Folia Microbiologica (2019) 64:143–151
https://doi.org/10.1007/s12223-018-0637-9

ORIGINAL ARTICLE

Characterization of clarithromycin heteroresistance among *Helicobacter pylori* strains isolated from the antrum and corpus of the stomach

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Received: 20 February 2018 / Accepted: 6 August 2018 / Published online: 10 August 2018

Abstract

Mixed infections and heteroresistance of *Helicobacter pylori* contribute to decreased efficacy of treatments. This study aimed to investigate frequency of clarithromycin heteroresistance and its link with mixed infections, medication history, and disease severity. A total of 40 pairs of *H. pylori* strains were isolated from the antrum and corpus of 97 patients. Susceptibility of the strains to clarithromycin was measured by agar dilution method. Site-specific mutations of *23S rRNA* at A2143G, A2142G, and A2142C positions were analyzed by PCR and genomic relatedness of pairs of the strains was determined by random amplified polymorphic DNA (RAPD)-PCR. The results showed a prevalence of 35% (14/40) clarithromycin resistance. Diversity of the antrum and corpus isolates in resistance to clarithromycin was detected among 17.5% (7/40) of the patients. Similarly, diversity in MIC value was also detected in two patients infected with the sensitive strains. Significant difference in frequency of resistance was detected among patients with peptic ulcer disease (PUD) (MIC90 32 μg/mL) and severe gastritis (MIC90 16 μg/mL), compared with those who suffered from non-ulcer dyspepsia (NUD) (MIC90 8 μg/mL) and chronic gastritis (MIC90 0.25 μg/mL). MIC values showed 8–32 folds increased levels in the corpus. A2142G, A2143G, and A2142C mutations were detected in three, two, and two patients, respectively, but not observed in 46% of the resistant strains. RAPD-PCR fingerprints showed identical molecular patterns for the isolates of the corpus and antrum in each patient. In conclusion, microevolution of *H. pylori* strains during chronic infection, rather than mixed infection, and inappropriate medication appear to be main reasons of treatment failure in adults.

Introduction

Helicobacter pylori (*H. pylori*) is a motile, curved, and Gram-negative bacterium that colonizes the human stomach. *H. pylori* is the major cause of gastric disorders ranging from chronic gastritis to gastric adenocarcinoma and its frequency accounts for nearly 50% in the world and as high as 80–90% in developing countries (Peek and Blaser 2002; Sgouras et al. 2015). Chronic infection with this bacterium promotes the multistep carcinogenic process of gastric cancer, which evolves through chronic gastritis, acute gastritis, gastric atrophy, intestinal metaplasia, dysplasia, and finally gastric adenocarcinoma (Kao et al. 2016; Shimizu et al. 2015; Wroblewski et al. 2014). Although eradication of *H. pylori* infection could not reverse histological changes of intestinal metaplasia and dysplasia, its significant effect on improvement of gastritis and gastric atrophy was shown by several studies (Kang et al. 2012; Kong et al. 2014). This improvement is not site specific and could occur in both the antrum and corpus (Norazah et al. 2009; Selgrad et al. 2014).

Failure of eradication therapy regimens is a therapeutic challenge in areas with high prevalence of antibiotic resistance rates. This failure could be explained by *H. pylori* genomic evolution in the stomach during chronic infection or colonization of this tissue with resistant strains (Alebouyeh et al. 2015;

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Shokrzadeh et al. 2015). Emergence of heteroresistance in *H. pylori* population was previously described (Farzi et al. 2015). Main causes of this emergence included consecutive evolution in *H. pylori* genome, high mutation rate and inefficient DNA mismatch repair systems (Huang et al. 2011; Teh et al. 2014), and high rate of recombination events among different strains within a host (Dorer et al. 2011). The patterns of *H. pylori* colonization in different gastric sites and the occurrence of associated disorders depend mainly on the level of acid output. In patients who have normal gastric acid production, bacterial colonization is predominantly confined to the antrum; however, involvement of gastric body and occurrence of corpus-predominant pangastritis could be observed in patients with decreased gastric acid output (Huang et al. 2017; Waldum et al. 2016). The colonized strains in the corpus generally evolved from the same lineage of strains that initially colonized the antrum. At these conditions, failures of eradication therapies can cause co-infection with genetically related or unrelated *H. pylori* strains displaying different antimicrobial susceptibilities in different parts of the stomach (Ayala et al. 2011).

Although efficacy of medication somewhat depends on host factors, selection of appropriate antimicrobial regimen should be based on knowledge about resistance rates of *H. pylori* strains and the presence of heteroresistance, especially in regions with a high infection rate. In developing countries, such as Iran, the prevalence of clarithromycin resistance rate altered during last years (Khademi et al. 2015; Shokrzadeh et al. 2015). Similarly, global clarithromycin resistance rates have increased from 9% in 1998 to 17.6% in 2008 in Europe and from 7% in 2000 to 27.7% in 2006 in Japan (Hu et al. 2017; Nishizawa and Suzuki 2014). This resistance rate was increased as high as > 40% in some countries, such as Turkey and China (Thung et al. 2016).

Failure of conventional regimens against *H. pylori* depends mainly on dominance of clarithromycin-resistant strains in each population. However, new cases of patients who have failed eradication regimens are emerging in areas with appropriate treatment history. Microevolution seems to be the main cause for this emergence. Resistance to clarithromycin is generally caused by point mutations in 23s rRNA gene, mainly through mutations at A2143G, followed by A2142G and A2142C positions (Boyanova 2017). These mutations can arise in the stomach of each patient during the chronic infection. In current study, to understand the microevolution of clarithromycin-resistant strains of *H. pylori* in a single host, we investigated development of these variants among *H. pylori* strains isolated from the antrum and corpus of the gastric tissue in each patient. Furthermore, differences of molecular fingerprints, minimal inhibitory concentrations (MIC) of the isolates, and their association with noted mutations were analyzed.

Patients and methods

Patients and data collection

The received biopsy samples were from patients that underwent endoscopy at Taleghani Hospital in Tehran, Iran, during the period from May 2014 to February 2015. All the patients with recent history of medication were excluded from the study. Demographic data and endoscopic findings of the patients were recorded in a standard questionnaire. This study was approved by the Ethics Committee of Shahid Beheshti University of Medical Sciences.

Microbiological and histopathological analyses

Three separate gastric biopsy specimens were taken from the antrum and from the corpus of the stomach. One of the biopsy samples from each site was sent to the pathology laboratory for histological analysis, and the other one was placed into a transport medium (thioglycolate agar plus yeast extract, 3%, Merck Co., Darmstadt, Germany, and Oxoid, Hampshire, UK, respectively) and sent to the microbiology laboratory for culture in a specific culture medium after homogenization. Growth of *H. pylori* colonies was screened on *Brucella* agar medium supplemented with 10% (*v*/*v*) fetal calf serum, 7% horse blood, and selective antimicrobials (vancomycin 10 mg/L; polymyxin B 0.25 mg/L; trimethoprim 5 mg/L; and amphotericin B 3 mg/L (Merck, Germany). The homogenized biopsies in BHI broth were inoculated and the plates were incubated at 37 °C for 3 to 5 days at microaerobic conditions (5% O_2, 10% CO_2, and 85% N_2). The suspected grown colonies were evaluated by rapid urease test, Gram staining, and biochemical tests, including oxidase and catalase reactions. Pure subcultures of *H. pylori* strains were prepared and stocked in − 70 °C for molecular investigation and antimicrobial susceptibility testing. *H. pylori* strain RIGLD 742 was used as a positive control strain in all the reactions.

Molecular characterization and mutation analysis

Genomic DNAs of the *H. pylori* strains were extracted by QIAamp DNA extraction kit according to the manufacturer's instructions (Qiagen, Hilden, Germany). Polymerase chain reaction (PCR) was done for molecular characterization of the *Helicobacter* strains at species level (*glmM* and *16S rRNA*) as described before (Farzi et al. 2015). To detect site-specific mutations of *23S rRNA* at A2143G, A2142G, and A2142C positions, a volume of 25-μL reaction mixture containing 1× PCR buffer, 0.3 μM of each primer, 1 μL of genomic DNA, 200 μM of dNTPs mix, 0.63 mmol of $MgCl_2$, and 0.2 U/μL of Taq DNA polymerase was used. PCRs were done separately in an automated thermal cycler (AG 22331; Eppendorf, Hamburg, Germany) under the following

conditions: 1 cycle of denaturation for 5 min at 94 °C, annealing for 5 min at 36 °C, extension for 5 min at 72 °C, followed by 30 cycles of denaturation for 1 min at 94 °C, annealing for 1 min at 36 °C, and extension for 2 min at 72 °C. Oligonucleotide sequences of the primers used in this study and size of the PCR products are shown in Table 1. DNA extracts of three reference strains encoding A2142G, A2143G, and A2142C (GenBank accession numbers JQ765438, JQ765441, and JQ765440) were used as control strains in this experiment.

Random amplified polymorphic DNA-PCR analysis

Random amplified polymorphic DNA (RAPD)-PCR typing was done for detection of related and distinct strains of *H. pylori* that dominantly colonized the antrum and corpus of the stomach in each patient. Accordingly, the more discriminative primer 1283 was used (Table 1). A volume of 25 μL containing 1× PCR buffer, 1 μmol/L of primer, 1 μL of genomic DNA (approximately 150 ng), 200 μmol/L of dNTPs mix, 2 mmol of $MgCl_2$, and 0.05 U/μL of Taq DNA polymerase was used for each reaction. RAPD-PCR amplification was performed in an automated thermal cycler (AG 22331; Eppendorf, Hamburg, Germany) as described before (Farzi et al. 2015). PCR products were electrophoresed in 1.8% agarose gel. Similarity of all RAPD banding profiles was analyzed by GelCompar II Software (TX, USA). Lack of polymorphisms or existence of ≤ 2 and > 2 different RAPD-PCR bands was considered as a definitive criterion for detection of identical or related and different strains, respectively.

Determination of MICs for clarithromycin

MIC of clarithromycin was examined by the agar dilution method based on the European Committee on Antimicrobial Susceptibility Testing (EUCAST). Different amounts of clarithromycin, at final concentration of 0.016 to 32 μg/mL (Sigma, St. Louis, MO), were added to Mueller-Hinton agar medium (Merck, Germany) containing 10% defibrinated horse blood. Freshly prepared *H. pylori* suspensions were prepared with turbidity equivalent to three McFarland standards, and then 10 μL of each suspension was inoculated on the prepared plates. The MIC values were determined after 72 h of incubation at 37 °C under microaerobic conditions. Epidemiological cut-off value > 0.25 μg/mL was used for detection of the resistant strains. Quality control was performed by using reference clarithromycin-resistant and clarithromycin-susceptible strains (RIGLD OC359 and RIGLD OC248, respectively) in each experiment.

Statistical analysis

SPSS for Windows (version 16.0; SPSS, Chicago, IL, USA) was used for the statistical analysis; baseline demographic data of the infected patients were analyzed and association of MIC values and site-specific mutations were compared by chi-square test. All the findings were considered statistically significant when the *p* values were less than 0.05.

Results

Prevalence of *H. pylori* infection

Out of 97 patients subjected to endoscopy, infection with *H. pylori* was detected among 42 (43.3%) patients. A total of 40 isolates from the antrum and 40 isolates from the corpus of the same patients were included in the study, and two patients were excluded, because of the lack of *H. pylori* colonization in one of the two stomach parts (the antrum or corpus). *H. pylori*-positive patients consisted of 20 males and 20 females, with their ages ranging between 10 and 70 years (mean age, 40–50 years). Identity of the isolates was confirmed as *H. pylori* by positive PCR results for *glmM* and *16S rRNA* genes.

Table 1 Oligonucleotide sequences of the primers used in this study and size of the PCR products

Primers	5′ → 3′	Size (bp)	Annealing Tm (°C)	Reference
Dp1:wild type Dp4:A2142G	F: ACGGCGGCCGTAACTATA R: AGGTCCACGGGGTCTTC	175	60.4 (56)	Pan ZJ 2002
Dp5:A2143G	R: AAGGTCCACGGGGTCTC		57.3 (56)	
DP6:A2142C	R: AGTAAAGGTCCACGGGGTCTTG		(56.6)	
16S rRNA	F:GGCTATGACGGGTATCCGGC R:GCCGTGCAGCACCTGTTTTC	764		Bohr UR 2002
glmM	F:GGATAAGCTTTTAGGGGTGTTAGGGG R:GCTTACTTTCTAACACTAACGCGC	296		Kauser F 2005
1283	GCGATCCCCA	–		Finger SA 2006

The infected patients suffered from severe gastritis (6/40, 15%), chronic gastritis (7/40, 17.5%), peptic ulcer disease (5/40, 12.5%), erythema (20/40, 50%), and erosion (1/40, 2.5%) (Table 2).

Diversity of clarithromycin resistance phenotype in the antrum and corpus

Resistance to clarithromycin was detected among 35% (14/40) of the patients. Diversity in resistance to clarithromycin between paired of the isolates from the antrum and corpus of a single patient was characterized among 17.5% of the patients (7/40). Similarly, diversity in MIC values was also detected in two patients infected with clarithromycin-sensitive strains (Table 3). A correlation was found between higher age groups and the resistance rate. A significant difference in frequency of resistance was detected among patients with PUD and severe gastritis compared with patients who suffered from NUD and chronic gastritis, respectively (Table 2). The resistant strains were mostly isolated from infected patients with recent history of clarithromycin-based therapeutic regimen.

Diversity of the minimum inhibitory concentration

Different MIC values were determined, as highest ones were characterized among patients with PUD (MIC90 32 μg/mL) and severe active gastritis (MIC90 16 μg/mL) compared with those who suffered from NUD (MIC90 8 μg/mL) and chronic gastritis (MIC90 0.25 μg/mL), respectively (Table 2). Diversity of MIC values for the isolates from the antrum and corpus was also confirmed in nine patients (Table 3). While analysis of MIC values showed 8–32 folds increased levels in the corpus, a higher MIC value was detected for one isolate in the antrum.

Clarithromycin mutation rates

Screening of three common mutations in *23S rRNA* (Dp4, Dp5, and Dp6) was done using site-specific primers. Results of the screening showed the existence of A2142G, A2143G, and A2142C mutations in three, two, and two patients respectively. Co-existence of A2142G and A2142C mutations was also detected in one patient. The A2142C mutation was detected in the corpus of one patient compared with wild-type allele in the *H. pylori* strain from the antrum (MIC 8 vs 0.25 μg/mL). All of these variants were obtained from patients with recent history of medication by clarithromycin-based regimens (Table 3).

RAPD typing

RAPD-PCR fingerprinting was performed on DNA extracts of the strains obtained from individual patients. Accordingly, the results showed identical molecular patterns for the isolates of the corpus and antrum in each patient. However, different RAPD patterns were detected among the strains from different patients (Fig. 1).

Discussion

In this study, our results showed that 17.5% of the patients harbored clarithromycin heteroresistance, including those with mutations at 23S rRNA gene (10%, 4/40). The observed

Table 2 Correlation between endoscopic and histological changes of the stomach and *H. pylori* clarithromycin resistance

	Resistance to clarithromycin *N* (%)		MIC_{50} (μg/mL)		Resistance rate to clarithromycin in each age group; *n*/*N* (%)						
	Antrum	Corpus	Antrum	Corpus	10–20	21–30	31–40	41–50	51–60	61–70	> 70
Endoscopic findings[a]											
Erythema	2/20 (10)	6/20 (30)	0.25	0.25		0/4	2/6	0/5	2/2	2/3	
PUD	3/5 (60)	3/5 (60)	16	0.25			2/3	0/1			1/1
NUD	9/35 (25.7)	13/35 (37.1)	0.25	8	0/1						
Erosion	0/1	0/1	0.25	0.25				0/1			
Histological findings											
Chronic gastritis	0/7	0/7	0.25	0.25		0/2	0/3	0/1	0/1		
Severe gastritis	4/6 (66.6)	4/6 (66.6)	0.25	8		0/1	0/1	1/1	2/2	1/1	
Intestinal metaplasia	0	0	0	0							
Dysplasia	0	0	0	0							
Total			0.25	0.25	0/1 (0)	0/7 (0)	4/13 (30.7)	1/9 (11.1)	4/5 (80)	3/4 (75)	1/1 (100)

[a] Endoscopic findings refers to disorders that were observed during endoscopy in each patient. Some of the disorders were concurrently detected in one single patient

Table 3 Diversity of minimal inhibitory concentration values, 23S rRNA mutations, and molecular fingerprints among pairs of *H. pylori* strains isolated from the antrum and corpus of a single patient

Pairs of *H. pylori* strains	Age group	Antrum				Corpus					
		R	S	MIC (μg/mL)	23S rRNA mutations[a]	R	S	MIC (μg/mL)	23S rRNA mutations[a]	RAPD profiles	Medication
HC748	31–40		S	0.25	No		S	0.25	No	Identical	Omeprazole, metronidazole, amoxicillin, bismuth
HC754	41–50		S	0.25	No		S	0.25	No	Identical	No
HC739	31–40	R		32	No	R		32	No	Identical	Metronidazole, pantoprazole, bismuth, ciprofloxacin, clarithromycin
HC749	> 70	R		16	No	R		16	No	Identical	Metronidazole, Nexium, levofloxacin, tetracycline, amoxicillin, clarithromycin
HC753	31–40	R		16	A2142G, A2142C	R		16	A2142G, A2142C	Identical	Ciprofloxacin, metronidazole, bismuth, clarithromycin
HC728	31–40		S	0.25	No		S	< 0.25	No	Identical	Metronidazole, pantoprazole
HC733	31–40		S	0.25	No		S	0.25	No	Identical	Metronidazole
HC737	21–30		S	0.25	No		S	0.25	No	Identical	Amoxicillin, metronidazole, bismuth, omeprazole
HC738	61–70		S	0.25	No		S	0.25	No	Identical	Omeprazole
HC740	21–30		S	0.25	No		S	0.25	No	Identical	Pantoprazole, domperidone, metronidazole
HC750	41–50		S	0.25	No		S	0.25	No	Identical	Omeprazole, amoxicillin
HC752	41–50		S	0.25	No		S	0.25	No	Identical	Ciprofloxacin, metronidazole, bismuth
HC755	21–30		S	0.25	No		S	0.25	No	Identical	Pantoprazole, metronidazole, ranitidine, domperidone
HC759	31–40		S	0.25	No		S	0.25	No	Identical	Omeprazole, pantoprazole,
HC760	21–30		S	0.25	No		S	0.25	No	Identical	Omeprazole, metronidazole, ceftriaxone
HC763	31–40		S	0.25	No		S	0.25	No	Identical	Pantoprazole, bismuth, metronidazole
HC764	41–50		S	0.25	No		S	0.25	No	Identical	Omeprazole, domperidone
HC725	61–70		S	0.25	No	R		4	No	Identical	Metronidazole, omeprazole
HC734	61–70	R		16	No	R		16	No	Identical	Omeprazole, metronidazole, ciprofloxacin
HC736	31–40		S	0.25	No	R		16	No	Identical	Metronidazole, pantoprazole
HC744	41–50		S	0.25	No		S	0.25	No	Identical	Omeprazole, ranitidine
HC761	41–50		S	0.25	No	R		0.5	No	Identical	Omeprazole, ranitidine
HC756	31–40	R		0.5	A2142G	R		4	A2142G	Identical	Omeprazole, domperidone, clarithromycin
HC727	51–60	R		0.5	A2143G	R		2	A2143G	Identical	Metronidazole, clarithromycin, bismuth, Nexium
HC747	51–60		S	0.25	No	R		8	A2142C	Identical	Omeprazole
HC729	31–40		S	0.25	No			< 0.25	No	Identical	Metronidazole, omeprazole, amoxicillin
HC731	21–30		S	0.25	No			< 0.25	No	Identical	Omeprazole
HC732	41–50	R		8	No	R		8	No	Identical	Metronidazole, levofloxacin clarithromycin
HC730	51–60	R		16	A2142G	R		16	A2142G	Identical	Metronidazole, clarithromycin, bismuth, omeprazole, ciprofloxacin
HC742	61–70		S	0.25	No	R		4	A2142G	Identical	Nexium/clarithromycin/ciprofloxacin
HC758	51–60	R		8	A2143G	R		8	A2143G	Identical	Omeprazole, tetracycline, levofloxacin, amoxicillin, clarithromycin

Table 3 (continued)

Pairs of *H. pylori* strains	Age group	Antrum				Corpus				RAPD profiles	Medication
		R	S	MIC (μg/mL)	23S rRNA mutations[a]	R	S	MIC (μg/mL)	23S rRNA mutations[a]		
HC726	10–20		S	0.25	No			< 0.25	No	Identical	Omeprazole
HC751	41–50		S	0.25	No		S	0.25	No	Identical	Pantoprazole, metronidazole
HC735	31–40		S	0.25	No		S	0.25	No	Identical	Metronidazole, bismuth, cefixime, Nexium
HC741	41–50		S	0.25	No		S	0.25	No	Identical	Metronidazole, pantoprazole, bismuth, ciprofloxacin, amoxicillin
HC743	31–40		S	0.25	No		S	0.25	No	Identical	Clidinium C, omeprazole
HC745	21–30		S	0.25	No		S	0.25	No	Identical	Pantoprazole, ceftriaxone, metronidazole
HC757	21–30		S	0.25	No		S	0.25	No	Identical	Ciprofloxacin, metronidazole
HC762	51–60		S	0.25	No		S	0.25	No	Identical	Omeprazole, levofloxacin, bismuth, metronidazole
HC746	31–40		S	0.25	No		S	0.25	No	Identical	Omeprazole, clidinium C

resistance rate of 35% in the studied population proposed 65% effectiveness for clarithromycin-based medication in these patients. This estimate was supported by recent studies, where clarithromycin-based regimens achieve eradication rates of approximately 79–88% in Iran (Fakheri et al. 2016; Mokhtare et al. 2015). While this is lower in developed countries, its worldwide rate is similar (75–82%) (25).

Colonization of heteroresistant *H. pylori* strains in the stomach plays a critical role in the failure of eradication. Long-term infection with *H. pylori* in this tissue, exposure with sub-lethal or ineffective doses of antibiotics, and the capacity for genomic evolution are possibly involved in the emergence of resistant variants in a single host. Mixed infection, i.e., co-infection with different strains in a single patient, also affects the efficacy of current regimens. In a study by Jae J. Kim et al., a frequency of 2.7% clarithromycin heteroresistance was reported among the two biopsy sites from each patient. This rate was lower compared with those obtained in our study. However, in patients infected with clarithromycin-resistant strains, the same rate of heteroresistance was described (46%) (Kim et al. 2003). Frequency of clarithromycin heteroresistance in our study was also higher compared with that of a study conducted by Cheng-Yen Kao et al. They showed difference in antibiotic resistance of *H. pylori* strains between the two samples of a single host in 26.3% of patients, which clarithromycin heteroresistance constituted 0.5% of them (Kao et al. 2014).

Infection with clarithromycin resistance strains is a predictive marker for prescription of alternative therapies, including concomitant and sequential therapy. Clarithromycin resistance is generally due to point mutations in the *23S rRNA*, mainly nucleotides A2142G and A2143G (Kao et al. 2014). In our study, while similar to other studies that mutation at A2143G nucleotide position was more common, no association was found between the mutation sites and the occurrence of heteroresistance phenotype (Vianna et al. 2016). Furthermore, increase in MIC values was not linked to the studied nucleotide changes. While in our study point mutations A2143G and A2142G were found in 53% of all isolates with CLA MIC > 2 mg/L, this frequency was reported in all CLA-resistant isolates by Zerbetto De Palma et al. (2017). Existence of other mutations in *23S rRNA* gene and their association with resistance phenotype of clarithromycin were reported in some countries (Rimbara et al. 2007). In addition, this difference could be explained by mutations in transcription initiation factor IF-2 (*infB*), 50S ribosomal protein L22 (*rpl22*), expression of efflux pumps of RND family (resistance modulation cell division), or alteration of outer membrane proteins (OMPs), which similarly confer resistance to clarithromycin in this bacterium (Kang et al. 2012; Hu et al. 2017; Hu et al. 2016). Further investigations are needed to find these relationships in the studied strains.

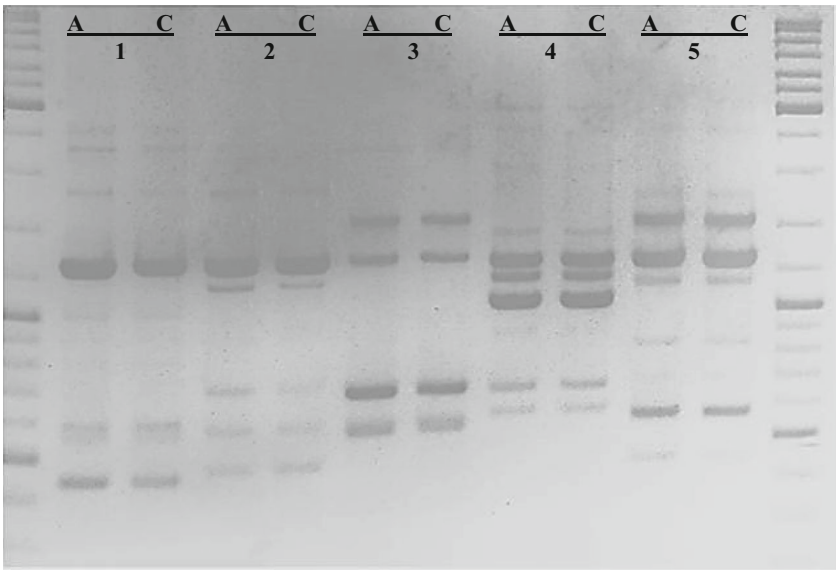

Fig. 1 RAPD-PCR fingerprinting patterns of *Helicobacter pylori* strains obtained from different parts of the stomach. Each number represents two strains from the antrum (A) and corpus (C) of a single patient. The primer 1283 was used in this analysis. Lanes 1–5 are related to pairs of strains HC748, HC754, HC739, HC749, and HC753, respectively. ZipRuler™ Express Mix DNA Ladder (Thermo Fisher Scientific, USA) was shown in the first and last lanes

Our results showed resistance to clarithromycin, most commonly among those patients who belonged to the older age groups (> 50 years old). The observed association between resistance to clarithromycin and age was described previously by Ji Z. et al. (Agudo et al. 2010; Ji et al. 2016). They showed the highest antibiotic resistance rate in patients' ages 71 to 80 years old. Extensive use of clarithromycin during childhood illnesses was proposed as the main reason. The higher rate of resistance in patients who were treated ineffectively compared with those not receiving medication supports this idea somewhat (Agudo et al. 2010). The observed link between antibiotic resistance and disease severity (e.g., PUD and severe active gastritis) is not well known. Exacerbation of gastric disease after ineffective treatment occurs in most of the patients, which accompanied with dominance of resistant strains in the stomach. In this view, the resistant strains may act as an innocent bystander, and their role as a trigger for severe disorders remains somewhat controversial.

Analyses of RAPD-PCR results did not show alteration in the fingerprints of pairs of the strains in each patient. This finding proposed that heteroresistant strains were mostly derived from pre-existing strains in each patient rather than from mixed-type infection. This finding was similarly supported by Jae J. Kim et al. and Cheng-Yen Kao et al. (Kao et al. 2014; Kim et al. 2003).

The presence of heteroresistant *H. pylori* infection suggested failure of current treatments for most of the studied patients. Administration of distinct non-standard regimens possibly plays an auxiliary role in this failure, since no internationally defined regimens were prescribed for the studied patients. Previous studies in Iran showed that triple therapy (PPI plus two antibiotics for 1 week) is not optimal, while furazolidone-based or clarithromycin-based quadruple therapy (furazolidone or clarithromycin plus PPI, bismuth subcitrate, amoxicillin for 2 weeks) was recommended as the first-line treatment regimen (Malekzadeh et al. 2004; Saberi-Firoozi and Nejabat 2006). Due to failure rates of 20–40% in Iran, second-line therapy that included tetracycline-based quadruple therapy (containing a PPI, bismuth, tetracycline, and two antibiotics, furazolidone or metronidazole, for 2 weeks) was recommended in the failed treatments. Prescription of this regimen was observed in only two of our patients. Inappropriateness of the medication was also observed by the administration of clarithromycin-based regimens for all the patients with 23S rRNA mutations. To eliminate the occurrence of untreatable infection through the emergence of highly resistant *H. pylori* strains, there is a need for harmonization of treatment regimens by the publication of a national guideline.

Conclusion

Our results showed the presence of clarithromycin heteroresistance in our patients. They were mostly derived from pre-existing strains in each patient. The results showed the same rate of *H. pylori* colonization at the antrum and the corpus of the stomach of most of the patients. While higher MIC levels were observed in the corpus, resistance to clarithromycin was observed in both sites of the stomach. The higher MIC levels were not correlated with mutations detected at nucleotide positions 2142 and 2143 of *23S rRNA* gene. Inappropriate medication can support recurrence of the infection in patients with resistant variants of clarithromycin. Upon failure of treatments, providing multiple biopsy specimens from different parts of the stomach seems to be necessary for doing the antimicrobial susceptibility testing.

Acknowledgments The authors of this article like to thank all staff of endoscopy unit of Ayatollah Taleghani Hospital and Foodborne and Waterborne Diseases Research Center for their sincere help and assistance.

Funding information This study was part of a fellowship dissertation and financially supported by a grant from Research Institute for Gastroenterology and Liver Diseases, Shahid Beheshti University of Medical Sciences, Tehran, Iran.

Compliance with ethical standards

This study was approved by the Ethics Committee of Shahid Beheshti University of Medical Sciences.

Conflict of interest The authors declare that they have no conflict of interest.

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