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

Karolina Klesiewicz[1], Paweł Nowak[1]✉, Elżbieta Karczewska[1], Iwona Skiba[1], Izabela Wojtas-Bonior[1], Edward Sito[2] and Alicja Budak[1]

[1]Department of Pharmaceutical Microbiology, Jagiellonian University Medical College, Kraków, Poland; [2]Falck Medycyna Outpatient Clinic of Gastroenterology, Kraków, Poland

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; Malfertheiner *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) (Malfertheiner *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) (Malfertheiner *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

✉e-mail: p.nowak@uj.edu.pl

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Abbreviations: EHSG, European Helicobacter Study Group; EUCAST, European Committee on Antimicrobial Susceptibility Testing; MALT, mucosa-associated lymphoid tissue lymphoma; MIC, minimal inhibitory concentration; PCR, Polymerase Chain Reaction; RFLP, Restriction Fragments Length Polymorphism

effectively blocked resulting in the inhibition of protein synthesis (Dzierżanowska, 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 (Malfertheiner *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 immunoassay, 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 rRNA gene, such as: A2143G, A2142G and A2142C (Occhialini *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 rRNA 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 Medycyna 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 Scheadler anaerobe broth (Oxoid, UK) supplemented with fetal bovine serum (Sigma-Aldrich, 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).

Mutation	Enzyme	Thermal profile	Products size	References
A2143G	Eco31I (BsaI)	37°C (30 min), 65°C (5 min)	304 bp and 101 bp	Agudo *et al.*, 2011
A2142G	BbsI	37°C (24 h)	332 bp and 93 bp	Occhialini *et al.*, 1997

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® Flexi Buffer, 1.5 µl of $MgCl_2$ (25 mM), 0.5 µl of PCR Nucleotide Mix (10 mM each), 0.125 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 (BsaI) enzyme (Thermo Scientific, USA) in order to detect A2143G mutation, while BbsI 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 (BsaI) and BbsI enzymes.

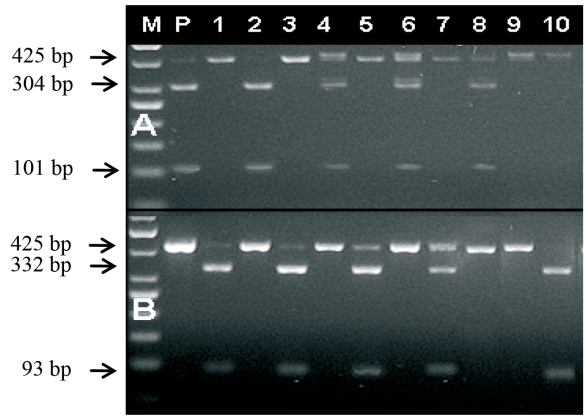

Figure 1. RFLP analysis of 23S rRNA amplicons (425 bp) digested with:
(**Part A**) Eco31I (BsaI) enzyme (digestion products of 304 and 101 bp — for A2143G mutants); (**Part B**) BbsI enzyme (digestion products of 332 and 93 bp — for A2142G mutants); Lines 1–10: clinical *H. pylori* isolates; Lines 1, 3, 5, 7, 10: A2142G *H. pylori* mutants; Lines 2, 4, 6, 8: A2143G *H. pylori* mutants; Line 9: clarithromycin-resistant isolate with negative results for both assayed mutations; P — *H. pylori* ATCC 700684; M — Molecular Weight Marker (50 bp O'Gene Ruler, Thermo Scientific, USA).

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 A2143G) 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 66.6% 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-

graud, 2004; Raymond *et al.*, 2007; Agudo *et al.*, 2011), we observed equal prevalence of both mutations in all analyzed strains. The discrepancies between our results and the results of other authors may have arisen from either geographical origin of the isolates or limited number of tested strains.

Our PCR-RFLP analysis revealed also the occurrence of three clarithromycin-resistant strains (14.2%) without 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; Mégraud & Lehours, 2007).

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; Gerrits *et al.*, 2006; Raymond *et al.*, 2007; Alvarez *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 according to EHSG recommendations, particularly in the regions of high clarithromycin resistance, such as Poland.

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

Mutation	No. (%) of strains		
	MIC≤32 mg/L n=18	MIC>32 mg/L n=3	Total n=21
A2142G	6 (33.3)	3 (100)	9 (42.9)
A2143G	9 (50)	0 (0)	9 (42.9)
Undetermined	3 (16.7)	0 (0)	3 (14.2)

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