNA gene are shown in **Table A3**.

4.2.4. rdxA and frxA: metronidazole resistance

The major cause of metronidazole resistance in *H. pylori* is the presence of point mutations in the *rdxA* and *frxA* genes, which encode two nitroreductases essential for the bioactivation of the drug, RdxA and FrxA respectively. To study the prevalence of resistance among the 52 strains included in this study, the sequences corresponding to these two genes were analysed and compared with the sequence of the reference strain 26695.

On the other hand, as mentioned above, point mutations are not the only alterations detected in these two genes (*see section 4.1.1.4)*. In the strains included in this study, however, no premature STOP were detected, so resistance was not due to protein truncation.

For the *rdxA* gene, four positions of the amino acid sequence were studied: 1, 16, 80 and 118. Mutations were detected at positions 16 and 118: R16C in strain B659-C2, R16H in strains B547F and MSL190 and A118T in strains B126, B297, B355, B360, B400, B444A, B448, B572A, JSS185-B120 and MSL190. R16C/H mutations are among the most frequently described in published studies. Arginine at position 16 (Arg16, R16) is one of the binding sites of the phosphoryl group of the FMN cofactor to the RdxA apoprotein **(68)**. Therefore, mutations at this position can weak this interaction, inducing inactivation of the protein and consequently promoting resistance. The R16C mutation has only been described in metronidazole-resistant *H. pylori* strains **(85, 114)**. The study by Goodwin *et al.* (1998) **(115)** demonstrated a link between the R16C mutation and elevated MIC values in resistant strains. Based on the data obtained, it was considered that strain B659-C2 might be resistant to the antibiotic. The R16H point mutation has been described in several studies (**67, 116, 117)** where it has been described only in resistant strains. Therefore, it was considered that strains B547F and MSL190 could be resistant to the antibiotic.

Similar to alterations at position 16, the A118T mutation has also been linked to *H. pylori* resistance to metronidazole in numerous studies as shown in the papers published by Acosta *et al*. (2017) and Rasheed *et al*. (2014) **(117, 118)**. Based on these results, it was concluded that strains with this mutation could be resistant to the antibiotic. The partial RdxA amino acid sequences of some of the analysed strains are shown in **Figure 16**.

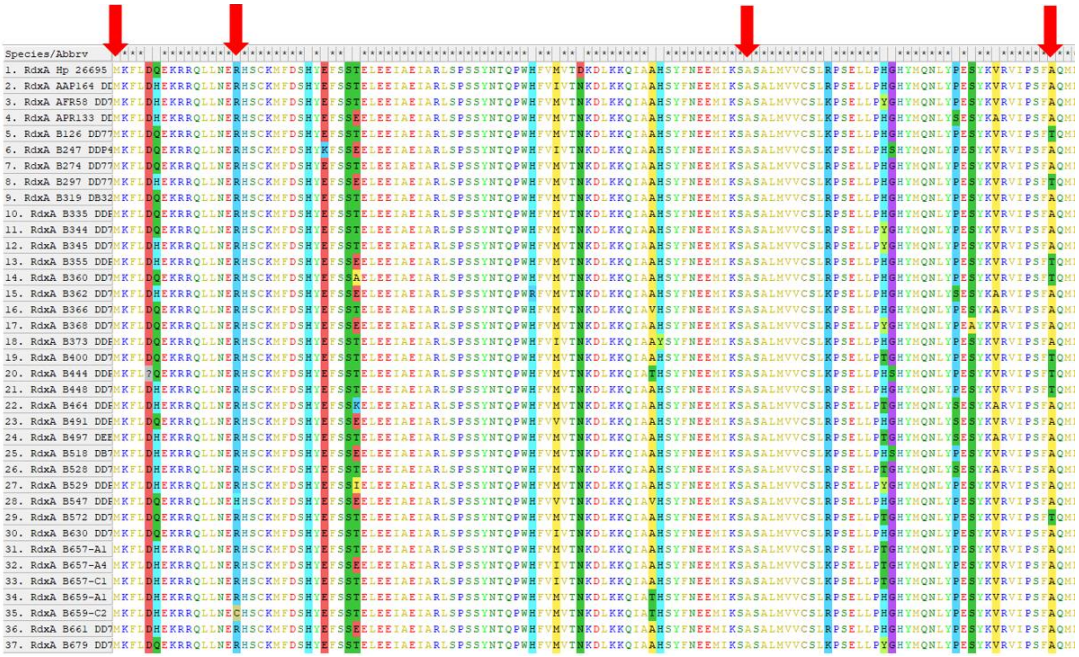

Figure 16. Part of the alignment of the RdxA amino acid sequences Red arrows indicate the positions 1, 16, 80 and 118, were mutations were detected.

On the other hand, the following mutations were observed in the *frxA* gene: A70V in strains B345 and B366, F72S in strains B355, B657-A4, B657-C1, B679 and JDX15, G73S in strains B335, B657-A4, B657-C1, B679 and JDX15, A138V in strain B547F and C193S in strains B335 and JDX15. Due to the controversy about the role of *frxA* gene mutations in relation to metronidazole resistance (*see section 4.1.1.4*), the data obtained from the literature were considered insufficient to evaluate these mutations and further studies, including phenotypic tests for these strains, should be performed to confirm their relationship with the development of resistance.

Mutations in both *rdxA* and *frxA* genes were observed in strains B355 and B547F, and further studies in these strains may help to determine more precisely the relationship between *frxA* gene mutations and metronidazole resistance in *H. pylori*. The mutations detected in the *rdxA/frxA* genes are shown in **Tables A4-A5**.

4.2.5. gyrA: levofloxacin resistance

Resistance of *H. pylori* to levofloxacin is primarily due to mutations in the QRDR region of the *gyrA* gene. For the analysis of the 52 strains included in the study, the sequences corresponding to the *gyrA* gene were compared with the reference strain 26695. Positions 87 and 91 of the amino acid sequence (corresponding to triplets AAC and GAT respectively) were selected as these positions have been most frequently associated with resistance. The mutations detected in the *gyrA* gene are shown in **Table A6.**

Mutations at position 87 involving an amino acid change were detected in 10/52 strains. The mutations detected were N87T (B126, B455, B518 and CRM21), N87I (B319, B345, B368) and N87K (B497A, B547F, BMG112). Regarding position 91, mutations were only detected in two strains: D91N in strain B362 and D91Y in strain B464A. N87K and D91N mutations have been associated with resistance in several previous studies and have been considered critical for the development of resistance **(85, 109, 116)**. Based on these results, it was concluded that strains B497A, B547F, BMG112 and B362 have mutations that would explain the antibiotic resistance. According to the studies by Lee *et al.* (2011) and Rimbara *et al.* (2012), the resistance to LEV of strains B497A, B547F and BMG112 would be expected to be higher that that of strain B362 (*see section 4.1.1.5*) **(80, 82)**. However, phenotypic studies are required to confirm it.

The D91Y substitution (B464A) has been described in resistant isolates in multiple studies. On the other hand, the N87T mutation has been described in several studies, but has been reported in sensitive isolates and is therefore not associated with resistance **(85, 109, 116).** In the study conducted by Mannion *et al.* (2021), the authors identified a resistant strain with the double N87T+D91Y mutation that would justify the role of the latter in the development of resistance **(119)**. Finally, studies published by Evariste *et al.* (2020) **(74)** and Lauener *et al.* (2019) **(85)** confirmed the role of the N87I mutation in the development of resistance. Therefore, based on the above results, it was concluded that strains B319, B345, B368, B497A, B547F, BMG112, B362 and B464A presented genetic mutations that would justify resistance to levofloxacin.

Although these are the most frequently described alterations, point mutations are not the only mutations described in *H. pylori* associated with levofloxacin resistance. The QDNSV insertion at the N-terminus was detected in 15 strains. This same insertion was detected using WGS (Whole Genome Sequencing) techniques in the study published by Qumar *et al.* in 2020 **(120)**. Based on the results of the study as well as published information, it was concluded that the 15 strains in which the insertion was observed were likely to be resistant to levofloxacin. However, phenotypic testing would be necessary to confirm such resistance. In strain BMG112, this insertion was detected together with the N87K mutation and, based on the results discussed in this section and in section 4.1.1.5, a higher degree of levofloxacin resistance would be expected compared to those strains with only the one of the two alterations individually. A five amino acid insertion was also detected in strain B464A. However, it was observed that the fourth amino acid did not match the one described in the literature, being in this case a leucine (L) instead of a serine (S). This single amino acid variation in the sequence of the insert has not been described previously. Therefore, its relation to the development of resistance to LEV could not be determined. Further studies on this strain are therefore required to establish the relation between this specific insertion and levofloxacin resistance. Partial GyrA amino acid sequences, including the 5 amino acid insertion, of part of the strains studied are illustrated in **Figure 17**.

Figure 17. Partial amino acid sequences of GyrA. The black frames highlight the presence of the 5 amino acid QDNSV insertion. Red oval indicates strain B464 harbouring the modified insertion.

4.2.6. rpoB: rifabutin resistance

For the study of rifabutin (RFB) resistance in the 52 *H. pylori* strains included in this study, the sequences corresponding to the *rpoB* gene were compared with the reference strain 26695. Codons 525, 530, 540 and 547 of the RRDR were determined and analysed for resistance-associated mutations. No strain showed alterations at these positions and it was therefore concluded that all strains were susceptible. These results can be justified considering that the use of rifabutin is limited due to its adverse effects and the potential risk of *M. tuberculosis* becoming resistant (*see section 1.7.1.6*). The mutations detected in the *rpoB* gene are shown in **Table A7.**

5. CONCLUSIONS

- Antibiotic resistance of *Helicobacter pylori* is a complex and multi-factorial process involving several mechanisms, some of which are still unknown.
- Genetic mutations are the most important mechanism of resistance.
    - Mutations in the conserved motifs of PBP1 are the main driver for amoxicillin resistance. Mutations in PBP2 and PBP3 appear to contribute to resistance, although their role is not fully defined.
    - Tetracycline resistance results from point mutations in triplet 926-928 of the 16S rRNA coding gene. The level of resistance depends on the number of nucleotides mutated in this triplet.
    - The major cause of clarithromycin resistance involves mutations along specific nucleotides in the macrolide binding pocket of the 23S rRNA. Mutations in the *infB* and *rplJ2* genes have a synergistic effect with those in the 23S rRNA.
    - Metronidazole resistance is due to inactivating mutations in the RdxA and FrxA reductases genes, which are the major enzymes involved in its activation.
    - The main cause of LEV resistance consists of mutations in the GyrA QRDR region. QDNSV insertion in the N-terminus leads to structural changes in the GyrA subunit and induces the development of resistance.
    - Resistance to rifabutin results from mutations at positions 525, 530, 540 and 547 of the RpoB RRDR region.
- The expression of β-lactamases is not the major mechanism of *H. pylori* amoxicillin resistance, which distinguishes it from most Gram-negative bacteria.
- The deregulation of OMPs, mainly those belonging to efflux pump RND supefamily and cellular adaptation processes (biofilms and coccoid morphology) are major contributors to the emergence of multidrug resistance in *H. pylori*.
- It has been possible to analyse the sequences corresponding to the 7 targeted genes (*pbp1*, 16S rRNA, 23S rRNA, *rdxA*, *frxA*, *gyrA* and *rpoB*).
- The N562Y and A369T point mutations in *pbp1* gene were predicted to confer amoxicillin resistance to strains AAP164, A491, B528 and B630.
- In 16S rRNA coding gene, point mutations were only detected at nucleotide 926. According to the results, strain B464A was predicted to be resistant to tetracycline.
- In seven strains (APR133, B319, B444A, B368, B491, B497 and JGF25) mutations were detected at positions 2146 and 2147 of the 23S rRNA gene. Due to the A2146G mutation, strain B497 was considered to be resistant.
- Strains harbouring R16C/H and A118T point mutations in the *rdxA* gene were predicted to be potentially resistant. Mutations detected in the *frxA* gene dit not allow no predict resistance to metronidazole because of the ongoing controversy on their role in the emergence of metronidazole resistance in *H. pylori*.
- Strains B319, B345, B362, B368, B464A, B497A, B547F and BMG112 presented point mutations in amino acids N87 and D91, and consequently it was considered that they might be resistant to levofloxacin. The strains with the QDNSV insertion in the N-terminal region of GyrA were considered to be resistant.
- No mutations were detected in the *rpoB* gene in any of the strains tested and it was therefore concluded that all the strains were susceptible to rifabutin.
- Potential predicted resistances to the different antibiotics shoud be confirmed by phenotypic tests
- For the remaining detected mutations, further studies, including phenotypic testing, will be required to confirm their role in *H. pylori* antibiotic resistance.

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7. ANNEXES

7.1. Global priority list of antibiotic resistant bacteria to guide Research, Discovery and Development of new antibiotics

* *Enterobacteriaceae* include: *Klebsiella pneumoniae*, *Escherichia coli*, *Enterobacter* spp., *Serratia* spp., *Proteus* spp., *Providencia* spp. and *Morganella* spp.

7.2. *Helicobacter pylori* strains

Table A.1. *H. pylori* strains included in the study. The GenBank Accession Number (GenBank AN) is given for each strain toguether with the locus tag for each of the genes analysed: *pbp1*, 16S rRNA, 23S rRNA, *rdxA*, *frxA*, *gyrA* and *rpoB*.

H. pylori strain	GenBank AN	*pbp1*	16S rRNA	23S rRNA	*rdxA*	*frxA*	*gyrA*	*rpoB*
AAP164	QDJV00000000	DDP52_03695	DDP52_07360	DDP52_07850	DDP52_05525	DDP52_RS03920	DDP52_04210	DDP52_06170
AFR58	QEHJ00000000	DD779_03020	DD779_07540	DD779_08080	DD779_04995	DD779_RS03250	DD779_03515	DD779_06310
APR133	QEHI00000000	DD777_02500	DD777_03505	DD777_06615	DD777_04320	DD777_RS02735	DD777_03030	DD777_05580
B126	QEHH00000000	DD776_03305	DD776_06305	DD776_08280	DD776_05150	DD776_RS03535	DD776_03820	DD776_06590
B247A	QDJU00000000	DDP46_02890	DDP46_05765	DDP46_08215	DDP46_04705	DDP46_RS03110	DDP46_03405	DDP46_06045
B274	QEHG00000000	DD773_02340	DD773_06745	DD773_08165	DD773_03910	DD773_RS02565	DD773_02830	DD773_05200
B297	QEHF00000000	DD775_02580	DD775_07125	DD775_08165	DD775_04635	DD775_RS03060	DD775_03345	DD775_05970
B314	QEHE00000000	DD774_02760	DD774_07175	DD774_08295	DD774_RS045	DD774_RS02985	DD774_03270	DD774_05845
B319	QELC00000000	DB320_03170	DB320_05530	DB320_08215	DB320_04955	DB320_RS03405	DB320_03700	DB320_05825
B335	QDJT00000000	DDP48_02575	DDP48_08255	DDP48_06850	DDP48_04405	DDP48_RS02825	DDP48_03105	DDP48_05780
B344	QEHD00000000	DD793_03640	DD793_05760	DD793_07110	DD793_05425	DD793_RS03865	DD793_04150	DD793_06040
B345	QEHC00000000	DD794_03575	DD794_05960	DD794_05640	DD794_05475	DD794_RS03790	DD794_04075	DD794_06240
B355	QDJS00000000	DDP51_03185	DDP51_05370	DDP51_08160	DDP51_04045	DDP51_RS03415	DDP51_04490	DDP51_05655
B360	QEHB00000000	DD769_01985	DD769_06520	DD769_03960	DD769_03780	DD769_RS02245	DD769_05245	DD769_05150
B362	QEHA00000000.1	DD771_03185	DD771_07510	DD771_02490	DD771_05060	DD771_RS03420	DD771_03705	DD771_06225
B366	QEGZ00000000	DD780_02980	DD780_05105	DD780_08085	DD780_04770	DD780_RS03200	DD780_03480	DD780_05370
B368	QEGY00000000	DD767_02650	DD767_06935	DD767_04630	DD767_04490	DD767_RS02875	DD767_03180	DD767_05805
B373	QDJR00000000	DDP47_02110	DDP47_08190	DDP47_08200	DDP47_03975	DDP47_RS02330	DDP47_02610	DDP47_05730
B400	QEGX00000000	DD778_02775	DD778_05535	DD778_04725	DD778_04575	DD778_RS03005	DD778_03295	DD778_05815
B444A	QDJQ00000000	DDP43_03095	DDP43_05805	DDP43_02415	DDP43_05055	DDP43_RS03330	DDP43_03625	DDP43_06070
B448	QEGW00000000	DD764_03090	DD764_07555	DD764_08185	DD764_04885	DD764_RS03315	DD764_03605	DD764_06225
B455	QEGV00000000	DD763_03125	DD763_06050	DD763_08105	DD763_03365	DD763_RS03360	DD763_03655	DD763_06330
B464A	QDJP00000000	DDP49_02650	DDP49_06940	DDP49_08135	DDP49_04465	DDP49_RS02870	DDP49_03165	DDP49_05790

B491	QDJO00000000	DDP44_02985	DDP44_07460	DDP44_08325	DDP44_04810	DDP44_RS03225	DDP44_03505	DDP44_06225
B497A	QEGU00000000	DEE33_02930	DEE33_08060	DEE33_05030	DEE33_04865	DEE33_RS03160	DEE33_03690	DEE33_06245
B508-S1	QDJN00000000	DDP45_03540	DDP45_05700	DDP45_07170	DDP45_RS05305	DDP45_RS03760	DDP45_04025	DDP45_05980
B508A-T2A	QDJM00000000	DDP35_07185	DDP35_05480	DDP35_07815	DDP35_RS05075	DDP35_RS03530	DDP35_03805	DDP35_05760
B508A-T4	QDJL00000000	DDP36_03785	DDP36_07385	DDP36_05740	DDP36_RS05565	DDP36_RS04005	DDP36_04270	DDP36_06015
B518	QELB00000000	DB721_02330	DB721_07455	DB721_07290	DB721_04270	DB721_RS09035	DB721_02875	DB721_06245
B528A	QEGT00000000	DD759_02965	DD759_06095	DD759_03255	DD759_05110	DD759_RS03200	DD759_03745	DD759_06385
B529	QDJK00000000	DDP37_03625	DDP37_07525	DDP37_08090	DDP37_05415	DDP37_RS03855	DDP37_04140	DDP37_06150
B547F	QDJJ00000000	DDP57_03025	DDP57_05890	DDP57_04980	DDP57_04820	DDP57_RS03250	DDP57_03530	DDP57_06155
B572A	QEGS00000000	DD754_03055	DD754_06640	DD754_08105	DD754_04865	DD754_RS03280	DD754_03570	DD754_05420
B630	QEGR00000000	DD752_03150	DD752_05965	DD752_07940	DD752_04980	DD752_RS03365	DD752_03665	DD752_06240
B657-A1	QDJI00000000	DDP42_03175	DDP42_05515	DDP42_08010	DDP42_04930	DDP42_RS03395	DDP42_03685	DDP42_05790
B657-A4	QDJH00000000	DDP34_03145	DDP34_05080	DDP34_02735	DDP34_04745	DDP34_RS03375	DDP34_03645	DDP34_05355
B657-C1	QDJG00000000	DDP38_03975	DDP38_06510	DDP38_06450	DDP38_04745	DDP38_RS03730	DDP38_03460	DDP38_05340
B659-A1	QDJF00000000	DDP39_02835	DDP39_05015	DDP39_07670	DDP39_04630	DDP39_RS03065	DDP39_03345	DDP39_05295
B659-C2	QDJE00000000	DDP41_03975	DDP41_07665	DDP41_05950	DDP41_05775	DDP41_RS04190	DDP41_04490	DDP41_06450
B661A	QEGQ00000000	DD750_02550	DD750_07595	DD750_08190	DD750_04355	DD750_RS02795	DD750_03080	DD750_05720
B679	QEGP00000000	DD756_02825	DD756_06025	DD756_08235	DD756_05115	DD756_RS03230	DD756_03505	DD756_06310
B712A	QEGO00000000	DD751_03575	DD751_05750	DD751_07770	DD751_05425	DD751_RS03810	DD751_04195	DD751_06025
BMG112	QEGN00000000	DD746_02960	DD746_04960	DD746_02220	DD746_02030	DD746_RS03195	DD746_03470	DD746_05240
CRL122	QDJD00000000	DDP32_03430	DDP32_07395	DDP32_00010	DDP32_04105	DDP32_RS03660	DDP32_04845	DDP32_06305
CRM21	QEGM00000000	DD741_03830	DD741_08025	DD741_08055	DD741_05670	DD741_RS04070	DD741_04335	DD741_06385
JDX15	QEGK00000000	DD743_02015	DD743_06375	DD743_07880	DD743_03840	DD743_RS02235	DD743_02515	DD743_05225
JGF25	QEGJ00000000	DD744_03145	DD744_07315	DD744_08050	DD744_05195	DD744_RS03375	DD744_03485	DD744_06140
JMM43	QEGI00000000	DD748_03630	DD748_07345	DD748_07930	DD748_05440	DD748_RS03860	DD748_04115	DD748_06155
JSS185	QEGH00000000	DD742_02225	DD742_06590	DD742_08130	DD742_04070	DD742_RS02460	DD742_02740	DD742_05405
MMV242	QEGG00000000	DD749_03170	DD749_07180	DD749_08130	DD749_04970	DD749_RS03405	DD749_03695	DD749_06425

MSL190	QEGF00000000	DD736_04450	DD736_06500	DD736_07895	DD736_04010	DD736_RS02455	DD736_02725	DD736_05270
VCT187	QEGD00000000	DD747_02470	DD747_05720	DD747_04440	DD747_04295	DD747_RS02690	DD747_02990	DD747_06265

7.3. Genetic Code and Amino acid codes

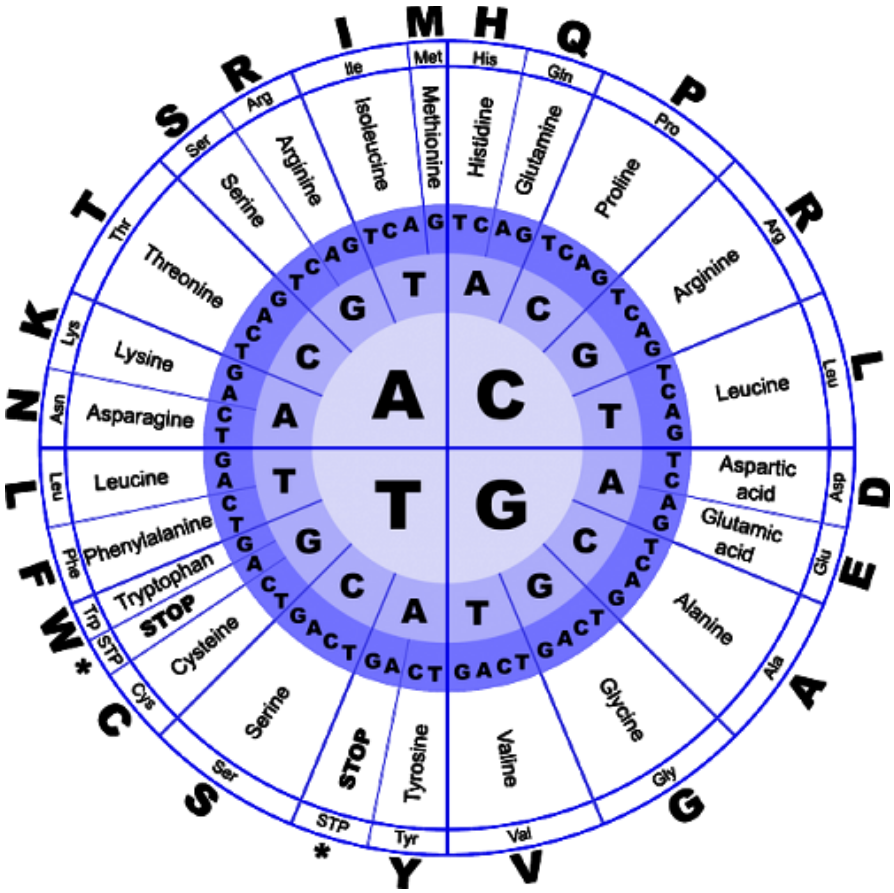

Figure A1. Genetic code. The innermst circle indicates the first Nucleotide of the codon, the middle circle indicates the second and the outermost circule the last Nucleotide of the codon. The amino acids encoded by each codon are shown around it (one- and three letter codes).

G	Glycine	Gly	**P**	Proline	Pro
A	Alanine	Ala	**V**	Valine	Val
L	Leucine	Leu	**I**	Isoleucine	Ile
M	Methionine	Met	**C**	Cysteine	Cys
F	Phenylalanine	Phe	**Y**	Tyrosine	Tyr
W	Tryptophan	Trp	**H**	Histidine	His
K	Lysine	Lys	**R**	Arginine	Arg
Q	Glutamine	Gln	**N**	Asparagine	Asn
E	Glutamic Acid	Glu	**D**	Aspartic Acid	Asp
S	Serine	Ser	**T**	Threonine	Thr

Figure A2. Amino acids, one- and three letter-codes

7.4. Results of the detection of mutations in the screening of the 7 resistance associated genes of the 52 strains selected

Table A2. Results of mutation detection in the 16S rRNA gene (TET)

Nucleotides	926	927	928
Resistance	C, G, T	T	C
ATCC 26695 (TET-S)	A	G	A
AAP164	A	G	A
AFR58	A	G	A
APR133	A	G	A
B126	A	G	A
B247A	A	G	A
B274	A	G	A
B297	A	G	A
B314	A	G	A
B319	A	G	A
B335	A	G	A
B344	A	G	A
B345	A	G	A
B355	A	G	A
B360	A	G	A
B362	A	G	A
B366	A	G	A
B368	A	G	A
B373	A	G	A
B400	A	G	A
B444A	A	G	A
B448	A	G	A
B455	A	G	A
B464A	C	G	A
B491	A	G	A
B497A	A	G	A
B508-S1	A	G	A
B508A-T2A	A	G	A
B508A-T4	A	G	A
B518	A	G	A
B528A	A	G	A
B529	A	G	A
B547F	A	G	A
B572A	A	G	A
B630	A	G	A
B657-A1	A	G	A
B657-A4	A	G	A
B657-C1	A	G	A
B659-A1	A	G	A
B659-C2	A	G	A
B661A	A	G	A
B679	M	G	A
B712A	A	G	A
BMG112	A	G	A
CRL122	A	G	A
CRM21	G	G	A
JDX15	A	G	A
JGF25	A	G	A
JMM43	A	G	A
JSS185-B120	A	G	A
MMV242	A	G	A
MSL190	A	G	A
VCT187	A	G	A

Mutations described in the references **(108, 109)**
M = A/C
Red Key mutations that confers resistance to tetracycline

Table A3. Results of mutation detection in the 23S rRNA gene (CLR)

Nucleotides	2146	2147	2186	2199	2227
Resistance	G	G	C	T	G
ATCC 26695 (CLR-S)	A	A	T	C	A
AAP164	A	A	T	C	A
AFR58	A	A	T	C	A
APR133	A	G	T	C	A
B126	A	A	T	C	A
B247A	A	A	T	C	A
B274	A	A	T	C	A
B297	A	A	T	C	A
B314	A	A	T	C	A
B319	A	G	T	C	A
B335	A	A	T	C	A
B344	A	A	T	C	A
B345	A	A	T	C	A
B355	A	A	T	C	A
B360	A	A	T	C	A
B362	A	A	T	C	A
B366	A	A	T	C	A
B368	M	A	T	C	A
B373	A	A	T	C	A
B400	A	A	T	C	A
B444A	A	G	T	C	A
B448	A	A	T	C	A
B455	A	A	T	C	A
B464A	A	A	T	C	A
B491	A	T	T	C	A
B497A	G	A	T	C	A
B508-S1	A	A	T	C	A
B508A-T2A	A	A	T	C	A
B508A-T4	A	A	T	C	A
B518	A	A	T	C	A
B528A	A	A	T	C	A
B529	A	A	T	C	A
B547F	A	A	T	C	A
B572A	A	A	T	C	A
B630	A	A	T	C	A
B657-A1	A	A	T	C	A
B657-A4	A	A	T	C	A
B657-C1	A	A	T	C	A
B659-A1	A	A	T	C	A
B659-C2	A	A	T	C	A
B661A	A	A	T	C	A
B679	A	A	T	C	A
B712A	A	A	T	C	A
BMG112	A	A	T	C	A
CRL122	A	A	T	C	A
CRM21	A	A	T	C	A
JDX15	A	A	T	C	A
JGF25	A	G	T	C	A
JMM43	A	A	T	C	A
JSS185-B120	A	A	T	C	A
MMV242	A	A	T	C	A
MSL190	A	A	T	C	A
VCT187	A	A	T	C	A

Mutations described in the references **(7, 43, 62, 85, 112, 113,)**
M = A/C
Red Key mutations that confers resistance to tetracycline

Table A4. Results of mutation detection in the *rdxA* gene (MTZ)

Nucleotides	1-2-3	46-7-8	238-39-40	352-3-4
Triplet (sensitive)	ATG (=M)	CGC (=R)	GCT (=A)	GCT (=A)
Amino acid position	1	16	80	118
Resistant	I	C, H	T	T
ATCC 26695 (MTZ-S	ATG (=M)	CGC (=R)	GCT (=A)	GCT (=A)
AAP164	ATG	CGC	GCT	GCT
AFR58	ATG	CGC	GCT	GCT
APR133	ATG	CGC	GCT	GCT
B126	ATG	CGC	GCT	ACT (=T)
B247A	ATG	CGC	GCT	GCT
B274	ATG	CGT (=R)	GCT	GCT
B297	ATG	CGC	GCT	ACT (=T)
B314	ATG	CGC	GCT	GCT
B319	ATG	CGC	GCT	GCT
B335	ATG	CGC	GCT	GCT
B344	ATG	CGC	GCT	GCT
B345	ATG	CGC	GCT	GCT
B355	ATG	CGC	GCT	ACT (=T)
B360	ATG	CGC	GCT	ACT (=T)
B362	ATG	CGC	GCT	GCT
B366	ATG	CGC	GCT	GCT
B368	ATG	CGC	GCT	GCT
B373	ATG	CGC	GCT	GCT
B400	ATG	CGC	GCT	ACT (=T)
B444A	ATG	CGC	GCT	ACT (=T)
B448	ATG	CGC	GCT	ACT (=T)
B455	ATG	CGC	GCT	GCT
B464A	ATG	CGC	GCT	GCT
B491	ATG	CGC	GCT	GCT
B497A	ATG	CGC	GCT	GCT
B508-S1	ATG	CGC	GCT	GCT
B508A-T2A	ATG	CGC	GCT	GCT
B508A-T4	ATG	CGC	GCT	GCT
B518	ATG	CGC	GCT	GCT
B528A	ATG	CGC	GCT	GCT
B529	ATG	CGC	GCT	GCT
B547F	ATG	CAC (=H)	GCT	GCT
B572A	ATG	CGC	GCT	ACT (=T)
B630	ATG	CGC	GCT	GCT
B657-A1	ATG	CGC	GCT	GCT
B657-A4	ATG	CGC	GCT	GCT
B657-C1	ATG	CGC	GCT	GCT
B659-A1	ATG	CGC	GCT	GCT
B659-C2	ATG	TGC (=C)	GCT	GCT
B661A	ATG	CGC	GCT	GCT
B679	ATG	CGC	GCT	GCT
B712A	ATG	CGC	GCT	GCT
BMG112	ATG	CGC	GCT	GCT
CRL122	ATG	CGC	GCT	GCT
CRM21	ATG	CGC	GCT	GCT
JDX15	ATG	CGC	GCT	GCT
JGF25	ATG	CGC	GCT	GCT
JMM43	ATG	CGC	GCT	GCT
JSS185-B120	ATG	CGC	GCT	ACT (=T)
MMV242	ATG	CGC	GCT	GCT
MSL190	ATG	CAC (=H)	GCT	ACT (=T)
VCT187	ATG	CGC	GCT	GCT

Red Mutations described in the references **(67, 85, 114-118)**
Key mutations that confers resistance to the antibiotic

Table A5. Results of mutation detection in the *frxA* gene (MTZ)

Nucleotides	94-5-6	202-3-4	208-9-10	214-5-6	217-8-9	412-3-4	454-5-6	457-8-9	577-8-9
Triplet (Sensitive)	GCT (=A)	TGG (=W)	GCG (=A)	TTT (=F)	GGT (=G)	GCT (=A)	GCA (=A)	GCG (=A)	TGT (=C)
Amino acid position	32	68	70	72	73	138	152	153	193
Resistance	V/T/S	STOP	G/V/T	S	S	T/V	V/T	V/G/Q/T	S/F/V
ATCC 26695 (MTZ-S)	GCT	TGG	GCG	TTT	GGT	GCT	GCA	GCG	TGT
AAP164	GCT	TGG	GCG	TTT	GGT	GCT	GCA	GCG	TGT
AFR58	GCT	TGG	GCG	TTT	GGT	GCT	GCA	GCG	TGT
APR133	GCT	TGG	GCG	TTT	GGT	GCT	GCA	GCG	TGT
B126	GCT	TGG	GCA (=A)	TTT	GGT	GCT	GCA	GCG	TGT
B247A	GCT	TGG	GCG	TTT	GGT	GCT	GCA	GCG	TGT
B274	GCT	TGG	GCG	TTT	GGT	GCT	GCA	GCG	TGT
B297	GCT	TGG	GCG	TTT	GGT	GCT	GCA	GCG	TGT
B314	GCT	TGG	GCG	TTT	GGT	GCT	GCA	GCG	TGT
B319	GCT	TGG	GCG	TTT	GGT	GCT	GCA	GCG	TGT
B335	GCT	TGG	GCG	TTT	AGC (=S)	GCT	GCA	GCG	AGT (=S)
B344	GCT	TGG	GCG	TTT	GGT	GCT	GCA	GCG	TGT
B345	GCT	TGG	GTG (=V)	TTT	GGT	GCT	GCA	GCG	TGT
B355	GCT	TGG	GCG	TCT (=S)	GGT	GCT	GCA	GCG	TGT
B360	GCT	TGG	GCG	TTT	GGT	GCT	GCA	GCG	TGT
B362	GCT	TGG	GCG	TTT	GGT	GCT	GCA	GCG	TGT
B366	GCT	TGG	GTG (=V)	TTT	GGT	GCT	GCA	GCG	TGT
B368	GCT	TGG	GCG	TTT	GGT	GCC (=A)	GCA	GCG	TGT
B373	GCT	TGG	GCG	TTT	GGT	GCT	GCA	GCG	TGT
B400	GCT	TGG	GCA (=A)	TTT	GGT	GCT	GCA	GCG	TGT
B444A	GCT	TGG	GCG	TTT	GGT	GCT	GCA	GCG	TGT
B448	GCT	TGG	GCG	TTT	GGT	GCT	GCA	GCG	TGT
B455	GCT	TGG	GCG	TTT	GGT	GCT	GCA	GCG	TGT
B464A	GCT	TGG	GCG	TTT	GGT	GCT	GCA	GCG	TGT
B491	GCT	TGG	GCG	TTT	GGT	GCT	GCA	GCG	TGT
B497A	GCT	TGG	GCG	TTT	GGT	GCT	GCA	GCG	TGT
B508-S1	GCT	TGG	GCG	TTT	GGT	GCT	GCA	GCG	TGT
B508A-T2A	GCT	TGG	GCG	TTT	GGT	GCT	GCA	GCG	TGT
B508A-T4	GCT	TGG	GCG	TTT	GGT	GCT	GCA	GCG	TGT
B518	GCT	TGG	GCG	TTT	GGT	GCT	GCA	GCG	TGT
B528A	GCT	TGG	GCG	TTT	GGT	GCC (=A)	GCA	GCG	TGT
B529	GCT	TGG	GCG	TTT	GGT	GCT	GCA	GCG	TGT
B547F	GCT	TGG	GCG	TTT	GGT	GTT (=V)	GCA	GCG	TGT
B572A	GCT	TGG	GCG	TTT	GGT	GCT	GCA	GCG	TGT
B630	GCT	TGG	GCG	TTT	GGT	GCT	GCA	GCG	TGT
B657-A1	GCT	TGG	GCG	TTT	GGT	GCT	GCA	GCG	TGT
B657-A4	GCT	TGG	GCG	TCT (=S)	AGC (=S)	GCT	GCA	GCG	TGT
B657-C1	GCT	TGG	GCG	TCT (=S)	AGC (=S)	GCT	GCA	GCG	TGT
B659-A1	GCT	TGG	GCG	TTT	GGT	GCT	GCA	GCG	TGT
B659-C2	GCT	TGG	GCG	TTT	GGT	GCT	GCA	GCG	TGT
B661A	GCT	TGG	GCG	TTT	GGT	GCT	GCA	GCG	TGT
B679	GCT	TGG	GCG	TCT (=S)	AGC (=S)	GCT	GCA	GCG	TGT
B712A	GCT	TGG	GCG	TTT	GGT	GCT	GCA	GCG	TGT
BMG112	GCT	TGG	GCG	TTT	GGT	GCT	GCA	GCG	TGT
CRL122	GCT	TGG	GCG	TTT	GGT	GCC (=A)	GCA	GCG	TGT
CRM21	GCT	TGG	GCG	TTT	GGT	GCT	GCA	GCG	TGT
JDX15	GCT	TGG	GCG	TCT (=S)	AGC (=S)	GCT	GCA	GCG	AGT (=S)
JGF25	GCT	TGG	GCG	TTT	GGT	GCT	GCA	GCG	TGT
JMM43	GCT	TGG	GCG	TTT	GGT	GCT	GCA	GCG	TGT
JSS185-B120	GCT	TGG	GCG	TTT	GGT	GCT	GCA	GCG	TGT
MMV242	GCT	TGG	GCG	TTT	GGT	GCT	GCA	GCG	TGT
MSL190	GCT	TGG	GCG	TTT	GGT	GCT	GCA	GCG	TGT
VCT187	GCT	TGG	GCG	TTT	GGT	GCT	GCA	GCG	TGT

Mutation not described in the references

Table A6. Results of mutation detection in the *gyrA* gene (LEV)

Nucleotides	259-60-61 / 274-5-6	271-2-3 / 286-7-8	Insertion
Triplet (sensitive)	AAC (=N)	GAT (=D)	—
Amino acid position	87	91	—
Resistance	I/K/Y	G/N/Y	QDNSV
ATCC 26695 (LEV-S)	AAC (=N)	GAT (=D)	No
AAP164	AAC	GAT	QDNSV
AFR58	AAC	GAT	—
APR133	AAC	GAT	—
B126	ACT (=T)	GAT	—
B247A	AAT (=N)	GAT	—
B274	AAC	GAT	QDNSV
B297	AAT (=N)	GAT	—
B314	AAT (=N)	GAT	—
B319	ATT (=I)	GAT	—
B335	AAT (=N)	GAT	—
B344	AAT (=N)	GAT	QDNSV
B345	ATC (= I)	GAT	QDNSV
B355	AAT (=N)	GAT	—
B360	AAT (=N)	GAT	—
B362	AAT (=N)	AAT (=N)	—
B366	AAT (=N)	GAT	—
B368	ATC (=I)	GAT	—
B373	AAC	GAT	—
B400	AAC	GAT	QDNSV
B444A	AAC	GAT	—
B448	AAC	GAT	—
B455	ACC (=T)	GAT	—
B464A	AAC	TAT (=Y)	QDNLV
B491	AAC	GAT	QDNSV
B497A	AAA (=K)	GAT	—
B508-S1	AAC	GAT	—
B508A-T2A	AAC	GAT	—
B508A-T4	AAC	GAT	—
B518	ACC (=T)	GAT	—
B528A	AAC	GAT	—
B529	AAC	GAT	QDNSV
B547F	AAA (=K)	GAT	—
B572A	AAT (=N)	GAT	—
B630	AAT (=N)	GAT	—
B657-A1	AAC	GAT	QDNSV
B657-A4	AAC	GAT	QDNSV
B657-C1	AAC	GAT	QDNSV
B659-A1	AAC	GAT	QDNSV
B659-C2	AAC	GAT	QDNSV
B661A	AAC	GAT	—
B679	AAT (=N)	GAT	—
B712A	AAC	GAT	—
BMG112	AAA (=K)	GAT	QDNSV
CRL122	AAC	GAT	—
CRM21	ACC (=T)	GAT	—
JDX15	AAT (=N)	GAT	—
JGF25	AAT (=N)	GAT	—
JMM43	AAC	GAT	QDNSV
JSS185-B120	AAC	GAT	—
MMV242	AAC	GAT	QDNSV
MSL190	AAT (=N)	GAT	—
VCT187	AAT (=N)	GAT	—

Mutations described in the references **(74, 82, 85, 109, 116)**
Insertion described in the references **(67)**
Insertion not described in the references
Red Key mutations that confers resistance to the antibiotic

Table A7. Results of mutation detection in the *rpoB* gene (RFB)

Nucleotides	1573-4-5	1588-89-90	1618-19-20	1639-40-41
Triplet (sensitive)	CTC (=L)	GAT (=D)	CAC (=H)	CTC (=L)
Amino acid position	525	530	540	547
Resistance	I	G/E/V	N	F
ATCC 26695 (RFB-S)	L	D	H	L
AAP164	CTC	GAT	CAC	CTC
AFR58	CTC	GAT	CAC	CTC
APR133	CTC	GAT	CAC	CTC
B126	CTC	GAT	CAC	CTC
B247A	CTC	GAT	CAC	CTT (=L)
B274	CTC	GAT	CAC	CTC
B297	CTC	GAT	CAC	CTC
B314	CTC	GAT	CAC	CTC
B319	CTC	GAT	CAC	CTC
B335	CTC	GAT	CAC	CTC
B344	CTC	GAT	CAC	CTC
B345	CTC	GAT	CAC	CTC
B355	CTC	GAT	CAC	CTC
B360	CTC	GAT	CAC	CTC
B362	CTC	GAT	CAC	CTC
B366	CTC	GAT	CAC	CTC
B368	CTC	GAT	CAC	CTC
B373	CTC	GAT	CAC	CTC
B400	CTC	GAT	CAC	CTC
B444A	CTC	GAT	CAC	CTC
B448	CTC	GAT	CAC	CTC
B455	CTC	GAT	CAC	CTC
B464A	CTC	GAT	CAC	CTC
B491	CTC	GAT	CAC	CTC
B497A	CTC	GAT	CAC	CTC
B508-S1	CTC	GAT	CAC	CTC
B508A-T2A	CTC	GAT	CAC	CTC
B508A-T4	CTC	GAT	CAC	CTC
B518	CTC	GAT	CAC	CTC
B528A	CTC	GAT	CAC	CTC
B529	CTC	GAT	CAC	CTC
B547F	CTC	GAT	CAC	CTC
B572A	CTC	GAT	CAC	CTC
B630	CTC	GAT	CAC	CTC
B657-A1	CTC	GAT	CAC	CTC
B657-A4	CTC	GAT	CAC	CTC
B657-C1	CTC	GAT	CAC	CTC
B659-A1	CTC	GAT	CAC	CTC
B659-C2	CTC	GAT	CAC	CTC
B661A	CTC	GAT	CAC	CTC
B679	CTC	GAT	CAC	CTC
B712A	CTC	GAT	CAC	CTC
BMG112	CTC	GAT	CAC	CTC
CRL122	CTC	GAT	CAC	CTC
CRM21	CTC	GAT	CAC	CTC
JDX15	CTC	GAT	CAC	CTC
JGF25	CTC	GAT	CAC	CTC
JMM43	CTC	GAT	CAC	CTC
JSS185-B120	CTC	GAT	CAC	CTC
MMV242	CTC	GAT	CAC	CTC
MSL190	CTC	GAT	CAC	CTC
VCT187	CTC	GAT	CAC	CTC