PCR-RFLP detection of point mutations A2143G and A2142G in 23S rRNA gene conferring resistance to clarithromycin in Helicobacter pylori strains*

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Background. The occurrence of clarithromycin resistance among Helicobacter pylori strains is a major cause of the treatment failure. Resistance to this drug is conferred by point mutations in 23S rRNA gene and the most prevalent mutations are A2143G and A2142G. The aim of the study was to evaluate the occurrence of A2143G and A2142G mutations in a group of H. pylori strains resistant to clarithromycin. Materials and Methods. The study included 21 clarithromycin-resistant H. pylori strains collected between 2006 and 2009 in southern Poland. Resistance to clarithromycin was quantitatively tested with the E-test to determine the minimal inhibitory concentration (MIC value). The point mutations of H. pylori isolates were detected by PCR followed by RFLP analysis. Results. The MIC values for clarithromycin for the analyzed strains ranged from 1.5 mg/L to 64 mg/L. Nine H. pylori strains exhibited A2143G mutation and A2142G mutation was found in 9 isolates as well. The results of RFLP analysis of 3 clarithromycin-resistant strains were negative for both mutations. The average MIC values for A2143G and A2142G mutants were 6 and 30 mg/L, respectively. Conclusions. Frequencies of A2143G and A2142G mutations were the same in all isolates tested. Strains with A2143G mutation exhibited lower MIC values than A2142G mutants. Application of PCR-RFLP method for detection of clarithromycin resistance allows for better and more efficient management of H. pylori infections.

Key words: Helicobacter pylori, clarithromycin resistance, PCR-RFLP, point mutations

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INTRODUCTION

Helicobacter pylori is a Gram-negative, spiral-shaped, microaerophilic bacterium which colonizes gastric mucosa of 50% of the human population worldwide. Moreover, H. pylori is a major cause of upper gastrointestinal tract diseases such as: dyspepsia, type B gastritis, gastric ulcer disease, and duodenal ulcer disease (Perez-Perez et al., 2004; Malferttheimer et al., 2012; den Hollander, 2013). H. pylori is also considered as a class I carcinogen, involved in the formation of gastric cancer and mucosa-associated lymphoid tissue (MALT) lymphoma (IARC, 1994; Ando et al., 2006; Konturek et al., 2009; Gisbert et al., 2011; Figueiredo et al., 2013).

Current recommendations for the management of H. pylori infection were elaborated by the European Helicobacter Study Group (EHSG) and presented in Maastricht IV/Florence Consensus Report in 2012. According to these guidelines, an effective treatment requires combined therapy, and it is important to take into consideration H. pylori resistance to clarithromycin on the area the patient is coming from (areas of low (<20%) and high (>20%) prevalence) (Malferttheimer et al., 2012).

Clarithromycin is one of the most common components of H. pylori infection treatment schemes. It is included in the standard triple therapies, as well as in non-bismuth quadruple therapies (either sequential or concomitant) (Malferttheimer et al., 2012). Moreover, clarithromycin is found to be one of the most effective antimicrobial agents used in the treatment of H. pylori infection (de Francesco et al., 2006). However, it should be remembered, that the development of clarithromycin resistance is a major cause of H. pylori treatment failure (Mégraud, 2004; Giorgio et al., 2013).

Clarithromycin is a semisynthetic 14-membered macrolide antibiotic with bacteriostatic activity against broad-spectrum of microorganisms including H. pylori. Due to this fact, this drug is widely used in the treatment of respiratory tract and skin diseases, as well as upper-gastrointestinal tract diseases caused by H. pylori. While clarithromycin is relatively stable in gastric juice, the use of an acid-suppressive drug (e.g. proton pumps inhibitor (PPI)) enhances the therapeutic effect of this antimicrobial agent. Moreover, therapeutic activity of clarithromycin is also related to its delivery to the gastric mucosa (Erah et al., 1997; Dzierżanowska, 2009; Fuki et al., 2011).

Antibacterial activity of clarithromycin is related to inhibition of bacterial protein synthesis by reversible binding to the 50S ribosomal subunits. The target site of clarithromycin is the peptidyl transferase loop of V domain of 23S ribosomal RNA molecule. Because of this binding, the translocation of aminoacyl transfer-RNA is...
effectively blocked resulting in the inhibition of protein synthesis (Dzierzanowska, 2009; Gerrits et al., 2006).

Resistance to clarithromycin is associated with the target site modifications and efflux pumps. Target site modifications are mediated by two mechanisms: 1) point mutations in the peptidyl transferase-encoding region of V domain of 23S rRNA gene and 2) post-transcriptional methylation of 23S rRNA region (Gerrits et al., 2006; Mégraud & Lehours, 2007; Hirata et al., 2010).

The mechanism of clarithromycin resistance in H. pylori is mainly associated with the following point mutations in 23S rRNA gene: 1) A2143G (previously described as A2144G) occurring in 69.8% of strains; 2) A2142G (previously described as A2143G) occurring in 11.7% of isolates; 3) A2142C reported in 2.6% strains (Taylor et al., 1997; Mégraud, 2004; Gerrits et al., 2006; Karczewska et al., 2009; Francavilla et al., 2010).

Moreover, other mutations such as A2115G, G2141A, C2147G, T2190C, C2195T, A2223G, and C2694A have been identified among H. pylori strains resistant to clarithromycin, although their role in the mechanism of resistance remains unclear (Hao et al., 2004; Mégraud, 2004; Gerrits et al., 2006; Agudo et al., 2010).

One of the major factors contributing to clarithromycin resistance of H. pylori strains is high outpatient consumption of clarithromycin, especially in the treatment of respiratory tract diseases (Mégraud, 2004; Mégraud et al., 2013). Mean rate of H. pylori clarithromycin resistance in Europe amounted to 17.5%. However, diversity between the regions could be observed: 7.7% in the Northern European countries, 18.7% in Western/Central Europe, and 21.5% in Southern Europe (Mégraud et al., 2013).

In Poland, according to our recent studies conducted in the years 2006–2008 and 2009–2011, the rate of H. pylori clarithromycin resistance among adults was 34% and 22%, respectively (Karczewska et al., 2011; Karczewska et al., 2012). Therefore, according to the Maastricht IV/Florence Consensus Report, southern Poland should be classified as a high clarithromycin resistance region (Malferttheiner et al., 2012).

Over the years, numerous methods have been developed to detect the resistance of H. pylori to clarithromycin. These methods can be divided into two groups: phenotypic and genotypic. Antibiotic susceptibility testing of H. pylori is routinely performed with the use of phenotypic methods. This assay, preceded by culturing of bacteria, includes both agar dilution technique and the use of strips impregnated with antimicrobial agent gradient (e.g. E-test, bioMérieux, France). In addition, these methods are labor-intensive and time-consuming; full testing takes up to two weeks. Unfortunately, as estimated, culture based methods fail in about 10% of cases due to contamination of biopsy specimens or growth difficulties (Gerrits et al., 2006). While culture-based phenotypic methods are time consuming, nucleic acid-based techniques could offer a faster and more accurate alternative. Molecular detection of clarithromycin resistance among H. pylori strains is mostly based on analysis of the unique point mutations in 23S rRNA gene. These techniques make it possible to determine the resistance profiles of H. pylori strains from cultured isolates as well as directly from biopsies or stool samples (Oleastro et al., 2003; Booka et al., 2005; Rimbara et al., 2005; Ho et al., 2010). The most important methods used to detect mutations are PCR followed by RFLP (restriction fragment length polymorphism) and Real-Time PCR (using SYBR-Green and/or labeled probes), although other methods are applied as well: PCR-DNA enzyme immunooassay, mismatched PCR, hybridization and sequencing techniques (Chisholm et al., 2001; Mégraud, 2004; Schabereiter-Gurtner et al., 2004; Gerrits et al., 2006; Agudo et al., 2010).

While molecular methods are more reliable and less time-consuming than the phenotypic ones, they are still not routinely used for detection of clarithromycin-resistant H. pylori strains in Poland. PCR-RFLP is performed to detect the most common point mutations in 23S RNA gene, such as: A2143G, A2142G and A2142C (Occialini et al., 1997; Ménard et al., 2002; Agudo et al., 2011). This method is based on the presence or absence of the sites recognized by restriction enzymes within the amplified DNA fragment (Gerrits et al., 2006; Mégraud & Lehours, 2007).

The aim of the present study was to detect the two most prevalent point mutations: A2143G and A2142G in 23S RNA gene associated with clarithromycin resistance of H. pylori strains with the use of PCR-RFLP technique.

**MATERIALS AND METHODS**

**Gastric biopsy specimens and H. pylori strains.** Twenty-one clarithromycin-resistant H. pylori clinical strains subjected to the study were obtained from gastric biopsy specimens of patients with upper-gastrointestinal tract diseases, from Falck Medyczyna Outpatient Clinic of Gastroenterology (Krakow, Poland) between 2006 and 2009.

H. pylori strains were isolated according to the following procedure:

- homogeneous tissue was spread 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 and incubated up to 10 days under microaerophilic conditions at 37°C;
- identification of H. pylori strains was based on: microscopic appearance of colonies, negative Gram staining and positive biochemical tests for urease, catalase and oxidase;
- isolated strains were frozen and stored at −80°C for further analysis in Schaedler anaerobe broth (Oxoid, UK) supplemented with fetal bovine serum (Sigma-Alrich, Germany) and glycerol (POCH, Poland).

The design of the study was approved by the Bioethical Commission of the Jagiellonian University and each patient signed an informed consent before participation in the study.

**Determination of the clarithromycin resistance with the use of phenotypic method (E-test).** Clarithromycin resistance was determined quantitatively, as the minimal inhibitory concentration (MIC value), with the use of strips impregnated with the antibiotic gradient (E-test, bioMérieux, France). Colonies were taken from the pure H. pylori cultures and suspended in sterile saline (NaCl 0.85% Medium, bioMérieux, France) on an equivalent of 3.0 McFarland units. The inoculum was spread onto Schaedler agar medium with 5% sheep blood (bioMérieux, France) with sterile cotton swabs. E-test strips with clarithromycin gradient were separately placed on inoculated plates according to the manufacturer recommendations (E-test technical manual, bioMérieux, France). The inoculated plates with strips were incubated under microaerophilic conditions for 72 hours at 37°C.
**Table 1. Conditions of digestion with restriction enzymes (enzymes, thermal profiles and products size).**

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Enzyme</th>
<th>Thermal profile</th>
<th>Products size</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>A2143G</td>
<td>Eco31I (Bsal)</td>
<td>37°C (30 min), 65°C (5 min)</td>
<td>304 bp and 101 bp</td>
<td>Agudo et al., 2011</td>
</tr>
<tr>
<td>A2142G</td>
<td>Bbsl</td>
<td>37°C (24 h)</td>
<td>332 bp and 93 bp</td>
<td>Occhialini et al., 1997</td>
</tr>
</tbody>
</table>

_H. pylori_ strains were classified as resistant when the clarithromycin MIC values exceeded 0.5 mg/L (according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoint tables for interpretation of MICs and zone diameters).

**Bacterial genomic DNA extraction.** DNA was extracted from pure 72-hour _H. pylori_ cultures. Colonies were suspended in 1 ml of sterile saline (NaCl 0.85% Medium, bioMérieux, France) and centrifuged (12000 rpm/3 min). Total bacterial genomic DNA was isolated with the use of Sherlock AX isolation kit (A&A Biotechnology, Poland), according to the manufacturer’s recommendations. The eluted DNA was stored at −20°C until use.

**Determination of the clarithromycin resistance with the use of genotypic method (PCR-RFLP).** Detection of the most common point mutations (A2143G, A2142G) conferring resistance to clarithromycin in _H. pylori_ strains was performed by PCR followed by RFLP analysis.

PCR assay was conducted using primers and thermal profiles described by Agudo (Agudo et al., 2011) (K1 – sense: CCA CAG CGA TGT GGT CTC AG and K2 – antisense: CTC CAT AAG AGC CAA AGC CC). The reaction mixture of the final volume 25 µl contained: 2 µl of genomic DNA, 2 µl of each primer, 5 µl of GoTaq® DNA Polymerase (5 u/µl) and Nuclease-Free Water (Promega, USA).

Amplicon of 425-base-pairs was visualized after electrophoresis in 2% agarose gel stained with ethidium bromide.

The RFLP assay was carried out with Eco31I (Bsal) enzyme (Thermo Scientific, USA) in order to detect A2143G mutation, while Bbsl enzyme (New England Biolabs, USA) was used to detect A2142G mutation. Digestion thermal profiles are shown in Table 1. The analysis was carried out against ATCC 700684 reference clarithromycin-resistant _H. pylori_ strain possessing A2143G mutation.

**RESULTS AND DISCUSSION**

_H. pylori_ resistance to clarithromycin is predominantly related to the point mutations in the peptidyl transferase-encoding region of V domain of 23S rRNA gene. The most prevalent point mutations responsible for this process are A2143G, A2142G, and A2142C (Mégraud, 2004).

**Prevalence of point mutations**

PCR-RFLP analysis was performed on 21 _H. pylori_ strains resistant to clarithromycin in order to detect the two most common point mutations: A2143G and A2142G in 23S rRNA gene. Figure 1 shows the results of restriction fragment length polymorphism analysis of 23S rRNA amplicons (425 bp) with Eco31I (Bsal) and Bbsl enzymes.

Among 21 clarithromycin-resistant _H. pylori_ strains included in our study, more than 90% of isolates carried A2143G (9; 42.9%) or A2142G (9; 42.9%) mutation. Therefore, our research confirms the reports of several authors that the predominant mutations responsible for clarithromycin resistance in _H. pylori_ are A2143G and A2142G (Occhialini et al., 1997; Mégraud, 2004).

Prevalence of the particular point mutations varies across geographical areas. Versalovic et al. (1997) showed that the majority of _H. pylori_ isolates from American patients carried A2143G (previously A2144G) mutation (52.5%), whereas 39% of strains contained A2142G (previously A2142G) mutation. Also, among French patients, A2143G mutation was predominant in clarithromycin-resistant strains — 90% of A2143G vs. 10% of A2142G mutants (Raymond et al., 2007). Alvarez et al. (2009) confirmed that A2143G mutation was more frequent among patients from Colombia and could be found in 75% of _H. pylori_ clarithromycin-resistant strains. According to the review conducted by Mégraud (Mégraud, 2004), the most common mutation was A2143G that occurred in about 69.8% of strains resistant to clarithromycin, but its prevalence varied from 52.7% in Germany up to 95.6% in Hong Kong. In Poland, 72% of the clarithromycin-resistant strains isolated from children, were reported to carry A2143G mutation (Rożynek et al., 2002). The average prevalence of A2142G mutation amounted to 11.7% and ranged from 5% of the resistant strains in Iran to 66.0% in Japan (Mégraud, 2004). Twenty per cent of _H. pylori_ strains isolated from Polish patients were A2142G mutants (Rożynek et al., 2002). While most researchers concluded that A2143G mutation was more common than A2142G one (Versalovic et al., 1997; Mé-
Association between the type of mutation and the level of clarithromycin resistance

According to some authors, different types of mutations are associated with different MIC values (Raymond et al., 2007). Correlation between the type of mutation and the MIC value for 21 analyzed H. pylori isolates is presented in Table 2. In our study, a MIC value exceeding 32 mg/L was defined as high-level resistance to clarithromycin (Versalovic et al., 1997).

In our study, all of A2143G H. pylori mutants demonstrated low MIC values to clarithromycin (MICs ≤32 mg/L), while in A2142G mutants we observed strains of both phenotypes; with high- or low-level of resistance. However, the results of our study showed that the strains with A2143G mutation had lower average MIC values than strains with A2142G mutation (6 mg/L and 30 mg/L, respectively). These results are consistent with those reported by other researchers who concluded that A2143G point mutation was correlated with lower clarithromycin MIC values than A2142G one (Stone et al., 1997; Versalovic et al., 1997; de Francesco et al., 2006; G errits et al., 2006; Raymond et al., 2007; Alva rez et al., 2009).

To conclude, PCR-RFLP method performed from pure H. pylori culture reduces the time of determination of clarithromycin resistance by about 4 days comparing to phenotypic methods of susceptibility testing. Faster detection of clarithromycin resistance enables selection of treatment regimen without clarithromycin. Our PCR-RFLP analysis revealed also the occurrence of any of the assayed mutations. In these isolates, resistance to clarithromycin might be associated with other less common mutations or with the efflux mechanism. Therefore, further analysis are required. In future, these mechanisms could be detected using PCR-RFLP or Real-Time PCR (Menard et al., 2002; Megraud & Lehours, 2007).

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Table 2. Correlation between clarithromycin MIC values [mg/L] and the type of mutation in 23S RNA gene of H. pylori strains resistant to clarithromycin.

<table>
<thead>
<tr>
<th>Mutation</th>
<th>No. (%) of strains</th>
<th>MIC≤32 mg/L</th>
<th>MIC&gt;32 mg/L</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n=18</td>
<td>n=3</td>
<td>n=21</td>
<td></td>
</tr>
<tr>
<td>A2142G</td>
<td>6 (33.3)</td>
<td>3 (100)</td>
<td>9 (42.9)</td>
<td></td>
</tr>
<tr>
<td>A2143G</td>
<td>9 (50)</td>
<td>0 (0)</td>
<td>9 (42.9)</td>
<td></td>
</tr>
<tr>
<td>Undetermined</td>
<td>3 (16.7)</td>
<td>0 (0)</td>
<td>3 (14.2)</td>
<td></td>
</tr>
</tbody>
</table>

REFERENCES


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