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1. Introduction

1.1. General information

*Helicobacter pylori* (*H. pylori*) is a spiral-shaped, Gram-negative, microaerophilic bacterium inhabiting human gastric mucosa and playing an essential role in dyspepsia, gastritis, gastric ulcer disease and duodenal ulcer disease [1-6]. Moreover, in 1994 the International Agency for Research on Cancer of the World Health Organization (IARC/WHO) concluded that *H. pylori* is a class I carcinogen, involved in gastric cancer and mucosa-associated lymphoid tissue (MALT) lymphoma [2, 7-11]. Furthermore, increasing number of studies showed the association between *H. pylori* infections and pathogenesis of extragastric diseases. The most convincing data appeared in case of iron-deficiency anemia (IDA) of unknown origin and idiopathic thrombocytopenia purpura (ITP) [12]. Eradication of *H. pylori* in IDA patients resulted in normalization of hemoglobin level while in ITP patients lead to a favorable platelet response [13]. Moreover, the findings and ongoing research concerning *H. pylori* infections suggest its association with other diseases such as: heart or vascular diseases (ischemic heart disease, coronary artery disease, cardiac syndrome x), neurological diseases (stroke, Alzheimer’s or Parkinson’s diseases) as well as obesity, diabetes mellitus, asthma or diseases of oral mucosa [1, 14-18].
H. pylori infection is one of the most common bacterial infections, with the estimated prevalence reaching 50% of the human population worldwide. Nevertheless, the occurrence of H. pylori infections correlate with the socioeconomic status and are often reflected in their geographical distribution. The frequency of this infection is markedly higher in the developing countries (80-90%) rather than in the developed countries (<40%) [4]. In Europe the incidence of H. pylori varies and can be associated with geographical location and the percentage of compatriots of non-European origin [19]. Current studies show the declining trend in the prevalence of H. pylori infections in developed countries such as Denmark or Czech Republic [20, 21], while the frequency of this infection in Asia–Pacific region ranges from 15.5% in Australia, 71.7% in Shanghai China, to 94.3% in Eastern Siberia [6].

The Polish multi-center study performed between 2000 and 2003 revealed that the incidence of H. pylori infections was 84% in adult patients [22], whereas our research, conducted in southern Poland between 2009 and 2011, showed that the rate of H. pylori infection among adults was 23% [23].

H. pylori has evolved numerous strategies for survival in the gastric niche (especially due to the release of urease, an enzyme, which increases the pH value of the environment around the bacterial cells) [24]. Furthermore, the clinical course of H. pylori infection depends from both the bacterial virulence factors and the host susceptibility (e.g. diet, genetic predispositions, degree of the immune response to infection) [3, 7, 25].

It was concluded that untreated H. pylori infection led to acid-related disorders of the digestive system. Great majority of individuals colonized by H. pylori developed co-existing chronic gastritis, which in subsets of patients could evolve to duodenal or gastric ulcer as well as gastric cancer [7, 8, 26, 27]. Experiments performed in animal models (Mongolian gerbils) confirmed the progressive sequence of lesions from chronic gastritis to gastric cancer by gastric atrophy, intestinal metaplasia, and dysplasia [28]. Figure 1 illustrates the sequential steps of diseases resulting from H. pylori infection.

H. pylori infection significantly enhances the risk of development of peptic ulcer disease (up to 15 times) as well as gastric cancer (up to 6 times) [1, 7, 24]. Peptic ulcer disease (PUD) – gastric and/or duodenal ulcer – develops in about 15% of H. pylori infected patients, whereas the progression to neoplastic lesions may occur only in a small fraction of patients with H. pylori infection. Gastric cancer arises in about 1-5% of patients and MALT cancer in <1% of infected subjects [7, 8, 27, 29].

The information presented above highlights the importance of an effective treatment against H. pylori. Furthermore, the increasing resistance of H. pylori to antibiotics/chemotherapeutics currently used in empirical therapy justifies the need for continuous surveillance of antibiotic resistance as well as compliance to guidelines of the European Helicobacter Study Group (EHSG) [1].
1.2. Virulence factors (VF)

The pathogenesis of *H. pylori* is determined by wide spectrum of virulence factors, which can be divided into two major groups:

1. **VF secreted into the bacterial extracellular environment** (e.g. immunogenic protein CagA, vacuolating cytotoxin VacA, secreted enzymes: urease, aliphatic amidase, catalase, oxidase, superoxide dismutase, phospholipases, glycosulfatase and proteolitic enzymes).
2. **VF associated with the bacterial surface** (e.g. outer membrane proteins, adhesins, flagella, LPS) [24, 25].

**CagA.** One of the most important *H. pylori* virulence factors is highly immunogenic protein CagA. It is encoded by cytotoxin associated gene (*cagA* gene), which is located on the *cag* pathogenicity island (*cag PAI*) and carried by 56-90% of *H. pylori* strains [8, 24, 29-32]. CagA protein (after translocation into the host cells by the type IV cag secretion system) stimulates epithelial cells to produce the following cytokines: interleukin IL-8 (strong chemotactic factor and activator of inflammatory process on the mucous membrane), IL-6, IL-1β, and tumor necrosis factor α (TNF-α; enhances the inflammatory process) [31, 33, 34] and is also responsible for changes in the host cells such as proliferation, motility, and polarity [25,
Patients infected with cagA positive strains usually exhibit a higher inflammatory response and are at a higher risk of developing gastric ulcer disease, atrophic gastritis, or gastric cancer [3, 8, 24, 36, 37].

**VacA.** Another major *H. pylori* virulence determinant is vacuolating cytotoxin A (VacA). This protein is involved in various mechanisms of programmed cell death (including apoptosis and necrosis) in the target host cells [38]. While VacA encoding gene (vacA) occurs in almost all *H. pylori* strains, only approximately 50% of isolates express active protein. Moreover, variations in the vacA gene structure considering signal (s) as well as middle (m) region result in differences in vacuolating activity of this cytotoxin [7, 8, 24, 34, 39]. *H. pylori* strains carrying s1/m1 allelic form of vacA gene have higher vacuolating activity than isolates with s1/m2 or s2/m1 alleles. Furthermore, polymorphisms among the vacA signal region (s1, s1a, s1b, s2) and middle region (m1, m2) are also correlated with the intensity of inflammation process and the severity of gastric epithelial cell injury, respectively. While strains possessing vacA s1a genotype cause stronger inflammation process than isolates carrying vacA s1b or vacA s2 genotype, the strains harboring vacA m1 genotype cause more severe gastric epithelial cell injury than the vacA m2 strains [34, 40]. Moreover, the infection with *H. pylori* strains possessing vacA s1/m1 allele is strongly associated with peptic ulcer disease and gastric cancer [7, 40]. A third polymorphic determinant of vacuolating activity of vacA gene was described in mid region and termed the intermediate (i), but its role in the pathogenesis of *H. pylori* infection remains debatable [41].

*H. pylori* is described as one of the most important risk factors contributing to the development of gastric cancer in more than 50% of cases, while CagA and VacA as major virulence determinants play an important role in this process [2, 7, 8]. Due to the presence/absence of CagA and VacA, *H. pylori* strains were divided into two types: type I (possessing cagA gene and expressing vacuolating cytotoxin) and type II (lacking the cagA gene and with no expression of vacuolating cytotoxin). Strains belonging to the type I (CagA+, VacA+ phenotype) are more virulent, as their infections are associated with increased risk of peptic ulcer disease and gastric cancer. Strains of the type II (CagA−, VacA− phenotype) are less virulent. Furthermore, an intermediate type of *H. pylori* strain has been described. Isolates belonging to this group have independent expression of CagA and VacA cytotoxins and represent a moderate level of virulence [24, 34, 42].

**Urease and aliphatic amidase.** Remarkable capacity of *H. pylori* to colonize highly acidic environment of stomach is mostly attributed to the production of urease. This enzyme hydrolyzes urea (to ammonia and carbon dioxide), which results in an increase of pH value in the environment surrounding the bacterium. A similar effect is produced by aliphatic amidase, which also contributes to the pH value of the gastric environment [43]. Furthermore, ammonia which is the product of enzymatic hydrolysis of urea, shows toxicity against epithelial cells [24, 25, 32].

**Other pathogenic enzymes.** *H. pylori* produces many other groups of enzymes responsible for the pathogenesis. While catalase, oxidase, and superoxide dismutase play a role in protecting the bacterial cells from phagocytosis, phospholipase A2, phospholipases C, and glycosulfatase
damage the cells of the mucous membrane [44-48]. Furthermore, *H. pylori* secretes numerous proteolytic enzymes responsible for the gastric mucus degradation [49].

**Adhesins.** Adherence of *H. pylori* to the host cell receptors triggers cellular changes, which include signal transduction cascades, leading to infiltration of inflammatory cells and to persistence of the organism. *H. pylori* adhesins are proteins, glycoconjugates, or lipids, which are involved in the initial stages of colonization by mediating the interactions between the bacterium and the host cell surface (AlpA, AlpB, BabA, Hsp60, Hsp70, LPS core, LPS O antigen (Lewis X), Nap) [8, 50, 51].

**Outer membrane proteins.** Outer inflammatory protein (OipA) is an outer membrane inflammatory-related protein significantly associated with the development of duodenal ulcers and gastric cancer. OipA plays an important role in induction of the mucosal cytokines and in gastric mucosal inflammation [7, 8, 36]. The outer membrane proteins contributing to the adherence of *H. pylori* are represented by: SabA, AlpAB [32, 36].

**Flagella.** *H. pylori* possess up to 6 unipolar flagella composed of two proteins: FlaA and FlaB [24, 52]. Due to the flagella, *H. pylori* is motile and can move within stomach niche between regions of low pH value to regions with higher pH value, which is more hospitable for these bacteria [8, 53]. The motility is also necessary for the successful colonization of the stomach mucosa [52].

**LPS.** Lipopolysaccharide of *H. pylori* is a unique and a potent immunogen. LPS of *H. pylori* contains O-antigen structures, which resemble human glycosphingolipids due to the presence of common host carbohydrate residues [54]. LPS contains structures like Lewis X and Lewis Y antigens, which are similar to antigens of human blood groups, which occur also in the human gastric epithelium. Due to molecular mimicry, bacteria are able to escape the host immune response. Moreover, the specific structure of *H. pylori* LPS is crucial for the adhesion to epithelial cells and colonization process of the human stomach by *H. pylori* [24, 54, 55].

While in the majority of people *H. pylori* colonization is asymptomatic, long-term carriage significantly increases the risk of development of site-specific diseases [7, 8, 34, 36]. It was also concluded that untreated *H. pylori* infection may lead to serious implications such as gastritis, gastric ulcer disease and gastric cancer, therefore it is crucial to provide effective treatment. Moreover, it was proved that introduction of early effective treatment of *H. pylori* infection in cases of peptic ulcer disease can prevent these patients from developing gastric cancer [1, 26].

### 1.3. Treatment guidelines

Several recommendations concerning diagnosis and treatment regimen were issued as the infection treatment is often unsuccessful. The first guidelines on diagnostic and therapeutic approaches for the *H. pylori* infection were issued in the United States of America. The second recommendations were introduced by Polish Society of Gastroenterology *Helicobacter pylori* Working Group (PSG-E/HPWG) in 1996. The next guidelines of the PSG-E/HPWG on diagnostic and therapeutic approaches for the *H. pylori* infection were issued in 2000, 2004 and 2008 [22, 56, 57]. The European Helicobacter Study Group (EHSG) took the initiative of introducing
Recommendations for the clinical management of *H pylori* infection in 1996 in Maastricht and since then four consensus reports have been issued (1997, 2000, 2005, 2012) [1, 5, 58, 59]. Recommendations for the management of *H. pylori* infection are constantly evolving along with the development of knowledge concerning this bacterium and its infections [1]. Current guidelines on the management of *H. pylori* infections were produced by EHSG in 2012 (Maastricht IV/Florence Consensus Report) and should be introduced in the management of this infection in European countries. Despite that fact, the recent Polish recommendations issued in 2008 still support the Third Maastricht Consensus Report (2005). Nevertheless, the new Polish guidelines are under development and will be issued in the near future.

The European Helicobacter Study Group and the Polish Society of Gastroenterology recommend therapeutic regimens, which include three types of drugs: 1) antisecretory (proton pump inhibitors (PPI)), 2) cytoprotectants (drugs containing bismuth salts), and 3) antibiotics/chemotherapeutics (clarithromycin, amoxicillin, metronidazole, tetracycline, levofloxacin, and rifabutin) [1, 22].

The treatment regimens of *H. pylori* infections according to the PSG-E guidelines (2008) [22] are presented in Table 1.

<table>
<thead>
<tr>
<th>Treatment strategy</th>
<th>Time of treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>The First-Line Treatment (one of the following):</td>
<td></td>
</tr>
<tr>
<td>PPI, amoxicillin (1000 mg) and metronidazole (500 mg) twice a day</td>
<td>10–14 days</td>
</tr>
<tr>
<td>PPI, clarithromycin (500 mg), and metronidazole (500 mg) twice a day</td>
<td></td>
</tr>
<tr>
<td>PPI, amoxicillin (500 mg), and clarithromycin (500 mg) twice a day</td>
<td></td>
</tr>
<tr>
<td>The Second-Line Treatment (one of the following):</td>
<td></td>
</tr>
<tr>
<td>PPI, amoxicillin (1000 mg), and metronidazole (500 mg) twice a day and tetracycline (250 mg) three times a day</td>
<td>10–14 days</td>
</tr>
<tr>
<td>PPI, amoxicillin (1000 mg), and metronidazole (500 mg) twice a day and bismuth salts (120 mg) four times a day</td>
<td></td>
</tr>
<tr>
<td>The Third-Line Treatment:</td>
<td></td>
</tr>
<tr>
<td>Evaluation of the susceptibility of the strains to the currently used antimicrobial agents: amoxicillin, metronidazole, clarithromycin, and tetracycline</td>
<td>14 days</td>
</tr>
<tr>
<td>(prolonged if the previous treatments were shorter)</td>
<td></td>
</tr>
<tr>
<td>Possible introduction of levofloxacin</td>
<td></td>
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<tr>
<td>Adding a probiotic</td>
<td></td>
</tr>
</tbody>
</table>

**Table 1.** Proposed strategy of the first, second and third – line treatment of *H. pylori* infection (table based on the guidelines of PSG-E Consensus Report, 2008 [22])

According to the Maastricht IV/Florence Consensus Report guidelines, the treatment regimen should be selected according to the areas of low (<20%) and high (>20%) clarithromycin resistance, to provide the most effective therapy. Figure 2 presents the current EHSG guidelines.
**Figure 2.** Treatment schemes of *Helicobacter pylori* infection considering the regions of low and high prevalence of clarithromycin-resistant strains (based on the Maastricht/Florence IV Consensus Report) [1].

In regions with high prevalence of resistance to clarithromycin (>20%), it is recommended to abandon clarithromycin in the empirical treatment of *H. pylori* infections, if the antibiotic susceptibility cannot be tested. As the first line empirical treatment, the bismuth–containing quadruple therapy is recommended. If the bismuth salts are not available in certain countries (e.g., Poland), then non-bismuth quadruple therapies (sequential or concomitant) could be prescribed. Non-bismuth quadruple sequential therapy consists of 5-days of PPI plus amoxicillin, followed by 5-day period with PPI, clarithromycin, and metronidazole (or tinidazole), whereas non-bismuth quadruple concomitant therapy comprises simultaneous use of clarithromycin, amoxicillin, metronidazole, and PPI for 10-14 days. Although both non-bismuth quadruple therapies contain clarithromycin, these regimens could be applied only as targeted therapy based on testing of the bacterial susceptibility to clarithromycin. In regions, where
bismuth salts are not available and high clarithromycin resistance occurs, targeted therapy with clarithromycin seems to be the best option.

After failure of the first line treatment, the second line treatment regimen with PPI, levofloxacin, and amoxicillin should be prescribed. It should be emphasized that levofloxacin cannot be used in patients with chronic bronchitis or pneumonia, who may have taken fluoroquinolones, hence it is recommended to perform susceptibility testing to levofloxacin. Moreover, increasing resistance rate to antimicrobial agents should be taken into account [1], for instance in Poland we noted an increasing resistance rate to levofloxacin [23]. Susceptibility testing conducted before treatment, can protect against increasing resistance rate.

If the second line regimen failed, a third line treatment based on antimicrobial susceptibility testing should be introduced. If susceptibility testing is not available, the empirical third line regimen should contain antimicrobials not used previously. Except for the drugs already mentioned, rifabutin is a therapeutic option (its use is limited to mycobacterial infections) [1].

Taking into account the EHSGG recommendations, the rationale is to perform susceptibility testing whenever possible to prevent the increase of drug resistance, especially in regions where the resistance to clarithromycin is high.

1.4. Antimicrobial agents and bacterial resistance

Recent data show that combination of standard therapeutic strategies have lost some efficacy. This fact is attributed to increasing antimicrobial resistance of H. pylori strains.

**Clarithromycin.** Among antimicrobial agents used in the treatment of H. pylori infections, clarithromycin was considered to be one of the most effective drugs; nonetheless emergence of clarithromycin resistance became the major risk factor of the treatment failure. In susceptible strains, the eradication rates amounted to 87.7%, whereas in resistant strains the rates decreased to 18.3% [5]. One of the most significant factors contributing to clarithromycin resistance is the extensive use of macrolides, particularly in the treatment of upper and lower respiratory tract infections in children [60]. Furthermore, studies conducted between 2001 and 2008 in 18 European countries confirmed the correlation between antibiotic resistance of H. pylori strains and outpatient antibiotic consumption in adults. According to this multi-center study, H. pylori clarithromycin resistance rates in Europe amounted to 17.5% and were considerably higher in western/central and southern Europe (>20%) than in the northern European countries (<10%) [61]. In Poland, the prevalence of H. pylori resistance to clarithromycin was 28% (data published by PSG-E) [22]. Nevertheless, according to our recent study conducted in the years 2006-2008 and 2009-2011, resistance of H. pylori strains isolated in southern Poland was also high and amounted to 34% and 22%, respectively [23, 62].

The major mechanism responsible for H. pylori clarithromycin resistance is associated with the decrease in the binding of the antibiotic to the ribosome which is caused by the occurrence of the point mutations within the peptidyltransferase-encoding region of 23S rRNA. The most frequently reported point mutation is A-G transition at position 2143 (A2143G), which occurs in 69.8% of H. pylori strains resistant to clarithromycin, while the less common mutations are represented by A2142G and A2143C. Furthermore, other mutations, such as A2115G, G2141A,
C2147G, T2190C, C2195T, A2223G, and C2694A, might also be associated with clarithromycin resistance. Molecular assays have a significant impact on improving the availability and accuracy of the data concerning *H. pylori* clarithromycin resistance, resulting in faster and better selection of effective therapy [60, 63].

**Metronidazole.** Another antimicrobial agent, recommended in the therapy of *H. pylori* infection is metronidazole. Metronidazole has a higher resistance rate than clarithromycin, but it is of secondary importance and can be partly overcome. Treatment with this antimicrobial agent is effective, however, with the exception of the regions where metronidazole resistance reaches 40%. The resistance of *H. pylori* strains to metronidazole amounted to 34.9%, in Europe [5, 61]. In Poland, the resistance rate to metronidazole is higher (46%), according to PSG-E data (2008) [22], whereas in southern Poland, according to our recent study, it is 44% [62].

Common use of metronidazole in parasitic as well as gynecological diseases results in high resistance rates of *H. pylori* strains isolated from both tropical residents and female patients and contributes to overall resistance of the bacterium population [60].

Mechanism of metronidazole resistance in *H. pylori* is not completely understood and, according to some authors, it is related to mutations in *rdxA* (which codes an oxygen-insensitive NADPH nitroreductase) and *frxA* genes (which encode NADPH flavin oxidoreductase) [60, 64].

**Levofloxacin.** Levofloxacin is another antimicrobial agent recommended in *H. pylori* treatment, however rapid acquisition of levofloxacin resistance may contribute to the reduction of its future efficiency. In Europe, the resistance rate of *H. pylori* to levofloxacin amounted to 14.4% between 2001 and 2008. Increasing rates of quinolone resistance are attributed to frequent use of these agents in the therapy of the respiratory and urinary tract infections. While in countries with lower quinolone consumption (e.g. Norway) the resistance rate of *H. pylori* strains remains lower, in countries with high use of quinolones (e.g. Italy) the resistance rate is higher [61]. According to data published by PSG-E, the population of *H. pylori* strains in Poland is susceptible to ciprofloxacin [22]. Nevertheless, we reported that levofloxacin resistance rate in southern Poland amounted to 16% in 2011 and in comparison with previous years, the figure had grown [23].

The resistance to fluoroquinolones in *H. pylori* strains is due to the point mutations occurring in *gyrA* gene at the 87 and 91 positions. Furthermore, some studies revealed a correlation between the resistance to levofloxacin and norfloxacin and the point mutation in the position 463 of *gyrB* gene [60, 65].

**Amoxicillin and Tetracycline.** According to current surveys, the resistance to amoxicillin as well as tetracycline is either very low or even absent, indicating their present clinical irrelevance. While amoxicillin resistance is most likely associated with mutational changes in the penicillin-binding encoding gene (*pbpD*), the tetracycline resistance is based on changes in three nucleotides in the 16S rRNA (AGA 926-928→TTC) [22, 60, 62].

**Rifabutin.** Rifabutin is one of the antimicrobial agents recommended as an alternative in the third line treatment, after two treatment failures. Prevalence of *H. pylori* resistance to this
antibiotic is not well known but it is presumed to be very low, because its use is limited to mycobacterial infection [1]. Moreover, rifabutin is very expensive and causes adverse events like leukopenia, thrombocytopenia or clinically important impairment of liver functions [66, 67]. However, increase in rifabutin consumption may contribute to rapid emergence of resistance. The mechanism of resistance to this antibiotic is mediated by mutations in the rpoB gene [1, 60].

1.5. Resistance rate in Poland

Summing up the current and previous data concerning the prevalence of H. pylori resistance in Poland, we revealed some differences. The results presented in Polish multi-center study performed between 2000 and 2003 revealed that 28% of the analysed H. pylori strains were resistant to clarithromycin, 46% to metronidazole, while all strains were susceptible to amoxicillin and tetracycline [22].

Our studies conducted in the years 2006-2009 and 2010-2012 showed that the resistance rates of H. pylori to clarithromycin in southern Poland were 34% and 22%, respectively [23, 62]. Moreover, we reported that the percentage of H. pylori strains resistant to metronidazole and levofloxacin accounted for 44% and 6%, respectively. Furthermore, we confirmed the coexistence of resistance to more than one antimicrobial agent: a) resistance to metronidazole and clarithromycin in 23% of the strains, b) resistance to metronidazole, clarithromycin, and levofloxacin in 4% of the strains [62]. Additionally, in our recent studies we demonstrated that in southern Poland the number of H. pylori strains resistant to levofloxacin increased significantly; from 5% in the years 2006-2008 to 16% in 2009-2011 [23].

As it was previously emphasized, the Maastricht IV/Florence Consensus Report recommends that treatment regimen should be selected according to areas of low (<20%) and high (>20%) clarithromycin resistance [1]. Referring to available data concerning clarithromycin resistance rates in Poland, this country should be classified as a high clarithromycin resistance region [22, 23, 62].

Although the current Polish guidelines (PSG–E, 2008) on diagnostic and therapeutic approaches to the H. pylori infection recommend clarithromycin in the first line treatment, the rationale is to either abandon clarithromycin in an empirical treatment or apply clarithromycin in therapy, but only with prior susceptibility testing.

2. Problem statement

Dynamic growth of H. pylori resistance to antimicrobial agents used in empirical treatment becomes an increasingly important problem. While H. pylori resistance is often associated with the antibiotic consumption and may vary according to region, it is recommended to constantly monitor the resistance rates of this bacterium. Furthermore, in the areas of high clarithromycin resistance rates as well as increasing resistance to other antibiotics/chemotherapeutics (used in H. pylori treatment), it is worth consideration to replace empirical therapy with targeted
therapy based on prior susceptibility testing. Moreover, early effective treatment of \textit{H. pylori} infection in patients with peptic ulcer disease can prevent gastric cancer.

3. Application area

Data concerning \textit{H. pylori} resistance rates enable to introduce the most effective schemes of infection treatment [5].

4. Research course

1. Monitoring of \textit{H. pylori} resistance to antimicrobial agents used in empirical therapy (according to the Maastricht IV/ Florence Consensus Report) in southern Poland.

2. Selection of the appropriate treatment regimen in \textit{H. pylori} infections in southern Poland, according to the current recommendations and local clarithromycin resistance rates (Maastricht IV/ Florence Consensus Report).

3. Introduction of treatment guided by antimicrobial susceptibility testing in \textit{H. pylori} infections in southern Poland, taking into account: a) high level of clarithromycin resistance, b) increasing resistance rates to levofloxacin, c) persisting high metronidazole resistance rate.


5. Materials and methods

\textbf{Clinical specimens.} The study covered a group of 316 adult dyspeptic patients with therapeutic indications for gastroscopy, who applied to the Falck Medycyna Outpatient Clinic of Gastroenterology (Krakow, Poland) and the Chair of Gastroenterology, Hepatology and Infectious Diseases, Jagiellonian University Medical College (Krakow, Poland).

All patients enrolled to the study underwent gastroscopy in the years 2010-2013. Two biopsy specimens (one from antrum and the second from the body of the stomach) were taken from each patient during gastroscopy. The biopsy specimens were transferred into transport medium (Portagerm Pylori, bioMérieux, France) and transported to the laboratory (Department of Pharmaceutical Microbiology, Jagiellonian University Medical College, Krakow, Poland) for further investigations.

Analysis of endoscopy and histopathological examination enabled to qualify patients into two groups:

1. Subjects with peptic ulcer diseases (PUD) including individuals with peptic and/or duodenal ulcers.
2. Subjects with non-ulcerous dyspepsia (NUD) comprising patients without any pathological changes found in endoscopy and patients with erosive and non-erosive diseases including esophagitis, gastritis or duodenitis.

**H. pylori microbiological culture and identification.** Gastric biopsy specimens were homogenized in glass sterile mortars and inoculated onto both non selective (Schaedler agar with 5% sheep blood, bioMérieux, France) and selective (Schaedler agar with 5% sheep blood and selective supplement Dent, Oxoid, UK) media. Simultaneously, direct Gram-stained slides and urease tests were performed from the biopsy specimens to confirm the presence of *H. pylori*. The culture was carried out for 3 to 10 days under microaerophilic conditions at 37°C. The cultured strains were identified according to:

1. Macroscopic appearance of the colonies;
2. Gram–stained slides prepared from bacterial colonies;
3. Biochemical tests (urease, catalase, and oxidase).

The study was approved by the Bioethical Commission of the Jagiellonian University (Krakow, Poland). Before entering our study, each patient signed the informed consent document.

**Susceptibility testing.** Susceptibility of *H. pylori* strains to the recommended antimicrobial agents (clarithromycin, metronidazole, levofloxacin, tetracycline, and amoxicillin) was quantitatively assessed by the use of strips impregnated with antimicrobial agents gradient (E-test, bioMérieux, France), which enabled to determine the minimal inhibitory concentration (MIC). The colonies from pure *H. pylori* culture were suspended in 0.85% aqueous NaCl solution in order to obtain an equivalent of 3.0 McFarland units. The bacterial suspension was spread onto Schaedler agar medium with 5% sheep blood (bioMérieux, France) with sterile cotton swabs. Single E-test stripes were applied onto the separate, inoculated agar plates and incubated according to the manufacturer recommendations (E-test technical manual, bioMérieux, France).

Results of the susceptibility testing were interpreted according to EUCAST guidelines (breakpoint values were presented in Table 2) [68].

Quality control of the susceptibility testing was performed with *H. pylori* ATCC 43504 strain.

**DNA extraction.** *H. pylori* culture (incubated for 72 hours on solid medium) was suspended in 1 ml of 0.85% aqueous NaCl solution and centrifuged (12000 rpm/3 min.). Genomic DNA was extracted using the Sherlock AX isolation kit (A&A Biotechnology, Poland) according to the manufacturer’s recommendations. DNA quantification was performed by spectrophotometry at 260 nm. The purity of DNA was evaluated by the ratio of the absorbance at 260 and 280 nm (A260/A280). The obtained DNA was stored at -20°C.

**PCR identification of *H. pylori*.** To confirm *H. pylori* microbiological identification, all analysed strains were submitted to 16S rRNA PCR assay using primers HP-1 and HP-2 described by Kargar et al. [69]. PCR reaction was performed in a final volume of 25 µl containing: 2 µl of genomic DNA, 2 µl of each primer, 5 µl of GoTaq® Flexi Buffer, 1.5 µl of
MgCl₂ (25 mM), 0.5 µl of PCR Nucleotide Mix (10 mM each), 0.125 µl of GoTaq® DNA Polymerase (5u/µl) and Nuclease-Free Water (Promega, USA). The following amplification conditions were used: the initial denaturation at 95°C for 2 min., 35 cycles of 95°C for 60 s, 58°C for 60 s, 72°C for 60 s and the final elongation at 72°C for 5 min. The expected 109-base-pair PCR product was visualized after electrophoresis on a 2% agarose gel stained with ethidium bromide.

cagA and vacA genes detection. The DNA from analysed H. pylori strains was subjected to PCR assay detecting the cagA gene with the use of primers D008 and R008 described previously by Chen et al. [70]. The PCR reaction was carried out as described above for the 16s rRNA PCR assay. PCR cycling conditions consisted of initial denaturation at 94°C for 5 min., 33 cycles: 94°C for 60 s, 60°C for 60 s, 62.5°C for 60 s, 72°C for 60 s and the final elongation at 72°C for 5 min. The expected product of 298-base-pairs was visualized after agarose gel electrophoresis. Furthermore, we performed the PCR assay to detect the vacA gene. A 229-base-pair fragment of vacA was amplified with the primer pair VAC3624F and VAC3853R described previously by Chisholm et al. [71]. The PCR reaction was carried out in the total volume of 25 µl as described above. The following amplification conditions were used: the initial denaturation at 95°C for 2 min., 35 cycles of 95°C for 30 s, 53°C for 30 s, 72°C for 30 s and the final elongation at 72°C for 5 min. The expected product was visualized after agarose gel electrophoresis.

Detection of clarithromycin resistance by RFLP-PCR analysis. The most prevalent point mutations responsible for clarithromycin resistance (A2143G and A2142G) were detected by PCR followed by RFLP analysis. A 425-base-pair fragment of the 23S rRNA was amplified with the primers K1 and K2 described by Agudo et al. [63]. PCR amplification of DNA was performed in a final volume of 25 µl as described above for the 16S rRNA PCR assay. The cycling program was: 1 cycle at 95°C for 2 min.; 35 cycles of 95°C for 30 s, 60°C for 30 s, 72°C for 30 s and a final elongation step at 72°C for 5 min. The amplicon was digested with Eco31I (BsaI) enzyme (Thermo Scientific, USA) for 30 min. at 37°C and 5 min. at 65°C to detect A2143G mutation [63] or with BbsI enzyme (New England Biolabs, USA) for 24 h at 37°C to detect A2142G mutation [72]. The restriction products were analysed by electrophoresis on an 2% agarose gel. Enzyme Eco31I digested the A2143G-positive H. pylori PCR amplicon into 304- and 101-base-pair fragments, while BbsI enzyme cut the A2142G-positive H. pylori PCR product into 332- and 93-base-pair fragments.

<table>
<thead>
<tr>
<th>Antibiotic agent</th>
<th>MIC breakpoints (mg/L)</th>
<th>S ≤</th>
<th>R &gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amoxicillin</td>
<td>0.12</td>
<td>0.12</td>
<td></td>
</tr>
<tr>
<td>Clarithromycin</td>
<td>0.25</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>Levofloxacin</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Metronidazole</td>
<td>8</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>Rifampicin</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Tetracycline</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>

Table 2. EUCAST clinical breakpoint for H. pylori [68].
6. Results

Prevalence of *H. pylori* infection. Among 316 patients with dyspeptic symptoms admitted to the study between November 2010 and June 2013, the presence of *H. pylori* infection was confirmed in 73 cases (according to rapid urease test and bacterial culture). Therefore, the prevalence of *H. pylori* infection in patients with dyspeptic symptoms from southern Poland amounted to 23.1% (73/316). Taking into account the analysis of endoscopy and histopathological examination among 73 *H. pylori*-positive patients, 26% (19/73) were qualified into the I group (patients with PUD), whereas 74% (54/73) patients were qualified into the II group (patients with NUD).

Susceptibility testing. The susceptibility testing of 73 *H. pylori* strains to the recommended antimicrobial agents showed that 51.7% of isolates were resistant to one or more drugs, while 49.3% of strains were susceptible to all tested antibiotics/chemotherapeutics. The resistance rates of *H. pylori* strains to the assayed antimicrobial agents were presented in Table 3 (data include strains resistant to one, two or four antibiotics/chemotherapeutics).

The rate of resistance of *H. pylori* strains to clarithromycin amounted to 23.3% (17/73). According to the Maastricht/Florence IV Consensus Report, southern Poland should be qualified as an area of high clarithromycin resistance. Consequently, the use of clarithromycin in *H. pylori* infections in this region should be either abandoned (as an empirical treatment) or applied but only with prior susceptibility testing. Due to this fact, it seems reasonable to change the current Polish recommendation (PSG-E, 2008) concerning clarithromycin use in the treatment of *H. pylori* infections.

Furthermore, the resistance rates to metronidazole and levofloxacin were 39.7% (29/73) and 12.3% (9/73), respectively.

<table>
<thead>
<tr>
<th>Antimicrobial agents</th>
<th>N (%) of resistant strains (n = 73)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clarithromycin</td>
<td>17 (23.3)</td>
</tr>
<tr>
<td>Metronidazole</td>
<td>29 (39.7)</td>
</tr>
<tr>
<td>Levofloxacin</td>
<td>9 (12.3)</td>
</tr>
<tr>
<td>Amoxicillin</td>
<td>1 (1.4)</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>

Table 3. Resistance rates of *H. pylori* to clarithromycin, metronidazole, levofloxacin, amoxicillin, and tetracycline in southern Poland (the data include strains resistant to one, two or four antibiotics/chemotherapeutics).

We also reported the presence of one strain (1.4%; 1/73) resistant to amoxicillin. Resistance to amoxicillin is an uncommon phenomenon and it is the first report of an amoxicillin-resistant strain in southern Poland. All examined *H. pylori* strains were susceptible to tetracycline.

Moreover, we analysed the co-occurrence of resistance to more than one antimicrobial agent among *H. pylori* isolates (Table 4).
Our studies showed the presence of 35.6% (26/73) of single-resistant, 8% (13/73) of double-resistant and 1.4% (1) of quadruple-resistant strains (susceptibility testing for each tested isolate included: clarithromycin, metronidazole, levofloxacin, and amoxicillin).

<table>
<thead>
<tr>
<th>Antimicrobial agents</th>
<th>N (%) of resistant strains n=73</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>single-resistant strains</strong></td>
<td></td>
</tr>
<tr>
<td>Clarithromycin</td>
<td>6 (8.2)</td>
</tr>
<tr>
<td>Metronidazole</td>
<td>16 (21.9)</td>
</tr>
<tr>
<td>Levofloxacin</td>
<td>4 (5.4)</td>
</tr>
<tr>
<td><strong>double-resistant strains</strong></td>
<td></td>
</tr>
<tr>
<td>Clarithromycin + Metronidazole</td>
<td>9 (12.3)</td>
</tr>
<tr>
<td>Metronidazole + Levofloxacin</td>
<td>3 (4.1)</td>
</tr>
<tr>
<td>Clarithromycin + Levofloxacin</td>
<td>1 (1.4)</td>
</tr>
<tr>
<td><strong>quadruple-resistant strains</strong></td>
<td></td>
</tr>
<tr>
<td>Clarithromycin + Metronidazole + Levofloxacin + Amoxicillin</td>
<td>1 (1.4)</td>
</tr>
</tbody>
</table>

Table 4. Co-occurrence of resistance to various antibiotics/chemotherapeutics among H. pylori strains isolated in southern Poland.

The MICs obtained in our study ranged from 0.016 mg/L to 256 mg/L for clarithromycin [Figure 3], from 0.016 mg/L to 256 mg/L for metronidazole [Figure 4], from 0.002 mg/L to 12 mg/L for levofloxacin [Figure 5], and from 0.016 mg/L to 0.125 mg/L for both amoxicillin [Figure 6] and tetracycline [Figure 7].

The mean MIC values were as follows: 19.2 mg/L for clarithromycin, 75.2 mg/L for metronidazole, 0.61 mg/L for levofloxacin, 0.018 mg/L for amoxicillin, and 0.024 mg/L for tetracycline.

Figure 3. The distribution of clarithromycin MICs among H. pylori strains (Clinical breakpoints according to EUCAST: resistant MIC>0.5 mg/L; susceptible MIC≤0.25 mg/L); * strains with MIC value of 0.5 mg/L were considered as resistant to clarithromycin according to the results of PCR-RFLP analysis.
Figure 4. The distribution of MICs among *H. pylori* strains (Clinical breakpoints according to EUCAST: resistant MIC>8 mg/L; susceptible MICs ≤8 mg/L).

Figure 5. The distribution of levofloxacin MICs among *H. pylori* strains (Clinical breakpoints according to EUCAST: resistant MIC>1 mg/L; susceptible MICs ≤1 mg/L).

Figure 6. The distribution of amoxicillin MICs among *H. pylori* strains (Clinical breakpoints according to EUCAST: resistant MIC>0.12 mg/L; susceptible MICs ≤0.12 mg/L).
Figure 7. The distribution of tetracycline MICs among *H. pylori* strains (Clinical breakpoints according to EUCAST: resistant MIC>1 mg/L; susceptible MIC≤1 mg/L).

The quantitative E-test method allowed us to observe the dynamically increasing resistance of *H. pylori* to antimicrobials during the monitoring.

In Poland, *H. pylori* resistance rate to clarithromycin exceeded 20% [22, 23, 62]. Our recent study revealed that this rate remained at the same high level or even increased to 23.3%. Therefore the current Polish guidelines (PSG–E, 2008) should be changed according to EHSG (2012). EHSG recommended abandoning clarithromycin in empirical treatment or susceptibility testing prior to the administration of this drug in regions with high prevalence of resistant *H. pylori* strains (>20%).

Moreover, it is worth emphasizing that our study showed that 6.8% of analysed strains demonstrated high clarithromycin MIC values: 256 mg/L.

Furthermore, we revealed persistent, high-level resistance to metronidazole (39.7% of isolates). Twenty-six percent of *H. pylori* strains displayed the metronidazole MIC values as high as 256 mg/L. Additionally, the resistance rate to levofloxacin increased in comparison to our previous studies (5% in the years 2006-2008 [62] vs. 12.3% currently).

Although the amoxicillin MIC values are still low, emergence of a resistant *H. pylori* isolate was a new phenomenon. It was the first amoxicillin-resistant *H. pylori* strain isolated in southern Poland.

The reason as to why *H. pylori* resistance to antibiotics/chemotherapeutics has to be monitored, are the results of our study. Moreover, in order to obtain effective regimen for the cure of *H. pylori* infection, it seems reasonable to perform susceptibility testing prior to the treatment.

**PCR identification of *H. pylori*.** Microbiological identification of all 73 *H. pylori* isolates was confirmed by PCR targeting the 16S rRNA gene (representative samples are presented in Figure 8).
**cagA and vacA genes distribution.** cagA and vacA genes have been identified as being virulence-associated and may have important clinical and epidemiological implications [73]. While cagA is carried by 56-90% of H. pylori strains [8, 24, 29-32], vacA is present in all identified H. pylori isolates [74]. The occurrence of cagA and vacA genes was tested in all 73 strains.

The cytotoxin associated gene - cagA - was detected in 80.8% of H. pylori strains (59/73), whereas cagA-negative strains accounted for 19.2% (14/73) (representative samples are shown in Figure 9). The occurrence of vacA gene was confirmed in 89% (65/73) of strains, while 11% (8/73) of strains were vacA-negative. The absence of vacA gene in 11% of assayed H. pylori strains was associated with limited sensitivity of the method used in this study [71].
Table 5 shows the relationship between the distribution of cagA and vacA genes and the clinical diagnosis.

Among 19 patients with peptic ulcer disease (PUD), 78.9% (15/19) were infected with H. pylori cagA-positive strains, while 21.1% (4/19) with strains cagA-negative. Moreover, 84.2% (16/19) of patients with PUD were infected with H. pylori vacA-positive strains and 15.8% (3/19) with strains vacA-negative. Double positive H. pylori strains (cagA-positive and vacA-positive) occurred in 63.2% (12/19) of patients with peptic ulcer disease.

Among the group of patients with NUD, 81.5% (49/54) of isolated H. pylori strains carried cagA gene, but only 18.5% (10/54) were cagA-negative. Although none of these patients developed ulceration during the study, it is probable that these infections, if untreated, may lead to serious consequences such as peptic ulcer disease or even gastric cancer, due to the fact that the cagA-positive H. pylori strains increase the likelihood of developing these diseases.

The cagA-positive, vacA-positive H. pylori genotype was the most common one in both groups of patients: with PUD as well as with NUD, whereas the double-negative genotype was not found.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>N (%) of strains</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PUD n=19</td>
</tr>
<tr>
<td>cagA +</td>
<td>15 (78.9)</td>
</tr>
<tr>
<td>cagA -</td>
<td>4 (21.1)</td>
</tr>
<tr>
<td>vacA +</td>
<td>16 (84.2)</td>
</tr>
<tr>
<td>vacA -</td>
<td>3 (15.8)</td>
</tr>
<tr>
<td>cagA + vacA +</td>
<td>12 (63.2)</td>
</tr>
<tr>
<td>cagA + vacA -</td>
<td>3 (15.8)</td>
</tr>
<tr>
<td>cagA - vacA +</td>
<td>4 (21.1)</td>
</tr>
<tr>
<td>cagA - vacA -</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>

PUD – peptic ulcer disease
NUD – non-ulcer dyspepsia

Table 5. Relationship between distribution of cagA and vacA genes and the clinical diagnosis.

**PCR-RFLP analysis of H. pylori resistance to clarithromycin.** Molecular analysis of clarithromycin resistance mechanism was conducted on 73 H. pylori strains. The results of PCR-RFLP analysis are shown in Figure 10 and Table 6.

Among the 17 clarithromycin-resistant isolates, Eco31I (BsaI) digested PCR amplicons of 12 (70.6%) strains to 304-base-pair and 101-base-pair, indicating that the strains contained A2143G mutation in the 23S rRNA gene. Furthermore, BbsI enzyme cut products of 3 (17.6%) strains to 332-base-pair and 93-base-pair suggesting that the isolates had A2142G mutation.
RFLP analysis of 11.8% (2/17) clarithromycin-resistant isolates gave negative results for both assayed mutations. Resistance to clarithromycin in these isolates (negative for both A2413G and A2142G) might be associated with other mutations in the 23S rRNA such as A2142C, T2245C, T2717C, A2115G, G2141A, T2182G, or T2182C as well as efflux pump [63].

Digestion of PCR products of 100% (56/56) strains not resistant to clarithromycin revealed the absence of the tested mutations. Moreover, our studies suggest that the presence of A2142G mutation is more related to the high clarithromycin MIC level than the A2143G mutation.

<table>
<thead>
<tr>
<th>Type of mutation</th>
<th>Clarithromycin–resistant n=17</th>
<th>Clarithromycin–susceptible n=56</th>
</tr>
</thead>
<tbody>
<tr>
<td>A2143G</td>
<td>12 (70.6)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>A2142G</td>
<td>3 (17.6)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>not detected</td>
<td>2 (11.8)</td>
<td>56 (100)</td>
</tr>
</tbody>
</table>

Table 6. Prevalence of A2143G and A2142G mutations in the group of 73 tested *H. pylori* strains.

7. Conclusions

Results of our studies concerning the resistance rates of *H. pylori* strains isolated from subjects in southern Poland suggest the need for constant monitoring of the resistance to a set of...
antimicrobial agents routinely used for empirical therapy. We observed high level of clarithromycin resistance rate (>20%), which resulted in the necessity of either to abandon clarithromycin in an empirical treatment or to perform susceptibility testing prior to application of clarithromycin-containing treatment.

We also noted the increase in *H. pylori* resistance to levofloxacin and persistent high level resistance to metronidazole, which resulted in the necessity of treatment guided by antimicrobial susceptibility testing.

To conclude, antimicrobial susceptibility testing can improve treatment outcomes and reduce the outpatient antibiotic consumption, while the introduction of molecular methods for clarithromycin resistance testing allows better and more efficient management of *H. pylori* infections.

8. Further research

Our further investigations will be concerned with the application of the PCR-RFLP method in detection of less frequent 23S rRNA gene mutations associated with clarithromycin resistance. Furthermore, we will focus on direct detection of *H. pylori* in biopsy specimens as well as characterization of the resistance determinants.

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References


