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Screening for *Helicobacter pylori* infection and Clarithromycin resistance using Real-Time Polymerase Chain Reaction

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Abstract. – OBJECTIVE: *Helicobacter pylori* (*H. pylori*) infection is spread worldwide and affects at least half of the world's population. Infected people are at increased risk of several diseases' development, including gastric adenocarcinoma. The aim of this study was to screen patients with dyspeptic symptoms for *H. pylori* infection and assess Clarithromycin resistance prevalence among the infected patients.

PATIENTS AND METHODS: Screening for *H. pylori* infection was performed in all patients using molecular test based on Real-Time Polymerase Chain Reaction (RT-PCR) in feces after RNA-DNA extraction. Stool samples from all participants were collected 1 to 3 days after patients' hospitalization. The positive results were furthermore assessed for confirmation by breath test and stool antigen test. By point mutations detection in 23S rRNA gene was possible to detect Clarithromycin resistance. Statistical analysis was performed via SPSS 22.0 (IBM Corp., Armonk, NY, USA).

RESULTS: This study enrolled 50 patients (18 males) at mean age 46.46±15.10 years. Using molecular test based on RT-PCR in feces we identified *H. pylori* infection in 24 patients (48.00%). Clarithromycin resistance was observed in 7 of them (29.17%). None of those patients was eradicated before. There was no significant difference by age and gender between infected and non-infected patients. Gastrointestinal symptoms were more often reported in infected patients ($p<0.05$). The molecular test showed 85.71% sensitivity and 100% specificity, with a diagnostic accuracy of 92.00%.

CONCLUSIONS: *H. pylori* screening by molecular test based on RT-PCR in feces might be beneficial as the test's accuracy is high and include Clarithromycin resistance assessment, which could improve the outcome of eradication therapy.

Key Words:
Helicobacter pylori, Screening, Clarithromycin resistance, Real-time PCR.

Introduction

Helicobacter pylori (H. pylori) is a gram-negative, spiral, microaerophilic bacterium form the family *Helicobacteriaceae* of the order *Campylobacterales*[1]. Currently, at least half of the world's population is infected with *H. pylori*[2]. The bacterium is mainly isolated from biopsy histological material from the stomach, but it can also be detected in saliva, gastric emesis and feces[3]. *H. pylori* causes a number of diseases and in 1994 the International Agency for Research on Cancers by the World Health Organization (WHO) classified *H. pylori* as Group 1: Carcinogenic to humans[4,5]. Gastric adenocarcinoma is the fifth most frequent and third malignant neoplasm. Nearly 100,000 new cases are registered worldwide each year. *H. pylori* causes distal gastric adenocarcinoma in about 89% of cases[6,7]. For proper diagnosis of the infection, invasive and non-invasive tests are performed[8]. It has been demonstrated the high sensitivity and specificity of a non-invasive molecular test based on real-time polymerase chain reaction (PCR) in feces. In respect to molecular tests on stool samples, the bacterial 23S ribosomal RNA subunit gene is defined as the most accurate diagnostic marker for *H. pylori* infection with 82% (95% CI: 77%-86%) sensitivity and 99% (95%CI: 98%-100%) specificity according to a recent meta-analysis[7]. Significant advantage of this method is the possibility to identify bacterial DNA mutations associated with antibiotic resistance.

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Guidelines recommend antibiotics and adequate PPI dose for the treatment of the infection[9]. The last two decades are marked with the increased resistance rates of *H. pylori* to macrolides, such as Clarithromycin. Early diagnosis of *H. pylori* and Clarithromycin resistance genes is mandatory for proper antibiotic therapy and resistance epidemiology follow-up[10].

Patients and Methods

Study Design and Assessment

This study enrolled 50 patients aged 18 years or older, who were admitted at the Gastroenterology Department due to dyspeptic symptoms, defined as the presence of at least one of the following: early satiation, post-prandial fullness, epigastric pain/burning. Symptom's duration was defined as for at least one day (early satiation and post-prandial fullness) or three days (epigastric pain/burning) per week during the last three months with symptoms onset at least six months previously[11]. Exclusion criteria were presence of diarrhea, any pathological findings by upper endoscopy (polyps, ulcers, malignancies, bleeding), as well as treatment with proton pump inhibitors and/or antibiotics or bismuth salts in the previous two and four weeks respectively, as these medications may reduce the bacterial load and lead to false negative results of current diagnostic tests for *H. pylori* infection[12]. Screening for *H. pylori* infection was performed in all patients by molecular test based on real-time PCR in feces including Clarithromycin resistance evaluation using VIASURE *H. pylori* real-time PCR Detection Kit (CerTest Biotec S.L. Zaragoza, Spain). Stool samples from all participants were collected 1 to 3 days after patients' hospitalization. Following the manufacturer's instructions, RNA-DNA extraction was first conducted according to the following workflow: transfer of 200 mcl of sample into 2.0 ml collection tube and stool sample lysis, binding of DNA and RNA using binding buffer; incubation at room temperature and centrifuging; washing with wash buffer I and II, centrifuging; ethanol removal and elution of the DNA/RNA. The DNA/RNA sample was stored at -20°C. After DNA isolation, the identification of *H. pylori* and Clarithromycin resistance was performed by the amplification of a conserved region of the ureB and 23S rRNA genes respectively, using specific primers and a fluorescent-labeled probe. The method is based on the 5' exonuclease activity of DNA polymerase, which cleaves the probe bounded to the complementary DNA sequence, separating the quencher dye from the reporter. This reaction generates an increase in the fluorescent signal, which was measured on real-time PCR platforms. The software of the used real-time PCR equipment performed the analysis of the samples according to manufacturer's instructions. By detection of point mutations in the 23S rRNA gene (A2142G and A2143G) was possible to detect Clarithromycin resistance. The positive results were furthermore assessed for confirmation by standard non-invasive test stool antigen test, which is highly sensitive (94%) and specific (97%) for *H. pylori* detection[13]. We used a colored chromatographic immunoassay for the qualitative detection of *Helicobacter pylori* in fresh stool samples (CerTest Biotec S.L. Zaragoza, Spain), following the manufacturer's instructions.

Ethics Approval

The study was approved by our Ethics Committee. All patients participated voluntarily in the study after the study protocol had been explained properly by the investigator and signed consent form.

Statistical Analysis

Quantitative data of the statistical analysis were presented as mean, standard deviations (SD), range or percentages. Quantitative data were analyzed by Student's *t*-test and Mann-Whitney U-test. Measurement data were compared using ANOVA after confirming normal distribution by the Kolmogorov-Smirnov test. Statistical significance was assumed at p-value of <0.05. Analyzes were performed using the SPSS 22.0 statistical package (IBM Corp., Armonk, NY, USA).

Results

Study enrolled 50 patients at mean age 46.46±15.10 years. 18 of them were men. 4 patients (12.90%) were treated for *H. pylori* infection in the last 6 years. Using molecular test based on real-time PCR in feces we identified *H. pylori* infection in 24 patients (48.00%). Furthermore, Clarithromycin resistance was observed in 7 of them (29.17%). None of those patients was eradicated before. Demographic and clinical data according to the *H. pylori* presence are presented at Table I.

Table I. Demographic and clinical data according to the *H. pylori* presence.

	H. pylori (+)	*H. pylori* (-)	*p*-value
n (%)	24 (48.00)	26 (52.00)	
Age: mean ± SD	47.74 ± 15.54	48.18 ± 12.97	NS
Females: n, (%†)	18 (75.00)	14 (53.85)	NS
Early satiation: n, (%)	13 (54.17)	16 (61.54)	NS
Post-prandial fullness: n, (%)	17 (70.83)	11 (42.31)	0.038
Epigastric pain: n, (%)	21 (87.50)	13 (50.00)	0.045
Epigastric burning: n, (%)	16 (66.67)	12 (46.15)	NS
1-2 symptoms: n (%)	5 (20.83)	17 (65.38)	0.04
3-4 symptoms: n (%)	19 (79.17)	9 (34.62)	0.037
Clarithromycin resistance: n (%)	7 (29.17)	-	

n: number; SD: standart deviation; *H. pylori*: *Helicobacter pylori*; †Percent of the subgroup; NS: not significant.

Females were the dominated gender in *H. pylori* positive group. However, there was no significant difference by age and gender between both groups (*H. pylori* positive and negative). According to the reported symptoms we observed significant more frequent post-prandial fullness and epigastric pain in infected patients. Furthermore, patients with *H. pylori* infection experienced more often 3 or 4 symptoms (p=0.037) compared to those without infection, who reported more often 1 or 2 symptoms (p=0.04).

To evaluate the accuracy of the molecular real-time PCR test we performed stool antigen test in our patients. The results of both methods are presented at Table II.

Furthermore, we evaluated the sensitivity and specificity of the real-time PCR detection of *H. pylori* (Table III).

Discussion

H. pylori is the most common chronic bacterial infection[2]. *H. pylori* was discovered in 1983 by Barry Marshall and Robin Warren during the evaluation of gastric mucosal tissue from patients with gastritis and peptic ulcers[1]. The highest risk of *H. pylori* transmission is during childhood and adolescence via the fecal-oral and oral-oral routes[3]. The bacterium causes several diseases, such as chronic atrophic gastritis, peptic ulcer, functional dyspepsia and is associated with a number of malignancies (gastric adenocarcinoma, MALT lymphoma, diffuse large B cell lymphoma), autoimmune diseases and extraintestinal manifestations (iron deficiency anemia and idiopathic thrombocytopenic purpura), which radically change the understanding and approach to

Table II. *H. pylori* presence or absence determinenation by the stool antigen test and real-rime PCR.

	H. pylori (+)†	n	*H. pylori* (-)†	n
Real-time PCR (+)	True positive	24	False positive	0
Real-time PCR (-)	False negative	4	True negative	22

†: *H. pylori p*resence or absence determination by the stool antigen test; *H. pylori*: *Helicobacter pylori*; "+": positive test; "-": negative test; n: number; PCR: Polymerase Chain Reaction.

Table III. Sensitivity and specificity of the real-time PCR detection of *H. pylori*.

Real-time PCR detection	Value	95% CI
Sensitivity	85.71%	67.33% to 95.97%
Specificity	100.00%	84.56% to 100.00%
Positive Predictive Value	100.00%	
Negative Predictive Value	84.62%	68.94% to 93.16%
Accuracy	92.00%	80.77% to 97.78%

PCR: Polymerase Chain Reaction.

these diseases[4,13]. For proper diagnosis of the infection, invasive and non-invasive tests are available, but none of them can be considered as gold standard alone[8]. Invasive methods include histology, culture and rapid urease testing, which require gastric biopsy specimens obtained by gastroduodenoscopy. Non-invasive approaches include urea breath testing, stool antigen test and serologic testing[14]. Recent studies have demonstrated the high sensitivity and specificity of a new non-invasive molecular test based on real-time PCR in feces[11]. 23S rRNA subunit gene has been shown to be the most accurate marker for detecting H. pylori in feces using molecular analyses based on real-time PCR[11]. In our study, we evaluated 50 patients using the real-time PCR detection of *H. pylori* in feces. By the screening, we observed *H. pylori* infection in 48.00% of all patients. The infection did not depend on age or gender. However, we found significant association between the reported post-prandial fullness and epigastric pain as well as the greater number of symptoms and the presence of *H. pylori* infection, which advert the importance of several symptoms by *H. pylori* screening inducement. By assessing the accuracy of the non-invasive molecular test, we observed high performance for the diagnosis of H. pylori infection among patients with dyspeptic symptoms. Our results are promising as the real-time PCR test showed 85.71% sensitivity and 100% specificity, with a diagnostic accuracy of 92.00%, 100% positive predictive value and 84.62% negative predictive value, which are comparable to the previous reported studies[11,15].

Therapeutic management of H. pylori is still problematic as many patients remain infected despite several standard drug regimens[4,9,16,17]. Clarithromycin is a bacteriostatic antibiotic that belongs to the macrolide family. The main role of Clarithromycin in *H. pylori* therapy is to prevent protein translation. Clarithromycin resistance due to point mutations in the 23SrRNA component of ribosomes is continuously increasing and is supposed to be the leading cause of eradication regimen failures. However, two major mutations A2142G and A2143G are listed as main cause of antibiotic resistance in clinical isolates[18,19]. A recent review[20] shows unacceptably low treatment success for first-line empirical treatment containing Clarithromycin, with only 18% exceeding 85% and approximately 60% failing to reach 80% eradication rates. About 19% of the European population is resistant to Clarithromycin[16]. Resistance is higher in Central/Eastern Europe [9.3% (95%CI: 0-22)] and is at its highest in Southern Europe [18% (95%CI: 2.1-34.8)]. In Eastern Europe, the situation is similar to that in Southern Europe. Several Bulgarian studies have reported a resistance rate of 18.4%-23.4%[18-21]. The estimated Clarithromycin resistance in our study was 29.17%, which is rather high compared to previous studies[21,22]. However, these results might be due to the more accurate method for detection of Clarithromycin resistance that we used in our study, namely real-time PCR.

Conclusions

H. pylori resistance doubled in Europe during the last two decades. Today culture and antimicrobial susceptibility testing are not routinely performed in the clinical practice, therefore studies on the resistance status of *H. pylori* and needed in order to improve the treatment strategies. The main advantage of the novel molecular test based on real-time PCR is the possibility to both accurately diagnose *H. pylori* infection in a non-invasive way and to detect Clarithromycin resistance, which benefits patients' compliance and early and proper treatment, leading to successful *H. pylori* eradication and reduced risk of related complication.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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