**Helicobacter pylori** resistance to antibiotics

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*Helicobacter pylori* is the human pathogen responsible for the development of gastritis that, in about 15% of the patients may further progress to more severe conditions, peptic ulcer disease and gastric cancer. Despite the ongoing discussion on which *H. pylori* infected patients should be treated, all colonized patients or just those with overt symptoms of disease, the high rates of prevalence of infection worldwide demand the need for good strategies for eradication. In fact, depending on the socioeconomic status of the country, the prevalence of infection varies from 40 to over 80% of the population, with higher rates for developing countries. Currently, the eradication of *H. pylori* is managed by the use of a triple therapy, involving the co-administration of two antibiotics and a proton pump inhibitor (PPI) or ranitidine bismuth during seven days. The antibiotic resistance is a transversal problem to all bacteria, gaining importance if leads to treatment failure. *H. pylori* is no exception of that, leading to eradication failure in about 20% of the patients. This chapter focuses on the current therapy options against *H. pylori* and on the problem of its antibiotic resistance. Included in the most prescribed species are the macrolides, fluoroquinolones, amoxicillin, nitroimidazoles, tetracycline among others. The laboratorial phenotypic and genotypic susceptibility testing methods are described, as well as the mechanism of acquisition of antibiotic resistance and the cellular mechanisms that result in antimicrobial resistance. Epidemiological considerations on the worldwide prevalence of each antibiotic resistance are analyzed. Strains presenting multiple resistances and new approaches to the management of *H. pylori* infection are also discussed.

**Keywords** *Helicobacter pylori,* antibiotic resistance; epidemiology

### 1. General remarks

*Helicobacter pylori* is Gram-negative bacterium belonging to the phylum *Proteobacteria*, *Epsilonproteobacteria* class, *Campylobacteres* order and *Helicobacteraceae* family. The genus *Helicobacter* is presently composed by 32 validly named species and characterized by bacteria that inhabit the interface between mucosa and lumen of the gut. These bacteria are microaerophilic, flagellated, spiral and the majority of them are urease positive, allowing the colonization of ruthless stomach environment. *H. pylori* is the human major pathogen type species of *Helicobacter* genus. Non-*pylori* *Helicobacter* species have mammals and birds as theirs hosts, and are sporadically isolated in clinical specimens, being unclear if these are associated with human diseases[41, 71]. Stomach mucosa colonization by *H. pylori* is for life if left untreated. The infectious persistence is assured namely by bacterial outer proteins and adherence to host epithelial cells. The most well-known virulence factors are the vacuolating toxin (VacA), which induces host cell vacuolation, membrane channel formation, disruption of endosomal / lysosomal function, apoptosis, and immunomodulation, and cytotoxin-associated gene A (CagA), which is transferred to epithelial cells via a Type IV secretion system, with subsequent CagA induced IL-8 secretion, actin rearrangements, tight junctions disruption and abnormal proliferation of the host cells[14]. During the typical lifelong chronic infection, two important diseases can occur, peptic ulcer and gastric cancer. *H. pylori* induces an alteration in the gastric physiology leading to acid hypersecretion and duodenal peptic ulcer disease. Alternatively, *H. pylori* damages the acid secreting mucosa leading to atrophic gastritis, gastric ulcer and/or gastric cancer [71].

Since the introduction of clinical medicine seventy years ago, antibiotics have become the main mean to control bacterial infections and *H. pylori* therapy, described below, is no exception, involving the administration of multiple antibiotics. However, antibiotic therapy fails in about 20-30% of the patients, mainly due to antibiotic resistance (primary and secondary), making urgent the development of new drugs or of alternative approaches [112]. Primary antibiotic resistance is ensured by the presence of a resistant organism previous to the administration of the drug and can be detected by pre-treatment susceptibility testing. Secondary resistance consists on emergency of a resistant organism after sub-optimal or unsuccessful treatment. Frequently *H. pylori* treatment selects for a resistant sub-population rather than producing resistant organisms [90]. Besides bacterial resistance, the non-patient compliance contributes to treatment failure in *H. pylori*.

The crisis of antibiotic resistance is a major public health problem. Antibiotic resistance has an important economic impact worldwide. In USA, antibiotic resistance is estimated to cost each year between US$5 billions and US$24 billions[64]. Bacteria that are resistant to new antibiotics appeared few years after the introduction of these drugs into clinical use. Moreover, besides rapid development of antibiotic resistance, its spread is also very rapid. There are several mechanisms for antibiotic resistance spreading, but the most concerning are those that involve plasmids and/or other mobile genetic elements, such as transposons and integrons, containing resistance genes, due to their rapid horizontal transfer between individual bacteria of the same or of different species [37]. Horizontal gene transfer by transformation, conjugation and transduction is rare among bacteria (10E-6 to 10E-9), but exposition to an antibiotic,
especially at low concentration, works as a selective agent for resistant bacteria [98]. Spontaneous mutations, although not occurring very often, may also turn bacteria resistant to a particular class of drugs. The mechanism for antibiotic resistance may be achieved by preventing the access of the antibiotic to its target, changing the structure of the target, degrading or changing the antibiotic, and/or by rapid extrusion of the antibiotic. However, a particular type of resistance is not confined to a single class of drugs and two bacteria may use different resistance mechanisms to withstand the same antibiotic [120]. Reducing or eliminating an antibiotic reduces the frequency of resistant strains, but resistance does not disappear from bacterial population. Thus, the continuous application of antibiotic therapy is dependent on ongoing development of new drugs. In the recent years, however, new drugs are often modifications of the existing ones and most of pharmaceutical companies reduced their efforts on programs for antibiotic discovery [37]. This chapter reviews the current therapeutic options against \textit{H. pylori} and the problem of antibiotic resistance against the most frequent prescribed drugs. The antibiotic susceptibility testing, the prevalence of specific antibiotic resistance, acquisition and molecular mechanism of resistance and alternative therapeutic options against resistant strains are discussed.

### 2. Eradication therapy for \textit{H. pylori}

Eradication of \textit{H. pylori} is recommended in case of duodenal ulcer, gastric ulcer, atrophic gastritis, gastric MALT (mucosa-associated lymphoid tissue) lymphoma, non-ulcer dyspepsia, uninvestigated dyspepsia, following resection of gastric cancer, first-degree relatives of patients with gastric cancer, unexplained iron-deficiency anemia, idiopathic thrombocytopenic purpura, before starting non-steroid anti-inflammatory drugs (NSAID) therapy in NSAID-naive patients, patients receiving long-term aspirin who develop gastrointestinal bleeding, and on patient request after discussion [67].

For eradication of \textit{H. pylori} a triple therapy is commonly used [77], which applies a proton pump inhibitor (PPI) or ranitidine bismuth and two antibiotics, most frequently amoxicillin and clarithromycin, during 7 days in Europe and 10 days in USA [56, 75, 112]. The most common used antibiotics are macrolides (clarithromycin or azithromycin), imidazole (metronidazole or tinidazol), amoxicillin and tetracycline. Antibiotic therapy fails in about 20% of the patients, but depending on the antibiotic it can result unsuccessful in 70% of the cases [56, 91]. Thisis mainly due to bacterial antibiotic resistance [75], but also because bacteria may be in a protective environment like the stomach mucus layer or even inside the epithelial cells [19]. Failure in this therapy may also arise because of the lack of patient compliance [90]. Clarithromycin resistance leads to the substitution of this antibiotic by metronidazole. Developed countries experiencing increasing rates of antibiotic resistance use alternative antibiotic regimens. These are quadruple therapy, sequential therapy and triple therapy using other antibiotics, such as levofloxacin, rifabutin, and furazolidone [112]. The quadruple therapy uses bismuth, PPI, metronidazole and tetracycline, and has the advantage of being inexpensive and effective in areas with high prevalence rate of clarithromycin resistance. The most prescribed antibiotic against \textit{H. pylori} used as a part of the triple therapy is clarithromycin. However, according to Consensus Conferences this antibiotic is not recommend when the percentage of resistance reaches 15% to 20% [74]. A recent phase 3 trial in Europe showed that quadruple therapy, two antibiotics, a PPI and bismuth, should be considered for first-line treatment in view of the rising prevalence of clarithromycin-resistant strains of \textit{H. pylori} [66].

### 3. \textit{H. pylori} antibiotic resistance

\textit{H. pylori} resistance to antibiotics is uneven worldwide, being higher in developed countries and lower in developing countries in agreement with prescription frequency. Increasing antibiotic selective pressure is triggering antibiotic resistance among strains. Being so, performance of susceptibility test before the initiation of therapeutic regimen has been suggested [118]. Table 1 shows information about the antibiotics more commonly used against \textit{H. pylori}. The use of the same antibiotics to deal with different diseases also potentiates the development of resistance, especially within the population group more exposed to these antibiotics [100].

#### 3.1 Susceptibility testing methods

Due to the worrying increase in prevalence of \textit{H. pylori} resistant strains, antibiotic susceptibility testing is becoming more and more important, either before prescribing treatment in high resistance rate areas, or after therapy failure. \textit{In vitro} susceptibility testing for \textit{H. pylori} can be performed either by phenotypic or genotypic methods. Phenotypic testing is challenging because the organism grows slowly even under optimal culture conditions. Owing to these difficulties and because antibiotic resistance in this microorganism is essentially due to point mutations, genotypic methods are an attractive alternative to the phenotypic methods. Moreover, these methods offer the advantage of testing directly from biopsy material, allowing a faster response, and their application on stool samples would be an important advance in the approach to susceptibility testing. Finally, these methods are more sensible in detecting mixed susceptible and resistant populations than phenotypic methods.
### Table 1  General information about antibiotics used against *H. pylori* infection.

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Antibiotic group</th>
<th>Cell target/mode of action</th>
<th>Use against other diseases [100]</th>
<th>World %resistance [16]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metronidazole</td>
<td>Nucleic acid synthesis inhibition</td>
<td>Metronidazole (pro-drug) is reduced by the pyruvate:ferredoxin:oxidoreductase; interact with host cell DNA, resulting in DNA strand breakage and fatal destabilization of the DNA helix [21]</td>
<td>Parasite related diseases, gynaecological, dental infections</td>
<td>26.7%</td>
</tr>
<tr>
<td>Clarithromycin</td>
<td>Protein synthesis inhibition</td>
<td>Bind to the 50S subunit of bacterial ribosomes, leading to inhibition of transpeptidation, translocation, chain elongation and, ultimately, bacterial protein synthesis [73]</td>
<td>Respiratory infections</td>
<td>17.2%</td>
</tr>
<tr>
<td>Levofoxacin</td>
<td>Nucleic acid synthesis inhibition</td>
<td>Inhibits bacterial DNA gyrase[73]</td>
<td>Bacterial conjunctivitis, nosocomial pneumonia, urinary tract infection</td>
<td>16.2%</td>
</tr>
<tr>
<td>Amoxicillin</td>
<td>Cell wall synthesis inhibition</td>
<td>Inhibit transpeptidation during cell wall synthesis [73]</td>
<td>Streptococcal pharyngitis, urinary tract infections</td>
<td>11.2%</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>Protein synthesis inhibition</td>
<td>Bind to the 30S subunit of bacterial ribosome, inhibit the ligation of aminoacyl-tRNA to ribosomal local A [7, 28]</td>
<td>Respiratory and bowel disease, prophylaxis of traveler’s diarrhea, cholera</td>
<td>5.9%</td>
</tr>
<tr>
<td>Rifabutin</td>
<td>Nucleic acid synthesis inhibition</td>
<td>Bind to the β-subunit of the RNA polymerase, inhibiting DNA transcription</td>
<td>Mycobacterium tuberculosis infections</td>
<td>1.4%</td>
</tr>
<tr>
<td>Furazolidone</td>
<td>Nucleic acid synthesis inhibition</td>
<td>Furazolidone binds to bacterial DNA which leads to the gradual inhibition of monoamine oxidase[73]</td>
<td>Giardiasis</td>
<td>Not reported</td>
</tr>
</tbody>
</table>

#### 3.1.1 Phenotypic methods

The agar dilution method is considered by the Clinical Laboratory Standard Institute (CLSI) the method of choice for testing *H. pylori* antibiotic susceptibility. Briefly, Mueller-Hinton agar supplemented with either horse blood (10% v/v) or sheep blood (5% v/v) is used to prepare plates with different antibiotic concentrations. The plates are inoculated with an inoculum corresponding to an opacity equivalent to the McFarland 4 opacity standard (approximately 10⁸ colony forming units (CFU)/ml), prepared from a 2-day old culture grown on a blood agar plate. The plates are then incubated at 37°C under sufficient microaerobic conditions, and read after 72h of incubation[32]. The breakpoints commonly used for the antibiotics are: clarithromycin, 0.25 μg/ml for susceptible strains, >0.5 μg/ml for resistant strains, and 0.5 μg/ml for intermediate strains; tetracycline, 2 μg/ml; rifabutin, 1μg/ml, ciprofloxacin (often tested instead of levofloxacin), 1 μg/ml; and amoxicillin, 0.25μg/ml for susceptible strains, >0.5 μg/ml for resistant strains, and 0.25-0.5 μg/ml for intermediate susceptible strains.

The broth dilution method has the advantage of being adaptable to automation; however, it is not frequently used for *H. pylori* because of the difficulty of growing this bacterium in broth. Supplementation of media such as brucella broth, brain heart infusion or Mueller Hinton broth with 10% horse serum and 0.25% yeast extract or 2% fetal calf serum may increase the performance of this test.

An alternative method is the breakpoint susceptibility testing, which is a simplified version of the agar dilution method. It consists of inoculating a streak of the strain to be tested on an agar plate containing an antibiotic concentration equal to the breakpoint concentration which defines resistance, for example, 1 μg/ml for clarithromycin, or twofold concentrations (0.25 and 1 μg/ml) to categorize the strains as susceptible, intermediary or resistant.

The disk diffusion method is the most simple and economic for routine susceptibility testing, however it is generally not recommended for slow growing bacteria. Nevertheless, disk diffusion has been validated to detect macrolides resistance, using erythromycin [36] and fluoroquinolones resistance, using ciprofloxacin (Comité de l’Antibiogramme de la Société Française de Microbiologie.). It is not recommend to test *H. pylori* susceptibility to metronidazole, amoxicillin and rifabutin.
Finally, the availability of strips impregnated with a gradient of antibiotic, of which the most popular is the Etest, has facilitated the antibiotic susceptibility testing in clinical practice, allowing a direct reading of the minimal inhibitory concentration (MIC). Furthermore, it is adapted to slow growing bacteria like *H. pylori*. A good correlation has been found between this method and the agar dilution method, with the exception of metronidazole, thus Etest is considered a reliable and alternative method for testing *H. pylori* susceptibility to a wide range of antimicrobial agents[32].

3.1.2 Genotypic detection of resistance

Up till now, molecular detection was developed for clarithromycin, fluoroquinolones and tetracycline, and the two main methods used are PCR-RFLP (polymerase chain reaction- restriction fragment length polymorphism) and real-time PCR.

Resistance to macrolides in *H. pylori* is due to point mutations within the peptidyltransferase-encoding region of the 23S rRNA. Three major mutations in two positions on the 23S rRNA have been described, in which an adenine residue is replaced by a guanine or a cytosine residue in different positions: A2142G, A2142C and A2143G [115]. These mutations can be detected by PCR-RFLP, using restriction endonucleases, such as BsaI for the A2142G mutation, BseI for the A2143G mutation and BceAI for detection of the A2142C mutation [79]. The detection of these mutations can also be performed by real-time PCR, and several types of probes have been described for detection of macrolide resistance in *H. pylori*, such as fluorescence resonance energy transfer (FRET) probes with melting curve analysis [89] and Scorpion probes [9]. Other genotypic methods include the Fluorescence in situ hybridization (FISH), which allows detection without performing DNA amplification [110], oligonucleotide ligation assay [103], DNA enzyme immunoassay [70], reverse hybridization-based line probe assay [114], and dual-priming oligonucleotide-based multiplex PCR [121].

Fluoroquinolones inhibit the A subunit of the DNA gyrase, encoded by the *gyrA* gene. The resistance of *H. pylori* against this class of antibiotics is caused by point mutations in theso-called quinolone resistance-determining region of the *gyrA* gene, mainly involve amino acid substitutions at amino acids 87 and 91[107]. A FRET and melting curve analysis-based real-time PCR approach was developed allowing a quick detection of fluoroquinolone-resistant *H. pylori* organisms [31].

Tetracycline resistance in *H. pylori* is caused by mutations in 16S rRNA corresponding to the helix 31 region of the 16S rRNA molecule. A triple mutation, AGA926-928TTC, has been shown to cause high-level tetracycline resistance, whilst single or double base mutations amongst these same three bases are associated with lower level resistance[111]. In the PCR-RFLP method, the 16S rRNA PCR product is digested by Hinfl, resulting in three bands for strains harboring the triple mutation leading to a high tetracycline resistance level, and only two for susceptible strains or for strains with a low resistance level[95]. The 16S rDNA polymorphisms are also excellent targets for real-time PCR detection of tetracycline resistance and recently a method was developed based on this technology[57]. Due to the complexity of the mechanisms underlying resistance to metronidazole and amoxicillin, molecular methods for detection of susceptibility profiles to these antibiotics are not yet available. Concerning rifabutin, no genotypic methods have up till now been developed probably because resistance to this antibiotic is seldom encountered.

3.2 Worldwide prevalence of antibiotic resistance

It is widely accepted that resistance to clarithromycin is the single most important factor in treatment failures, thus it is the prevalence which has been the most widely studied so far. Several studies document a rise in the frequency of strains resistant to this antibiotic all over the world, in the last few years. This fast worldwide emergence of clarithromycin-resistant *H. pylori* strains, contributes not only to a decrease up to 70% in effectiveness of the current treatment regimens, but also to the emergence of resistant strains to alternative antibiotics, such as fluoroquinolones, rifamycins or tetracyclines. As for other antibiotics, resistance rates to clarithromycin are highly variable over the world, with higher rates being reported in the USA, Europe, especially southern Europe, and other developed countries, compared to developing countries. Resistance rate to clarithromycin varies between 10-15% in the USA[20, 80], 1.0-11% in northern European countries [76, 92] and between 17-37.6% in southern Europe and France[10, 17, 94]. In Asia, the higher resistance rates has been reported for Japan (12-36.1%) [51, 52], and in south America, for Chile (>20%) [6]. The majority of the studies present data over several years and therefore it is possible to see the evolution of resistance, which in the case of clarithromycin illustrates an increasing trend. Even in northern European countries where the rates have traditionally been low, appear to be rising in the more recent years [88].

In developed countries, resistance rates are often higher in children than in adults, due the widespread use of macrolides to treat upper respiratory tract infections in paediatrics [55]. Besides patient’s age and geographic region, other risk factors for clarithromycin resistance are female gender (not consistently reported) and disease status. It has been reported that clarithromycin resistance is less common in ulcerogenic than in non-ulcerogenic strains, which can be related with their higher virulent profile, resulting in a shorter generation time and making them in closer contact with gastric cells and therefore more accessible to the antibiotics. On the other hand, it is possible that non-ulcer patients are greater consumers of antibiotics. Nevertheless, the main risk factor is the previous consumption of macrolides[33].
Resistance to metronidazole has less impact on the success of eradication, and in addition, there is a lack of reproducibility between different testing methods, impairing somehow the comparison of the results between studies. Nevertheless, most of the published data show that metronidazole resistance is very high in Africa (80-100%), Asia (50-95%), except in Japan where it is relatively rare (9-12%)[51], and South America (≈50%) (reviewed in [6, 16]). In Europe and USA, resistance to metronidazole varies from 20 to 40%, with a static trend [6, 16, 88]. The main risk factor for metronidazole resistance is the previous use of nitroimidazoles for treating parasitic diseases in tropical countries and for gynecological infections in developed countries [6, 73].

*H. pylori* resistance to fluoroquinolones is an emerging factor with the aggravating circumstance that it has a high impact on the eradication rate of *H. pylori* infection [86]. Overall, ciprofloxacin/levofloxacin resistance is much higher in Europe (24.1%) than in Asia (11.6%) and is almost absent in Africa[16]. Resistance to fluoroquinolones also mirrors the use of these drugs, which have been extensively used over the last decade in developed countries [23]. The increasing detection rate of fluoroquinolones-resistant strains over time warrants specific attention and continued surveillance, as it may compromise the efficacy of second line schemes for the treatment of *H. pylori* infection.

Data regarding amoxicillin resistance are highly conflicting. While the majority of studies report a very low resistant rate (<1%), observed in Europe and USA, others report prevalence as high as 85.6% in Africa[16, 85]. Also, a wide variation of amoxicillin resistance rates has been reported in Asian countries, ranging from 0% in Japan to 8.8% in Korea and 36.1% in Taiwan[16]. These discrepant results have to be interpreted with caution until the mechanism of resistance is fully understood. On the other hand, some strains with decreased susceptibility (MIC 0.5 µg/ml instead of 0.05 µg/ml) are sometimes encountered. Overall, it is assumed that resistance to amoxicillin in *H. pylori* is a rare event. Similarly, the *H. pylori* resistance to tetracycline is very low (<5%), except in a few countries like South Korea[54] and Cameroon[85].

A systematic review of studies concerning primary *H. pylori* antibiotic resistance published through January 2006 to December 2009 showed that resistance to rifabutin is rare, with an overall frequency of 1.4% (95% CI: 0.81-9%) [16]. This low prevalence is probably due to the fact that this antibiotic has a limited use. To date, no *H. pylori* resistant strains to furazolidone have been reported. Resistance to several combinations of antibiotics has been reported, such as the double combinations clarithromycin and metronidazole, clarithromycin and levofloxacin, and metronidazole and levofloxacin; the triple combinations clarithromycin, metronidazole and levofloxacin, and clarithromycin, metronidazole and rifabutin. Multiple resistant *H. pylori* strains is increasing worldwide, with an overall prevalence of 9.6% (95% CI: 8.5-10.7%), considering data of patients enrolled from 1993 to 2009 [16]. This prevalence varies according to the geographical region and the antibiotics combination [8, 17, 46, 104]. Quadruple-resistant strains to clarithromycin, metronidazole levofloxacin and rifabutin have also been reported [123]. Identified prior unsuccessful therapies are the key factor for the development of multiresistance in *H. pylori*.

### 3.3 Resistance against Macrolides

Clarithromycin is the most powerful antibiotic against *H. pylori*, ensuring the eradication of 96.4% of phenotypic and genotypic susceptible strains [18]. This macrolide exerts its bactericidal activity by impairing the normal functioning of the bacterial ribosome. Indeed, as indicated in Table 1, by interacting with the 23S rRNA within the 50S ribosomal subunit, clarithromycin stimulates the release of peptidyl-tRNA from the A site (aminoacyl accepter site), blocking the elongation of the nascent peptide chain during bacterial protein synthesis [115]. In *H. pylori*, primary resistance to clarithromycin is associated with chromosomal point mutations in either one or both copies of the 23S rRNA gene, specifically in the peptidyltransferase-encoding region in its domain[115]. As mentioned above, in Western countries, the most frequent detected point mutations are the transitions A2143G (69.8%) and A2142G (11.7%), and the transversion A2142C (2.6%), which are easily assessed by PCR-based tools. These point mutations change the spatial structure of the bacterial ribosome, preventing the efficient interaction between clarithromycin and the 50S rRNA. Thereby, they allow protein synthesis to proceed normally and confer resistance to clarithromycin[73]. Most frequently, strains have the same sequence for both copies of the 23S rRNA gene, but few having one wild type copy and one mutated copy have been described being expected to have an intermediate level of resistance to macrolides[108].

Even though, the impact of each of them on the normal function of the ribosome should not be the same. Albeit some controversial data in the literature[108], De Francesco et al. claimed that only A2143G reduces the eradication efficiency of clarithromycin of clinical isolates to levels below 30%, both A2142G and A2142C mutations having a marginal role in conferring real phenotypic resistance[18]. Therefore, genotypic assessment of *H. pylori* clarithromycin resistance overestimates the phenotypic resistance rates[16, 18], strongly affecting the therapeutic options.

No less important is the fact that among *H. pylori* strains harbouring genotypic susceptibility regarding those three point mutations, there are some clinical isolates presenting phenotypic resistance, as detected by phenotypic-methods. In such strains, clarithromycin resistance should be achieved by other less prevalent point mutations in the 23S rRNA gene (e.g., C2147G transversion and G1939A, T1942C, A2142G, T2182C and T2717C transitions[26, 27, 53], or perhaps, in a similar manner to other microorganisms, by methylation of the 23S rRNA[62] and efflux pumps[69]. Some
authors claim that mutation of the 23S rRNA gene for the acquisition of resistance to macrolides is an evolutionary event that occurred in bacterial species presenting a low number of copies of this gene, as is the case of *H. pylori*. In fact, resistance to this class of antibiotics in bacterial species containing multiple copies of this gene in their genome, namely *Escherichia coli*, is accomplished through the acquisition of a methylase enzyme, which introduces methyl groups to the adenosines residues at similar positions in 23S rRNA[108].

*In vitro* data show that selection of *H. pylori* resistant mutants may occur when sub-inhibitory concentrations of clarithromycin are present in the gastric mucosa. However, whether clarithromycin resistance is maintained when selection pressure stops is controversial. In fact, bacteria will maintain mutations that confer resistance only if they have no cost for its survival. Explaining their prevalence, *in vitro* assays have shown that only A2142G and A2143G mutations have no effect on *H. pylori* growth rates[108]. Although some claim that is not stable at long term, several studies have demonstrated that resistant mutants maintain their ability to survive in the presence of clarithromycin[73].

### 3.4 Resistance against Fluoroquinolones

Fluoroquinolones have a broad spectrum of activity against Gram-positive and Gram-negative strains, including *H. pylori*. For that reason, this class of antibiotics, namely levofloxacin, was recently considered for use as a second-line therapy for *H. pylori* eradication[30]. In general, susceptibility to fluoroquinolones results from inhibition of both DNA gyrase and topoisomerase IV, causing topological modifications in bacterial DNA status. Bacterial resistance to fluoroquinolones are attributed to mutations in genes that encode subunits of DNA gyrase (gyrA and gyrB genes) and/or of topoisomerase IV (parC and parE genes)[73, 117].

In *H. pylori*, only point mutations in quinolone resistance-determining-regions of gyrA gene alone[73] or, as recently reported for some clinical isolates, in combination with mutation in gyrB gene[117] were identified as responsible for primary resistance to fluoroquinolones. As far as we know, neither parC nor parE genes were detected in *H. pylori* genome. However, there are some clinical isolates showing no genotypic resistance regarding both gyrA and gyrB genes, but displaying phenotypic resistance, suggesting alternative mechanisms, such as additional mutations outside the quinolone resistance-determining-regions, or efflux systems[117].

### 3.5 Resistance against Amoxicillin

Amoxicillin is one of the first-line antibiotics used for eradication of *H. pylori*[30]. The mechanisms of resistance to amoxicillin are known for their complexity, which explains the large variations in its prevalence worldwide[16, 73]. β-Lactams, including amoxicillin, cross the outer cell membrane of *H. pylori* through porin channels and diffuse to the bacterial cytoplasm[22]. Once inside the cell, amoxicillin binds to penicillin binding proteins (PBPs) inhibiting their transpeptidase activity and, thereby, impairing the synthesis of the peptidoglycan wall. In general, bacterial resistance to amoxicillin and other β-lactams is due to hydrolysis by a β-lactamase, or by mutational modification in or around the penicillin-binding motifs of PBPs[102]. In the case of *H. pylori*, primary resistance to amoxicillin arises from a decrease in the affinity of PBPs to β-lactams antibiotics caused by mutations in *pbp1A* gene alone[72], or in combination with mutations in *php2* and *php3* genes[96]. Resistance to amoxicillin in *H. pylori* strains may be further enhanced by decreasing bacterial permeability to these molecules. Such event is achieved by mutations in genes encoding two members of a family of related porins (Hop, heat-modifiable outer membrane proteins), hopB and hopC genes[84].

### 3.6 Resistance against Nitroimidazoles

Over more than 45 years of clinical use, metronidazol is still described as the most cost-effective antibiotic against anaerobic bacteria and protozoa, and some microaerophiles, including *H. pylori*. Indeed, its low cost, good bactericidal activity, favourable pharmacokinetic and pharmacodynamic properties, and minor adverse effects, overcomes the problems of resistance[63]. This pro-drug passively diffuses into the bacterial cytoplasm, where it is activated by reduction of the low potential nitro group that is attached to its imidazole ring. This short-lived nitroso free radical is a cytotoxic form of the drug that, by interacting with bacterial DNA, causes oxidative DNA damage and cell death[63].

Resistant strains are deficient in metronidazolereduction in result to mutations of genes that encode proteins involved in this process. As these proteins are essential components for core metabolism, metronidazol resistance has low clinical significance among anaerobes. In contrast, the resistance to this antibiotic among clinical isolates of *H. pylori* is increasing worldwide, as mentioned above. In this microaerophile, primary metronidazol resistance is often achieved through mutational inactivation of either one or two non-essential genes, the *oxygen-insensitive NADPH nitroreductase (rdxA)* and the *NADPH-flavin-oxidoreductase (frxA)*, encoding RdxA[34] and FrxA and nitroreductases[48], respectively. In the absence of a clear panel of identified point mutations and notwithstanding the relevance of these two proteins, it is now accepted that resistance to metronidazol is a much more complex process, entirely dependent on the intracellular redox potential and oxygen levels. In fact, changes in the expression of other redox enzymes, namely thioredoxinreductase, alkyl hydroperoxidereductase, superoxide dismutase, and other enzymes involved in the
oxireduction of ferredoxin were shown to be implicated in the metronidazole resistance[50]. Supporting these findings, there are some resistant strains having no mutations in either rdxA or frxA genes[50]. Moreover, in vitro assays have clearly demonstrated that double mutant strains (strains lacking both rdxA and frxA-active genes) become susceptible to metronidazole under anaerobic conditions[29]. Finally, efflux pumps may also contribute to metronidazole resistance in clinical isolates of H. pylori[78].

3.7 Resistance against Tetracycline

Tetracycline resistance has been considered low in H. pylori, especially because this antibiotic is only used in second line treatment, even though it is increasing as mentioned above. The resistance to the bacteriostatic antibiotic tetracycline has been reported in recent years[100]. Tetracycline inhibits the protein synthesis by reversible binding to the 30S subunit of bacterial ribosomes, inhibit the ligation of aminoacyl-tRNA to ribosomal local A[7, 28]. Tetracycline molecules comprise a linear fused tetracyclic nucleus (rings designated A, B, C, and D), to which a variety of functional groups are attached. Bacterial resistance to tetracycline may be due to an energy-dependent efflux of tetracycline-associated efflux proteins. The exportation of this antibiotic out of the cell allows the protein synthesis to continue, since the ribosome is no longer inhibited[12]. Deletion in efflux genes increases the sensitivity to tetracycline. Resistance to tetracycline may also be mediated through ribosomal protection proteins. These proteins act by releasing the antibiotic from the ribosome or by reducing the affinity with the ribosome. These tetracycline resistance proteins (Tet) include TetM, TetO and TetS, which have homology with elongation factors and an initiation factor. Besides these two most frequent mechanisms of resistance, the enzymatic inactivation of tetracycline in the presence of NADPH, by the product of tetT gene and the presence of several mutations in the 16S rRNA genes that interfere with the binding site of tetracycline have been also described[12, 28].

In H. pylori the mechanisms for tetracycline resistance appear to be essentially based on mutations in the 16S rRNA gene. H. pylori triple-base-pair substitution AGA926-928TTC increases the resistance to tetracycline[28], which make this gene a good target for genotypic detection of resistance. Single and double mutations in this same area are associated with low-level of tetracycline resistance[12]. Single-nucleotide substitutions (CGA, GGA, TGA, AGC, or AGT) at the putative tetracycline binding site (AGA965-967) are also associated with tetracycline resistance. In fact, single-nucleotide substitutions (A965 to G or T substitutions and A967 to C or T substitutions) are associated with a reduction in tetracycline binding to ribosomes. Recently, the role of proton motive force-dependent efflux proteins was suggested to play a role in resistance of H. pylori to tetracycline[1], suggesting that resistance to tetracycline may be multi-factorial, involving alterations both in ribosomal binding and in membrane permeability[1, 122]. The selective pressure of tetracycline appears to be the driven force for selection of resistant strains[101].

3.8 Resistance to other antibiotics

Rifabutin belongs to the class of rifamycins, and is used in alternative therapeutic schemes to treat H. pylori infection. Rifabutin and other derivatives of rifampin are inhibitory against H. pylori at very low concentrations in vitro[42]. Resistance to rifabutin is caused by amino acid exchanges in the β-subunit of the DNA-directed RNA polymerase (RpoB). In H. pylori, mutations at codons 525 to 545 and 586 have been described for resistant mutants selected in vitro[42], while for a H. pylori clinical isolate that developed resistance to rifabutin during therapy the only mutation found was located at the beginning of the rpoB gene, affecting the codon 149[43].

5. Therapeutic alternatives against H. pylori

Motivated by the increasing prevalence of antibiotic resistant strains, several alternative treatments against H. pylori, not involving the use of antibiotics, are under investigation.

Photodynamic therapy is clinically used in treatment of various malignant and pre-malignant conditions, including Barrett’s oesophagus and gastric cancer[2, 5, 45, 93]. Harmless visible light, at the appropriated wavelength, is used for exciting non-toxic photosensitive molecules to highly reactive triplet states, which, by reacting with molecules in their immediate vicinity, originate free radicals and reactive singlet oxygen. These in turn cause oxidative cell destruction and death[2]. Porphyrin derivatives are among the most widely used photosensitizers[49]. Photodynamic inactivation (PDI) of bacteria, widely explored in the past two decades[49], have suggested that it does not induce bacterial resistance[38] and its efficiency is independent of the pattern of antibiotic resistance of the strain[106]. Although still limited, data on the in vitro and in vivo use of PDI in eradication of H. pylori is very promising[11, 38, 39, 59, 82]. PDI cannot be used to treat systemic infections[38], therefore the localization of the infection by H. pylori is favourable for endoscopic access for topical application of photosensitizer and light delivery. Because of the outer membrane, which impairs the access of the photosensitizer to the bacterial cytoplasm, Gram-negative bacteria are, in general, less sensitive to PDI[49]. H. pylori may be an exception to this because of its natural ability to accumulate photoactive porphyrins, namely protoporphyrin IX and coproporphyrin, as by-products of endogenous biosynthesis of heme, allowing efficient PDI eradication with low fluence of violet/blue light (375 - 425 nm)[38, 39]. In fact, a recent prospective pilot trial
indicated a significant reduction of H. pylori load in the stomach of a small group of infected patients, just by intragastric irradiation with violet light (408 nm) [59]. Similar results were obtained by oral administration of 5-aminoolevulinic acid, a metabolic precursor of protoporphyrin IX, followed by irradiation with blue light[119]. However, the use of this drug is not innocuous to the stomach lining, since it is also used by human cells. In both studies, none of the tested patients achieved complete and sustained eradication of H. pylori, stressing the need of further studies to search for photosensitizers for efficient eradication of this bacterium but ensuring the safety of the human gastric epithelium.

The use of bacteriophages to treat infectious diseases as also been considered. Bacteriophage therapy consists in the use of bacteriophages that specifically lyses target bacteria and was developed and applied in the pre-antibiotic era. In fact, antibiotics hold back this research field, but at present, with the emergence of antibiotic resistance, this old idea is coming back [40]. Bacteriophages are bacterial virus composed of a nucleic acid and a protein capsid that depend on the host cell to replicate. Lytic phages are more adequate for bacteriophage therapy, since they rapid promote cell lysis[3, 4, 13, 47]. The screening for H. pylori phages is a resumed topic in the literature [44, 99]. However, the recent identification on sequenced strains of a prophage reminiscent in H. pylori strain B38 [109] and a complete inducible prophage in H. pylori strain B45 [113], opens new perspectives of using phages to treat H. pylori infection.

Notwithstanding the previous approaches an anti-H. pylori vaccine for prophylaxis and therapy is expected to be the most cost-effective approach to deal with this microorganism, as in other infectious diseases [97]. Different formulations, including whole cell and recombinant-antigens’ based vaccines, have been successfully tested on animal models offering them protection against experimental infections[97]. Even considering that some have reached clinical trials[68], but none resulted yet effective for human use. These were based in H. pyloriky virulence factors, and on abundant and/or surface exposed proteins, namely urease[24, 105, 124, 127, 128], CagA in conjugation with VacA and neutrophil activating protein (NapA)[68]. Other examples of tested proteins are the antioxidant protein catalase (KatA)[83], some heat shock proteins (Hsp) (namely HspB)[125], the flagellar sheath protein putative N-acetyleneuraminylactose-binding hemagglutinin (hpA)[87], and also some adhesins[97]. Rendering difficult the development of effective vaccines is the mentioned high genetic variability of this organism[25]. In fact, by studying the immunoproteome of a group of heterogeneous H. pylori strains, isolated from Portuguese patients differing in age, gender and H. pylori-associatedgastric diseases, we have recently shown that genetic variability among strains is not reflected in their proteome but, instead, is implicated in the antigenicity of the proteins[116]. In other words, this study showed clear evidence of the variability of antigenic pattern among H. pylori strains.

Other alternative options include phytotherapy, in which plant products, that are active on biological systems, are used for infections’ treatment [58]. These include for instance red grapes [15], which are highly abundant in polyphenol, namely resveratrol[126], which is known to inhibit H. pylori and has anti-inflammatory, anti-cancer, cardioprotective and neuroprotective activities [65]. This protective activity may be due to modulation of inflammatory cytokines like interleukin-6, transcription factors like nuclear factor-κB, and regulatory enzymes like mitogen-activated protein kinases or may have multiple modulatory effects on H. pylori-induced IL-8 secretion, reactive oxygen species production, and morphological changes [35, 126].

Finally, probiotics, that are life organisms or produced substances that are orally administrated to promote health, have also been proposed for H. pylori therapy, essentially as adjuvants [60, 61, 81].

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Conclusion

During the last years we are observing a continuous increase of antibiotic resistance in H. pylori strains. This increase is related with the use of antibiotics that work as a selective agent of resistant strains. In fact, countries presenting highest prevalence are those in which antibiotic prescription is also more common. The prior to therapy application of sensitive test appears to be very important to control antibiotic resistance. The genotypic sensitivity test may play an important role, since it may be possible to apply in samples that do not require invasive tests. Continuous surveillance of antibiotic resistance is needed in order to achieve better treatment efficiency. The studies focusing on alternative therapies should be endured to assure different therapeutic approaches in multi-resistant H. pylori strains.

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