ORIGINAL PAPER

DOI: http://dx.doi.org/10.15403/jgld-758

Genetic Antibiotic Resistance of *Helicobacter pylori* in South-Eastern Romania

Eugen Dumitru[1,2], Luana Alexandrescu[1], Anda Carmen Hanu[1], Cristina Tocia[1], Georgeta Camelia Cozaru[2,3], Anca Florentina Mitroi[2,3], Costel Brînzan[2,3], Mariana Aşchie[3], Irina Magdalena Dumitru[1,4]

1) Faculty of Medicine, Ovidius University of Constanța
2) Research Center for the Morphological and Genetic Study in Malignant Pathology (CEDMOG), Ovidius University of Constanța
3) Pathology Department, Emergency County Clinical Hospital of Constanța
4) Clinical Infectious Diseases Hospital, Constanța, Romania

Address for correspondence:
Luana Alexandrescu
Faculty of Medicine, Ovidius University of Constanța, Romania
alexandrescu_l@yahoo.com

Received: 17.01.2020
Accepted: 29.02.2020

ABSTRACT

Background & Aims: *Helicobacter pylori* (*H. pylori*) infection is widespread, affecting about half of the world's population. The increasing prevalence of strains resistant to antimicrobial agents is a critical issue for the eradication rate of *H. pylori*. With the rapid development of molecular pathology, some molecular markers have been confirmed to be associated with antibiotic resistance. This study investigated the genetic antibiotic resistance of *H. pylori* in the south-eastern region of Romania.

Methods: The study was addressed to an adult population of patients from Constanța County, Romania with uninvestigated dyspepsia who had not previously received *H. pylori* eradication therapy. Out of 321 tested patients who met the inclusion and exclusion criteria, 90 (38.9%) had positive rapid urease test from gastric biopsy samples. Primary genetic resistance to antibiotics (fluoroquinolone and clarithromycin) was tested by the GenoType HelicoDR kit (Hain Lifescience GmbH, Germany).

Results: Out of 90 patients whose samples were tested, the majority were female, 59/90 (65.5%) and the mean age was 46.6 ± 14.7 years. Primary clarithromycin resistance mutations were detected in 18/90 (20%) of patients, the most common mutation in our study being A2147G (associated with a high level of clarithromycin resistance and lower cure rates); other mutations were A2147G, A2146G and A2146C. Primary fluoroquinolones resistance mutations were detected in 27/90 (30%) of patients, and the most common mutations were D91N, D91G, and N87K. There was no correlation with patients' gender or age.

Conclusions: Clarithromycin and fluoroquinolone resistance of *H. pylori* was moderately high in our study. The type of mutation responsible for clarithromycin resistance is the one with more chances of eradication failure (A2147G). There is a need for monitoring *H. pylori* resistance patterns in Romania to provide data that can guide empirical treatment. This is the first published study on the genetic resistance of *H. pylori* in Romania.

Key words: *Helicobacter pylori* – drug resistance – anti-bacterial agents – clarithromycin – fluoroquinolone.

Abbreviations: *H. pylori: Helicobacter pylori*; MALT: mucosa-associated lymphoid tissue; PCR: polymerase chain reaction.

INTRODUCTION

Helicobacter pylori (*H. pylori*) infection is widespread, affecting about half of the world's population; it is more common in developing countries where it can have a frequency of 70% to 90% in the population and less frequently encountered in western countries where the infection rate in the population is between 25% to 50% [1]. In Romania, studies on the prevalence of *H. pylori* infection are rare and quite old and show an infection rate of up to 70% in the population [2, 3]. *H. pylori* infection is the most common cause of chronic gastritis, gastric and duodenal ulcer, gastric adenocarcinoma, and mucosa-associated lymphoid tissue (MALT) lymphoma lymphoma [4]. The eradication rate of *H. pylori* infection is unanimously recognized as markedly decreasing in recent years and the major reason is antibiotic resistance. In the *H. pylori* eradication regimens, six antibiotics are commonly used, including amoxicillin, clarithromycin, levofloxacin, metronidazole, furazolidone and tetracycline [5]. Antibiotic *H. pylori* resistance has reached an alarming rate in all regions of the world, with clarithromycin-based regimens having an eradication rate below 80%, and fluoroquinolone-based regimens even lower [6, 7]. International guidelines recommend that clarithromycin should be used in the first line of therapy in those regions where its resistance is less than 15%;

where clarithromycin resistance is greater than 15%, quadruple therapy with bismuth salts should be used instead [5]. Romania lacks in studies on *H. pylori* resistance to antibiotics because cultivating *H. pylori* in culture media is a difficult method. On the other hand, it is known that phenotypic resistance to antibiotics is genetically determined. Recently, a new technique for testing antibiotic susceptibility has been studied, namely the detection of mutations in genes that confer antibiotic resistance. In the case of *H. pylori*, antibiotic resistance is based on point mutations, without involving more complex mechanisms such as plasmids, transposons and integrons [8].

In this study, we tested the *H. pylori* resistance to antibiotics (clarithromycin and fluoroquinolones) in the south-eastern region of Romania by characterizing the genotypes that confer resistance to these antibiotics.

METHODS

Study design, endoscopy and gastric biopsies

The study was addressed to a population of adult patients from Constanta County with uninvestigated dyspepsia defined by the presence of at least one of the following gastrointestinal complaints: epigastric pain, heartburn, fullness, discomfort, early satiety, nausea, regurgitation, vomiting and belching [9]. An important condition was the inclusion only of patients who had not previously received *H. pylori* eradication therapy so that the study addresses the primary antibiotic resistance of *H. pylori* infection in our geographical area.

The exclusion criteria were patients under 18 years of age, those who had used proton pump inhibitors or antibiotics during the last 30 days before endoscopy, patients who were unable to communicate, or admitted with a major emergency such as gastrointestinal bleeding.

Between January and March 2019, 318 patients, aged between 19 and 77 years underwent upper digestive endoscopy in the Department of Gastroenterology of the Clinical, County and Emergency Hospital of Constanța. The videoendoscope used was the Pentax EPK-i7010 system with a EG-2990i endoscope. Out of a total of 318 patients investigated, only 231 patients met the inclusion criteria and expressed their consent to be enrolled in the study. During endoscopy, gastric biopsy samples were taken from these patients following this protocol: from each patient, two samples were taken for rapid urease test (one from the antrum and the other one from the gastric body), and three samples for genetic determinations (one from the antrum, one from the gastric body and the third sample targeted the endoscopic aspect that most likely suggested the infection). If the rapid urease test was positive, the biopsy samples were sent to the molecular genetic laboratory. If the urease test was negative, the samples taken for the genetic tests were dropped without these tests being carried out. Genetic testing was performed exclusively in cases where rapid urease test was positive. The presence of *H. pylori* infection was detected by the urease test in 90 (38.9%) patients, out of the 231 patients tested.

The gastric biopsy samples intended for further processing in the genetics laboratory were collected and preserved in the DNA/RNA Shield (Zymo Research). The samples were weighed and frozen at -80 °C until further processing. Molecular tests were performed at the Research Center for the Morphological and Genetic Study in Malignant Pathology (CEDMOG) "Ovidius" University of Constanta.

DNA extraction

Genomic DNA extraction from the biopsy samples was carried out using the QIAmp DNA Mini Kit (Qiagen, Germany) following the manufacturer's instructions. About 15 mg of biopsies was homogenized with Tissue Ruptor II (Qiagen, Germany) thoroughly in 100 µl PBS for 3 min or until complete disruption. Thereafter, the homogenate was incubated at 56 °C for 1 hour in 100 µl of ATL buffer and 20 µl of protein kinase K and then for 10 min at 70 °C in 200 µl of AL buffer. Subsequently, after adding 200 µl of ethanol, the mixture was carefully applied to the QIAamp Mini spin column and then washed in the centrifugal field with two buffers AW1 and respectively AW2 at 12000 rpm for 1 min. Finally, the column was then placed in a new tapered collection tube and 150 µL of buffer AE was added on the column membrane and centrifuged at 8000 rpm for 1 minute to collect the eluate.

The DNA purified from samples were assessed for purity, concentration, and integrity using a NanoDrop One™ Spectrophotometer (Thermo Scientific™), Qubit®3.0 (Life Technologies).

GenoType HelicoDR molecular analysis

The patients detected to be positive for *H. pylori* strain by the rapid urease test were further subjected to detection of point mutations in genes responsible for fluoroquinolone and clarithromycin resistance using the GenoType HelicoDR kit (Hain Lifescience GmbH, Germany), according to the manufacturer's instructions. The molecular test is based on multiplex polymerase chain reaction (PCR) amplification and reverse-hybridization of biotinylated primers on a strip containing allele-specific oligonucleotide probes, that permits to identify the various mutation N87K, D91N, D91G, D91Y of the gyrA gene (codon 87 and 91) and A2146G, A2146C, A2147G of the 23S rRNA gene (positions 2146 and 2147).

Each 50 µl PCR reaction consists of 35 µl PNM (containing nucleotides and biotinylated primers), 5 µl 10 x polymerase incubation buffer, 4.75 µl of water for PCR, 0.25 µl HotStartTaq DNA polymerase (2 units), and 5 µl DNA template (concentration was set between 20 to 100 ng). Samples were incubated in a thermal cycler (Biometra, Germany) with the following parameters: 1 cycle at 95°C for 15 min for DNA polymerase activation, followed by 10 cycles at 95°C for 30 s and at 58°C for 2 min. Then, 20 cycles, where each cycle contains a denaturation step at 95°C for 25 s, an annealing step at 53°C for 40 s, and a polymerization step at 70°C for 40 s. The PCR reaction ended with the elongation of all amplicons for 8 min at 70°C.

The hybridization process involves denaturation of 20 µl of the PCR product mixed with 20 µl denaturation solution for 5 min at RT. Subsequently, the hybridization of single-stranded DNA with allele-specific oligonucleotides on a solid phase (strip) in 1 ml of HYB buffer at 45°C for 30 min was performed on a thermoshaker.

The stringent washing, conjugation and enzymatic visualization of labeled and denatured amplification products

were performed at 45°C for 15 min, at 30 min at RT and 10 min at RT, respectively. We did not use positive and negative controls because GenoType Helico DR (Hain Lifescience GmbH) is a validated test for in vitro diagnostic use from biopsy samples. The results concerning *H. pylori* detection and susceptibility to clarithromycin and fluoroquinolones were obtained by analyzing the positive and negative bands in DNA strips.

Statistical tests

Data were entered and computed using Excel, where continuous variables were expressed as mean +/- standard deviation, whereas categorical variables were expressed as number and percentage. Notably, all samples were tested before statistical analysis for their normal distribution. The association between resistance or susceptibility to clarithromycin, respectively fluoroquinolones and age was evaluated using Independent Samples t-test and the association between resistance and gender was evaluated using Pearson's chi-squared, where p-value < 0.05 was considered to be statistically significant.

Ethical approval

The study was conducted according to good laboratory practice and in accordance with the Declaration of Helsinki and national and institutional standards. Informed consent was obtained from all patients, and the study was approved by the Local Ethics Commission for the Approval of Clinical and Research Developmental Studies (approval no. 15 /05.12.2018).

RESULTS

Among the 90 patients whose samples were tested for the detection of point genetic mutations that confer antibiotic resistance, the majority were female, 59/90 (65.5%). The mean age of the group of patients tested was 46.6 ± 14.7 years (range between 19 and 77 years).

Mutations in the 23S rRNA locus (Supplementary Fig) conferring clarithromycin resistance were detected in 20.0% of cases (18/90), of which 72.2% (13/18) showed mutations in codon A2147G, 16.6% (3/18) in codon A2146G, and 11.1% (2/18) in codon A2146C). Out of these, 33.3% of samples (6/18) presented a heterozygous profile and 66.6% (12/18) were of homozygous type (Table I).

Mutations in the gyrA locus conferring fluoroquinolone resistance were detected in 30% of cases (27/90); these were of D91N type (16 cases, of which 10 homozygous cases), D91G type (5 cases, of which 3 homozygous cases), N87K type (5 cases, of which 2 homozygous cases), D91G/D91Y type (1 homozygous case), and D91N/D91Y type (1 homozygous case) (Table II).

A number of 6 (6.66%) samples from a total of 90 tested concomitantly showed genetic mutations in both the gyrA gene and the 23S rRNA gene, so antibiotic resistance was detected as follows:

a. Mutations that confer resistance only to clarithromycin = 12/90 (13.3%)

b. Mutations that confer resistance only to fluoroquinolone = 21/90 (23.3%)

c. Mutations that confer resistance to both antibiotics simultaneously = 6/90 (6.66%)

d. Mutations that confer resistance to at least one of the antibiotics (a+b+c) = 39/90 (43.3%)

e. Samples without genetic resistance mutations (samples with antibiotic susceptibility) = 51/90 (56.6%).

There was no significant difference between the ages of patients with clarithromycin resistance mutations and those without resistance mutations (45.4 ±12.0 years vs. 46.9 ± 15.4 years; p > 0.05) (Fig. 1) or between the ages of patients with resistance mutations to fluoroquinolones and those without resistance mutations (49.8± 13.8 years vs. 45.3 ± 15.0 years; p > 0.05) (Fig. 2).

Table I. Type of mutations conferring clarithromycin resistance

Antibiotic tested	Number of samples tested	The type of mutation detected	Number of samples detected with mutation	The homozygous type (*aa*)	The heterozygous type (*Na*)
Clarithromycin	90	A2147G	13/18	10/13	3/13
		A2146G	3/18	1/3	2/3
		A2146C	2/18	1/2	1/2

N = wild type allele; *a* – mutant allele

Table II. The type of mutations conferring fluoroquinolone resistance found

Antibiotic tested	Number of samples tested	The type of mutation detected	Number of samples detected with mutation	The homozygous type (*aa*)	The heterozygous type (*Na*)
Fluoroquinolone	90	D91N	16/27	10/27	6/27
		D91G	5/27	3/27	2/27
		N87K	5/27	2/27	3/27
		D91G/D91Y	1/27	1	0
		D91N/D91Y	1/27	1	0

N = wild type allele; *a* – mutant allele

There was no significant difference in the rate of clarithromycin resistance between males and females (Table III).

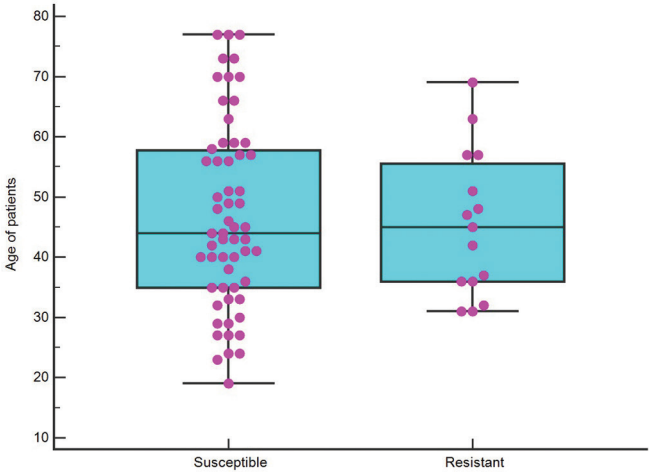

Fig. 1. Age distribution of patients according to *H. pylori* susceptibility to clarithromycin.

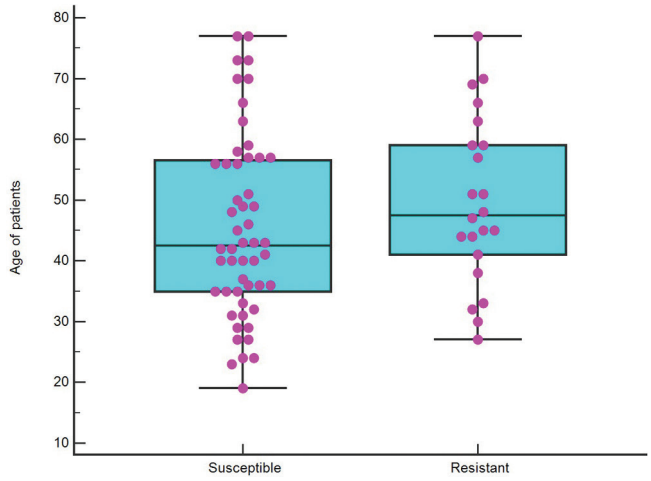

Fig. 2. Age distribution of patients according to *H. pylori* susceptibility to fluoroquinolones.

Although there was a tendency for fluoroquinolone resistance to be more common in females than males (23.3% vs. 6.7%), this difference did not reach statistical significance (p=0.11) (Table IV).

DISCUSSION

This study tested *H. pylori* resistance to two antibiotics (clarithromycin and fluoroquinolones). Clarithromycin belongs to the first line of therapy against *H. pylori* and many recent studies showed a decrease in its efficacy. Fluoroquinolones are part of the second line therapy, together with the therapy based on bismuth salts [5].

In this study, primary genetic resistance to antibiotics was tested by GenoType HelicoDR kit (Hain Lifescience GmbH, Germany). Compared to culture-based method, the previous reported sensitivity, specificity, positive, and negative predictive values of the GenoType HelicoDR for detecting *H. pylori* resistance were, respectively, 100, 86.2, 89.7%, and 100% to clarithromycin as well as 82.6, 95.1, 90.5%, and 90.7% to fluoroquinolones [10]. In another study, the sensitivity and specificity of detecting resistance were 94% and 99% for clarithromycin and 87% and 98.5% for levofloxacin, respectively, and the positive and negative predictive values for detecting resistance were 99% and 94% for clarithromycin and 96% and 96% for fluoroquinolones [11]. Owing to the restricted use of culture, GenoType HelicoDR is a very promising molecular test for diagnosis and detection of clarithromycin and fluoroquinolones resistance in *H. pylori* directly from gastric biopsy specimens.

H. pylori resistance to clarithromycin was associated with the presence of point mutations in domain V of the 23S rRNA gene, namely at nucleotide position 2146 and 2147 (positions according to *H. pylori* reference strain 26695). This domain is the most common binding site for antibiotics that inhibit translation like clarithromycin [12].

Clarithromycin resistance in *H. pylori* is attributable, in most cases, to three single point mutations (A2146C, A2146G and A2147G) in the 23S rRNA gene that can be accurately detected by PCR [13-16].

Table III. Distribution of mutations conferring clarithromycin resistance by gender

Variables	23S rRNA mutations				Wild-type allele	p
	A2146C	A2146G	A2147G	All mutations		
Male (n = 31)	-	1 (1.1%)	5 (5.6%)	6 (6.7%)	25 (27.8%)	0.91
Female (n = 59)	2 (2.2%)	2 (2.2%)	8 (8.9%)	12 (13.3%)	47 (52.2%)	
Total (n = 90)	2 (2.2%)	3 (3.3%)	13 (14.4%)	18 (20%)	72 (80%)	

Table IV. Distribution of mutations conferring fluoroquinolone resistance by gender

Variables	gyrA mutations						Wild-type allele	p
	D91G	D91N	D91G/ D91Y	N87K	N87K/ D91G	All mutations		
Male (n = 31)	1 (1.1%)	4 (4.4%)	-	1 (1.1%)	-	6 (6.7%)	25 (27.8%)	0.11
Female (n = 59)	4 (4.4%)	11 (12.2%)	2 (2.2%)	3 (3.3%)	1 (1.1%)	21 (23.3%)	38 (42.2%)	
Total (n = 90)	5 (5.6%)	15 (16.7%)	2 (2.2%)	4 (4.4%)	1 (1.1%)	27 (30%)	63 (70%)	

The most common mutation related to clarithromycin resistance in our study was A2147G, consistent with the results of other studies. Mutations with A2147G are associated with a high level of clarithromycin resistance and lower cure rates [17]. On the other hand, mutation with A2146G has higher reported minimal inhibitory concentrations but the clinical relevance is minimal [18]. Other mutations that can be found in other parts of the world that have been found to confer clarithromycin resistance in *H. pylori* strains are the T2289C, T2190C, T2182C, A2223G, C2195T, C2245T, C2694A, G2141A and G2224A, A2146C, A2146G, and A2147G [19–22].

Worldwide, the prevalence of primary *H. pylori* clarithromycin resistance varies, but it is generally between 12.5-23.5% in European countries, higher in Africa, where it can reach up to 30% [23-25] and very high in some Asian countries where it can reach values of 85% [26].

The results of our study showed the presence of 23S rRNA gene mutations that confer clarithromycin resistance in 20% of cases and classify us as a region with moderately increased resistance to clarithromycin. These data are consistent with the results reported in other studies on the same topic. In European regions, the lowest primary clarithromycin resistance was reported in Norway (5.9%), whilst the highest in Spain (32.01%) and Portugal (42.35%) [6, 21, 27].

The increased prevalence of clarithromycin resistance in our country may be related to the frequent use of macrolides, especially for the treatment of respiratory infections, as some recent reports suggest, although there are no studies on this topic [28-31].

Fluoroquinolone resistance, on the other hand, is associated with mutations in the quinolone resistance-determining region (QRDR) of the gyrA gene at codons 87 and 91 (positions according to *H. pylori* reference strain 26695); in some cases, they may be associated with mutations in the gyrB gene [32-34]. Mutations that have been found to cause quinolone resistance include N87H, N87I, N87K, N87Y, D91A, D91G, D91N, and D91Y [25, 35, 36]. In agreement with the previous results, the most frequent mutations in our study were D91N, D91G, and N87K.

Worldwide, the primary fluoroquinolone resistance rate varies depending on the geographical region; in Europe, with some exceptions (Italy, Portugal), it is 3.9%, in Africa it is 17.4% and in the USA it is 31.9% [37]. Our study showed a high percentage of *H. pylori* isolates resistant to fluoroquinolones (30%).

As in other studies, high resistance to fluoroquinolones in our population could be related to increased fluoroquinolone use for concurrent infections as well as its usage in the poultry industry leading to the emergence of resistance in *H. pylori*. Infections requiring fluoroquinolone usages, such as infectious diarrhea, urinary tract infections, pulmonary infections and tuberculosis, are common in our region [38, 39]. Fluoroquinolone resistance to *H. pylori* has been associated with second-line treatment failure.

In the present study, there was a tendency for fluoroquinolone resistance to be more common in females than males (23.3% vs. 6.7%), but without reaching statistical significance (p=0.11). A possible explanation for this phenomenon may be the fact that females have been more likely to use fluoroquinolones in their medical history (these antibiotics are commonly used to treat urinary tract infections that are more common in women).

In some studies, individuals with a dual population of *H. pylori* (heteroresistance, that means the coexistence of susceptible and resistant isolates in the same patient for the same antimicrobial agent) were identified [40]. In our study, the cases with heteroresistance were represented by the heterozygous profile of resistance (Tables I and II). However, in these special cases, it is worthwhile to mention that the test used in our study cannot differentiate between specimens with one strain that carries a resistance-mediating mutation only on one of its two chromosomes (true heterozygotes), and one specimen containing more than one *H. pylori* strain (true dual population), one with mutation bands and the other wild type.

Genetic mutations associated with *H. pylori* resistance to rifampicin, tetracycline, and metronidazole [41-43] have also been described, but they were not the subject of our study.

The importance of the results of this study for our region lies in the way of choosing the first intention therapy for the eradication of *H. pylori*. Thus, international guidelines recommend in areas with a high prevalence of clarithromycin resistance of >15%, the bismuth quadruple therapy and concomitant therapy for 14 days according to the Canadian guidelines and 10 days according to the North American and European guidelines [5, 43, 45].

The findings of our study strongly recommend pre-treatment susceptibility testing of clarithromycin as we were beyond the cutoff rates (more than 15%) where clarithromycin susceptibility testing is highly recommended [5].

Our study has several limitations. It was designed as a single center laboratory-based study using endoscopic gastric biopsy samples, on a relatively small number of patients. Then, the determination of point mutations by this method of molecular genetics can address only a limited number of nucleotide positions. Thus, the assessment of antibiotic resistance may be underestimated as there might be other genetic polymorphisms and even other pathophysiological mechanisms that confer antibiotic resistance that have not been studied by this method. Other recent studies have reported that clarithromycin resistance may be associated with other point mutations outside the V domain [22, 46]. Also, mutations of other genes such as rpl22 (encodes a ribosomal protein that interacts with the 23S rRNA domains) and infB (encodes translation initiation factor, IF-2) were associated with clarithromycin resistance [8].

In this study, we have not examined the possible role of active efflux mechanisms. Their involvement in intrinsic antibiotic resistance in *H. pylori* is still a matter of debate and would require transcriptional analyses of the genes encoding drug efflux systems, which was beyond the scope of this study.

Another limitation was the lack of studying the genetic changes associated with resistance to other antibiotics (metronidazole, tetracycline, rifampicin), due to the financial limitations. We chose to study only resistance to clarithromycin and fluoroquinolones because they are antibiotics widely used in our geographical area for various infections, possibly associated with an increased resistance rate.

Another limitation is a lack of a detailed history of antibiotics used by patients in the past, as medical history data are not electronically recorded in our country.

CONCLUSIONS

Clarithromycin resistance is moderately high in our study (20%), and the type of mutation responsible for its resistance in our population is the one with more chances of eradication failure (A2147G). Fluoroquinolone resistance was increased in our study (30%), more frequent in women (37.2%). Only 56.6% of patients presented susceptible *H. pylori* infection on both antibiotics.

There is a need for monitoring *H. pylori* resistance patterns in Romania to provide data that can guide empirical treatment to reduce associated morbidity and mortality for *H. pylori* infections or a strategy to individualize the therapy by previously determining antibiotic susceptibility. This is the first study in Romania published that has studied the sensitivity of *H. pylori* to antibiotics by determining the genetic mutations associated with antibiotic resistance.

Conflicts of interest: None to declare.

Authors' contributions: E.D. and I.M.D. conceived the study, wrote and revised the paper. E.D., L.A., A.C.H., C.T. performed endoscopies and biopsy samples. G.C.C., A.F.M., C.B. and M.A. performed genetic tests. All other authors critically revised the paper and approved the final version.

Acknowledgements: The study was carried out through a collaborative project between a private medical center (SC Gastromond SRL) that provided the research funding and the Faculty of Medicine, Ovidius University of Constanta. Genetic investigations were performed at The Research Center for the Morphological and Genetic Study in Malignant Pathology (CEDMOG).

Supplementary material: To access the supplementary material visit the online version of the *J Gastrointestin Liver Dis* at http://dx.doi.org/10.15403/jgld-758

REFERENCES

1. Atherton JC. The pathogenesis of Helicobacter pylori-induced gastro-duodenal diseases. Annu Rev Pathol. 2006;1:63-96. doi:10.1146/annurev.pathol.1.110304.100125
2. Sporea I, Popescu A, van Blankenstein M, Sirli R, Focşea M, Dănilă M. The prevalence of Helicobacter pylori infection in western Romania. Rom J Gastroenterol 2003;12:15-18.
3. Hooi JKY, Lai WY, Ng WK, et al. Global Prevalence of Helicobacter pylori Infection: Systematic Review and Meta-Analysis. Gastroenterology 2017;153:420-429. doi:10.1053/j.gastro.2017.04.022
4. Makola D, Peura DA, Crowe SE. Helicobacter pylori infection and related gastrointestinal diseases. J Clin Gastroenterol 2007;41:548-558. doi:10.1097/MCG.0b013e318030e3c3
5. Malfertheiner P, Megraud F, O'Morain CA, et al; European Helicobacter and Microbiota Study Group and Consensus panel. Management of Helicobacter pylori infection-the Maastricht V/Florence Consensus Report. Gut 2017;66:6-30. doi:10.1136/gutjnl-2016-312288
6. Savoldi A, Carrara E, Graham DY, Conti M, Tacconelli E. Prevalence of Antibiotic Resistance in Helicobacter pylori: A Systematic Review and Meta-analysis in World Health Organization Regions. Gastroenterology 2018;155:1372-1382.e17. doi:10.1053/j.gastro.2018.07.007
7. Arama SS, Tiliscan C, Negoita C, et al. Efficacy of 7-Day and 14-Day Triple Therapy Regimens for the Eradication of Helicobacter pylori: A Comparative Study in a Cohort of Romanian Patients. Gastroenterol Res Pract 2016;2016:5061640. doi:10.1155/2016/5061640
8. Lauener FN, Imkamp F, Lehours P, et al. Genetic Determinants and Prediction of Antibiotic Resistance Phenotypes in Helicobacter pylori. J Clin Med 2019;8:E53. doi:10.3390/jcm8010053
9. Ford AC, Marwaha A, Sood R, Moayyedi P. Global prevalence of, and risk factors for, uninvestigated dyspepsia: a meta-analysis. Gut 2015;64:1049-1057. doi:10.1136/gutjnl-2014-307843
10. Miendje Deyi VY, Burette A, Bentatou Z, et al. Practical use of GenoType® HelicoDR, a molecular test for Helicobacter pylori detection and susceptibility testing. Diagn Microbiol Infect Dis 2011;70:557-560. doi:10.1016/j.diagmicrobio.2011.05.002
11. Cambau E, Allerheiligen V, Coulon C, et al. Evaluation of a new test, genotype HelicoDR, for molecular detection of antibiotic resistance in Helicobacter pylori. J Clin Microbiol 2009;47:3600-3607. doi:10.1128/JCM.00744-09
12. Versalovic J, Shortridge D, Kibler K, et al. Mutations in 23S rRNA are associated with clarithromycin resistance in Helicobacter pylori. Antimicrob Agents Chemother 1996;40:477-480. doi:10.1128/AAC.40.2.477
13. Owen RJ. Molecular testing for antibiotic resistance in Helicobacter pylori. Gut 2002;50:285-289. doi:10.1136/gut.50.3.285
14. Redondo JJ, Keller PM, Zbinden R, Wagner K. A novel RT-PCR for the detection of Helicobacter pylori and identification of clarithromycin resistance mediated by mutations in the 23S rRNA gene. Diagn Microbiol Infect Dis 2018;90:1-6. doi:10.1016/j.diagmicrobio.2017.09.014
15. Schabereiter-Gurtner C, Hirschl AM, Dragosics B, et al. Novel real-time PCR assay for detection of Helicobacter pylori infection and simultaneous clarithromycin susceptibility testing of stool and biopsy specimens. J Clin Microbiol 2004;42:4512-4518. doi:10.1128/JCM.42.10.4512-4518.2004
16. Beckman E, Saracino I, Fiorini G, et al. A Novel Stool PCR Test for Helicobacter pylori May Predict Clarithromycin Resistance and Eradication of Infection at a High Rate. J Clin Microbiol 2017;55:2400-2405. doi:10.1128/JCM.00506-17
17. Francavilla R, Lionetti E, Castellaneta S, et al. Clarithromycin-resistant genotypes and eradication of Helicobacter pylori. J Pediatr 2010;157:228-232. doi:10.1016/j.jpeds.2010.02.007
18. van Doorn LJ, Glupczynski Y, Kusters JG, et al. Accurate prediction of macrolide resistance in Helicobacter pylori by a PCR line probe assay for detection of mutations in the 23S rRNA gene: multicenter validation study. Antimicrob Agents Chemother 2001;45:1500-1504. doi:10.1128/AAC.45.5.1500-1504.2001
19. Rajper S, Khan E, Ahmad Z, Alam SM, Akbar A, Hasan R. Macrolide and fluoroquinolone resistance in Helicobacter pylori isolates: an experience at a tertiary care centre in Pakistan. J Pak Med Assoc 2012;62:1140-1144.
20. Kim JM, Kim JS, Kim N, et al. Gene mutations of 23S rRNA associated with clarithromycin resistance in Helicobacter pylori strains isolated from Korean patients. J Microbiol Biotechnol 2008;18:1584-1589.
21. Agudo S, Pérez-Pérez G, Alarcón T, López-Brea M. High prevalence of clarithromycin-resistant Helicobacter pylori strains and risk factors associated with resistance in Madrid, Spain. J Clin Microbiol 2010;48:3703-3707. doi:10.1128/JCM.00144-10
22. Hao Q, Li Y, Zhang ZJ, Liu Y, Gao H. New mutation points in 23S rRNA gene associated with Helicobacter pylori resistance to clarithromycin in northeast China. World J Gastroenterol 2004;10:1075-1077. doi:10.3748/wjg.v10.i7.1075

23. Jaka H, Rüttgerodt N, Bohne W, et al. Helicobacter pylori Mutations Conferring Resistance to Fluoroquinolones and Clarithromycin among Dyspeptic Patients Attending a Tertiary Hospital, Tanzania. Can J Gastroenterol Hepatol 2019;2019:8481375. doi:10.1155/2019/8481375
24. Giorgio F, Principi M, De Francesco V, et al. Primary clarithromycin resistance to Helicobacter pylori: Is this the main reason for triple therapy failure? World J Gastrointest Pathophysiol 2013;4:43-46. doi:10.4291/wjgp.v4.i3.43
25. Jaka H, Rhee JA, **Östlundh** L, et al. The magnitude of antibiotic resistance to Helicobacter pylori in Africa and identified mutations which confer resistance to antibiotics: systematic review and meta-analysis. BMC Infect Dis 2018;18:193. doi:10.1186/s12879-018-3099-4
26. Quek C, Pham ST, Tran KT, et al. Antimicrobial susceptibility and clarithromycin resistance patterns of Helicobacter pylori clinical isolates in Vietnam. F1000Res 2016;5:671. doi:10.12688/f1000research.8239.1
27. Ghotaslou R, Leylabadlo HE, Asl YM. Prevalence of antibiotic resistance in Helicobacter pylori: A recent literature review. World J Methodol 2015;5:164-174. doi:10.5662/wjm.v5.i3.164
28. Special Eurobarometer 478: antimicrobial Resistance (in the EU), 2018-11–13, Version v1.00. Available from: https://www.gesis.org/eurobarometer
29. Surveillance of antimicrobial resistance in Europe. Annual report of the European Antimicrobial Resistance Surveillance Network (EARS-Net) 2016. Available from: https://ecdc.europa.eu/sites/portal/files/documents/AMR-surveillance-Europe-2016.pdf
30. Voidăzan S, Moldovan G, Voidăzan L, Zazgyva A, Moldovan H. Knowledge, Attitudes And Practices Regarding The Use Of Antibiotics. Study On The General Population Of Mureş County, Romania. Infect Drug Resist 2019;12:3385-3396. doi:10.2147/IDR.S214574
31. Topor G, Grosu IA, Ghiciuc CM, Strat AL, Lupuşoru CE. Awareness about antibiotic resistance in a self-medication user group from Eastern Romania: a pilot study. Peer J 2017;5:e3803. doi:10.7717/peerj.3803
32. Bogaerts P, Berhin C, Nizet H, Glupczynski Y. Prevalence and mechanisms of resistance to fluoroquinolones in Helicobacter pylori strains from patients living in Belgium. Helicobacter 2006;11:441-445. doi:10.1111/j.1523-5378.2006.00436.x
33. Cattoir V, Nectoux J, Lascols C, et al. Update on fluoroquinolone resistance in Helicobacter pylori: new mutations leading to resistance and first description of a gyrA polymorphism associated with hypersusceptibility. Int J Antimicrob Agents 2007;29:389-396. doi:10.1016/j.ijantimicag.2006.11.007
34. Wang LH, Cheng H, Hu FL, Li J. Distribution of gyrA mutations in fluoroquinolone-resistant Helicobacter pylori strains. World J Gastroenterol 2010;16:2272-2277. doi:10.3748/wjg.v16.i18.2272
35. Hung KH, Sheu BS, Chang WL, Wu HM, Liu CC, Wu JJ. Prevalence of primary fluoroquinolone resistance among clinical isolates of Helicobacter pylori at a University Hospital in Southern Taiwan. Helicobacter 2009;14:61-65. doi:10.1111/j.1523-5378.2009.00655.x
36. Wang G, Wilson TJ, Jiang Q, Taylor DE. Spontaneous mutations that confer antibiotic resistance in Helicobacter pylori. Antimicrob Agents Chemother 2001;45:727-733. doi:10.1128/AAC.45.3.727-733.2001
37. Thung I, Aramin H, Vavinskaya V, et al. Review article: the global emergence of Helicobacter pylori antibiotic resistance. Aliment Pharmacol Ther 2016;43:514-533. doi:10.1111/apt.13497
38. Umegaki N, Shimoyama T, Nishiya D, Suto T, Fukuda S, Munakata A. Clarithromycin-resistance and point mutations in the 23S rRNA gene in Helicobacter pylori isolates from Japan. J Gastroenterol Hepatol 2000;15:906-909. doi:10.1046/j.1440-1746.2000.02072.x
39. Dzierzanowska-Fangrat K, Rozynek E, Jozwiak P, Celinska-Cedro D, Madalinski K, Dzierzanowska D. Primary resistance to clarithromycin in clinical strains of Helicobacter pylori isolated from children in Poland. Int J Antimicrob Agents 2001;18:387-390. doi:10.1016/s0924-8579(01)00421-6
40. Mascellino MT, Porowska B, De Angelis M, Oliva A. Antibiotic susceptibility, heteroresistance, and updated treatment strategies in Helicobacter pylori infection. Drug Des Devel Ther 2017;11:2209-2220. doi:10.2147/DDDT.S136240
41. Hays C, Burucoa C, Lehours P, Tran CT, Leleu A, Raymond J. Molecular characterization of Helicobacter pylori resistance to rifamycins. Helicobacter 2018;23:e12451. doi:10.1111/hel.12451
42. Lee SM, Kim N, Kwon YH, et al. rdxA, frxA, and efflux pump in metronidazole-resistant Helicobacter pylori: Their relation to clinical outcomes. J Gastroenterol Hepatol 2018;33:681-688. doi:10.1111/jgh.13906
43. Gerrits MM, Berning M, Van Vliet AH, Kuipers EJ, Kusters JG. Effects of 16S rRNA gene mutations on tetracycline resistance in Helicobacter pylori. Antimicrob Agents Chemother 2003;47:2984-2986. doi:10.1128/aac.47.9.2984-2986.2003
44. Fallone CA, Chiba N, van Zanten SV, et al. The Toronto Consensus for the Treatment of Helicobacter pylori Infection in Adults. Gastroenterology 2016;151:51-69.e14. doi:10.1053/j.gastro.2016.04.006
45. Chey WD, Leontiadis GI, Howden CW, Moss SF. Correction: ACG Clinical Guideline: Treatment of Helicobacter pylori Infection. Am J Gastroenterol 2018;113:1102. doi:10.1038/s41395-018-0132-6
46. Garrido L, Toledo H. Novel genotypes in Helicobacter pylori involving domain V of the 23S rRNA gene. Helicobacter 2007;12:505-509. doi:10.1111/j.1523-5378.2007.00506.x

Supplementary Figure. Examples of mutations that correlate with clarithromycin and fluoroquinolone resistance of *H. pylori* in genotyped cases.

CC: conjugate control; AC: amplification control; HP: *Helicobacter pylori*; WT: wild type; MUT: mutant.