

Original Article

***Helicobacter pylori* 23S rRNA gene mutations associated with clarithromycin resistance in chronic gastritis in Vietnam**Van Huy Tran¹, Thi Minh Thi Ha¹, Phan Tuong Quynh Le¹, Viet Nhan Nguyen¹, Trung Nam Phan¹, Bianca Paglietti²¹ Hue University of Medicine and Pharmacy – Hue University, Hue, Vietnam² Department of Biomedical Sciences, University of Sassari, Sassari, Italy**Abstract**

Introduction: Data about the prevalence of the A2142C, A2142G, and A2143G mutations in 23S rRNA gene is still limited. The aim of this study was to determine the prevalence of these mutations in 23S rRNA gene of *H. pylori* vietnamese strains.

Methodology: One hundred and sixty-nine patients with *H. pylori*-positive chronic gastritis were examined. *H. pylori* was detected by rapid urease test and Polymerase chain reaction (PCR). Total DNA was extracted from gastric biopsy specimens. A2142C, A2142G, and A2143G mutations were detected by DNA sequencing and PCR-restriction fragment length polymorphism (PCR-RFLP).

Results: A2143G mutation was detected in 36.1% of samples, A2142G mutation in 3.6%, while A2142C mutation was not found in any case. The mixture of wild-type and mutation strains was found in 50% of specimens with A2142G, in 23% of specimens with A2143G mutation. There was no association of 23S rRNA gene point mutations with gender or age. However, an association between the heterogeneity of mutation and age was evidenced, with mean age of the group of pure A2143G higher than the group of wild-type/A2143G mixture, and rate of the wild-type/A2143G mixture higher in patients under 40 years of age.

Conclusion: A2143G mutation was prominent, while A2142C mutation was not found in the 23S rRNA gene. PCR-RFLP has revealed a reliable assay allowing a rapid and cost-effective detection of clarithromycin-resistant strains. This is useful in countries as Vietnam with high prevalence of clarithromycin-resistance before choosing optimal therapy for *H. pylori* eradication.

Key words: clarithromycin resistance; A2142G; A2143G; A2142C; 23S rRNA gene; *Helicobacter pylori*.

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Introduction

Helicobacter pylori (*H. pylori*) infection plays a major role in gastroduodenal diseases, and represents the primary cause of gastric cancer among patients with chronic gastritis. Clarithromycin is a key antibiotic in the standard triple therapy for eradication of *H. pylori* [1]. However, resistance against clarithromycin is increasing worldwide, reducing the success rate of standard triple therapies to mean values as low as 18% to 44% [2]. The diagnosis of clarithromycin-resistant *H. pylori* in patients with chronic gastritis is essential in order to select an effective therapy for eradication treatment. Clarithromycin-resistant *H. pylori* relying on antimicrobial susceptibility tests as disk diffusion or E-test, requires further subculturing steps for several days [3] and the successful rate varies on laboratories. In 1996, Versalovic observed the association of mutations at domain V of 23S rRNA gene with the status of clarithromycin resistance in strains of *H. pylori* [4]. These mutations are known as A2142G and A2143G,

defined by Taylor *et al.* in 1997 [5], which represent nowadays the molecular basis of the diagnosis of clarithromycin-resistant *H. pylori* through genotyping techniques. Recently, the mutations at 2142 and 2143 positions of 23S rRNA gene were responsible for more than 90% of clarithromycin resistance in developed countries [6], with the predominance of A2143G, A2142G and A2142C.

The prevalence of *H. pylori* infection in Vietnam is particularly high (74.6% of population) [7] and Vietnam is a country with an intermediate gastric cancer risk [8]. For this reason a successful eradication therapy appears essential not only to reduce the risk of developing gastric cancer but also to treat other related disorders.

The rate of clarithromycin resistance varies across our country, being 33% in two big cities (Ho Chi Minh city in the South Vietnam and Hanoi in the North Vietnam) [9] and 42.4% (35 out of 35 strains with A2143G mutation) in central Vietnam [10]. In a

previous study we highlighted a high rate of primary and secondary resistance to clarithromycin in the central region of Vietnam precluding the use of this drug for the treatment of *H. pylori* infection [11]. Its detection and surveillance is therefore mandatory in order to adapt the antibiotic combination to local resistance patterns. Data about the prevalence of the A2142C, A2142G, and A2143G mutations in 23S rRNA gene associated to clarithromycin resistance in Vietnam is still limited. For this reason, the aim of our study was to determine the prevalence of clarithromycin-resistance among *H. pylori* isolates in Vietnamese patients with chronic gastritis via the detection of mutations at 2142 and 2143 positions of domain V of 23S rRNA gene by using DNA sequencing and PCR-restriction fragment length polymorphism (PCR-RFLP) on DNA extracted from gastric biopsy specimens. Early detection of clarithromycin-resistance is necessary to select an optimal *H. pylori* eradication therapy.

Methodology

Case selection

Gastric biopsy specimens were obtained from patients with chronic gastritis via endoscopy at the Gastrointestinal Endoscopy Center, Hue University Hospital, Vietnam, between 2015 and 2016. Exclusion criteria were: treatment with antibiotics within 4 weeks prior to endoscopy, taking any PPI or H₂-blocker within

2 weeks prior to endoscopy, and history of antibiotic allergy. All participants signed the informed consent forms and our study was approved by the Ethics Committee of Hue University of Medicine and Pharmacy of Vietnam, under ethics certificate THH.2013-KC.09.

The *H. pylori* infection was screened by rapid urease test (RUT) on biopsy specimens. One hundred and eighty DNA samples were extracted from RUT-positive gastric biopsy specimens using Wizard Genomic DNA purification Kit (Promega, Madison, Wisconsin, USA), and *H. pylori* infection was confirmed by PCR using primers 5'-AAGCTTTTtaggggtgTTAGGGGTTT-3' (ureC-F) and 5'-AAGCTTACTTTCTAACACTAACGC-3' (ureC-R) targeting urease C (*ureC*) gene [12]. The amplification reaction was carried out in a final volume of 25 μL containing 12.5 μL of GoTaq Green MasterMix 2X (Promega, Madison, Wisconsin, USA), 10 pmol each of the primers and 100 ng of extracted DNA. The amplification reaction was performed in Applied Biosystems 2720 Thermocycler with an initial denaturation step of 95°C for 5 minutes followed by 30 cycles of 95°C for 1 minute, 55°C for 1 minute, 72°C for 1 minute; and a final cycle at 72°C for 8 minutes. The amplicons were analyzed by electrophoresis on a 1% agarose gel stained with RedView. The PCR yielded a fragment of 294 bp. All PCR reactions were conducted with a negative and a positive control

Figure 1. Alignment images of a sample with A2142G mutation (A) and a sample with A2143G mutation (B) obtained using BLAST with *H. pylori* U27270. (Mutations are highlighted in yellow).

Helicobacter pylori 23S and 5S ribosomal RNA genes, complete sequence
 Sequence ID: [gi|881379|U27270.1](#) Length: 3857 Number of Matches: 1
 Range 1: 2394 to 3054

A

Score	Expect	Identities	Gaps	Strand	Frame
1186 bits(642)	0.0()	656/662(99%)	3/662(0%)	Plus/Plus	
Features:					
Query 13	ATT-CTGTGCGGTT-AATACCGACCTGCATGAATGGCGTAAACGAGATGGGAGCTGCTCA	70			
Sbjct 2394	ATTCTTGTTCGGTTAAATACCGACCTGCATGAATGGCGTAAACGAGATGGGAGCTGCTCA	2453			
Query 71	ACCAGAGATTTCAGTGAATTTGAGTGGAGGTGAAAATTCCTCCTACCCGCGGCAAGACGG	130			
Sbjct 2454	ACCAGAGATTTCAGTGAATTTGAGTGGAGGTGAAAATTCCTCCTACCCGCGGCAAGACGG	2513			
Query 131	GAAGACCCCGTGGACCTTTACTACAACCTAGCACTGCTAACGGGAATATCATGCCGAGGA	190			
Sbjct 2514	GAAGACCCCGTGGACCTTTACTACAACCTAGCACTGCTAACGGGAATATCATGCCGAGGA	2573			
Query 191	TAGGTGGGAGGCTTTGAAGTAAGGGCTTTGGCTCTTATGGAGCCATCCTTGAGATACCAC	250			
Sbjct 2574	TAGGTGGGAGGCTTTGAAGTAAGGGCTTTGGCTCTTATGGAGCCATCCTTGAGATACCAC	2633			
Query 251	CCTTGATGTTTCGTTAGCTAACTGGCCCTGTTTATCCACAGGCAGGACAATGCTTGGTG	310			
Sbjct 2634	CCTTGATGTTTCGTTAGCTAACTGGCCCTGTTTATCCACAGGCAGGACAATGCTTGGTG	2693			
Query 311	GGTAGTTTGAATCGGCGGCTCGCCCTCTAAAAGTAACGGAGGCTTGCAGAGGTTGGCTC	370			
Sbjct 2752	GGTAGTTTGAATCGGCGGCTCGCCCTCTAAAAGTAACGGAGGCTTGCAGAGGTTGGCTC	2752			
Query 371	ATTGCGGTTGGAAATCGCAAGTTGAGTGTAAATGGCACAAGCCAGCCTGACTGTAAGACAT	430			
Sbjct 2812	ATTGCGGTTGGAAATCGCAAGTTGAGTGTAAATGGCACAAGCCAGCCTGACTGTAAGACAT	2812			
Query 431	ACAAGTCAAGCAGAGACGAAAGTCCGGTATAGTATCGGCTGTTCTGTGTGGAAGGGCC	490			
Sbjct 2872	ACAAGTCAAGCAGAGACGAAAGTCCGGTATAGTATCGGCTGTTCTGTGTGGAAGGGCC	2872			
Query 491	ATCGCTCAAAGGATAAAAAGTACCCCGGGGATAACAGGCTGATCTCCCCAAGAGCTCAC	550			
Sbjct 2932	ATCGCTCAAAGGATAAAAAGTACCCCGGGGATAACAGGCTGATCTCCCCAAGAGCTCAC	2932			
Query 551	ATCGACGGGGAGGTTGGCACCTCGATGTCGGCTCATCCGACCTCGGGGCTGGAGCAGGT	610			
Sbjct 2992	ATCGACGGGGAGGTTGGCACCTCGATGTCGGCTCATCCGACCTCGGGGCTGGAGCAGGT	2992			
Query 611	CCCAAGGGTATGGCTGTTCCGCATTTAAAGCGGTACCGAGCTGGGTTGAGAACCTCGTG	670			
Sbjct 2993	CCCAAGGGTATGGCTGTTCCGCATTTAAAGCGGTACCGAGCTGGGTTGAGAACCTCGTG	3052			

Helicobacter pylori 23S and 5S ribosomal RNA genes, complete sequence
 Sequence ID: [gi|881379|U27270.1](#) Length: 3857 Number of Matches: 1
 Range 1: 2394 to 3054

B

Score	Expect	Identities	Gaps	Strand	Frame
1181 bits(639)	0.0()	655/662(99%)	3/662(0%)	Plus/Plus	
Features:					
Query 13	ATT-CTGTGCGGTT-AATACCGACCTGCATGAATGGCGTAAACGAGATGGGAGCTGCTCA	70			
Sbjct 2394	ATTCTTGTTCGGTTAAATACCGACCTGCATGAATGGCGTAAACGAGATGGGAGCTGCTCA	2453			
Query 71	ACCAGAGATTTCAGTGAATTTGAGTGGAGGTGAAAATTCCTCCTACCCGCGGCAAGACGG	130			
Sbjct 2454	ACCAGAGATTTCAGTGAATTTGAGTGGAGGTGAAAATTCCTCCTACCCGCGGCAAGACGG	2513			
Query 131	AGAGACCCCGTGGACCTTTACTACAACCTAGCACTGCTAACGGGAATATCATGCCGAGGA	190			
Sbjct 2514	GAAGACCCCGTGGACCTTTACTACAACCTAGCACTGCTAACGGGAATATCATGCCGAGGA	2573			
Query 191	TAGGTGGGAGGCTTTGAAGTAAGGGCTTTGGCTCTTATGGAGCCATCCTTGAGATACCAC	250			
Sbjct 2574	TAGGTGGGAGGCTTTGAAGTAAGGGCTTTGGCTCTTATGGAGCCATCCTTGAGATACCAC	2633			
Query 251	CCTTGATGTTTCGTTAGCTAACTGGCCCTGTTTATCCACAGGCAGGACAATGCTTGGTG	310			
Sbjct 2634	CCTTGATGTTTCGTTAGCTAACTGGCCCTGTTTATCCACAGGCAGGACAATGCTTGGTG	2693			
Query 311	GGTAGTTTGAATCGGCGGCTCGCCCTCTAAAAGTAACGGAGGCTTGCAGAGGTTGGCTC	370			
Sbjct 2694	GGTAGTTTGAATCGGCGGCTCGCCCTCTAAAAGTAACGGAGGCTTGCAGAGGTTGGCTC	2752			
Query 371	ATTGCGGTTGGAAATCGCAAGTTGAGTGTAAATGGCACAAGCCAGCCTGACTGTAAGACAT	430			
Sbjct 2752	ATTGCGGTTGGAAATCGCAAGTTGAGTGTAAATGGCACAAGCCAGCCTGACTGTAAGACAT	2812			
Query 431	ACAAGTCAAGCAGAGACGAAAGTCCGGTATAGTATCGGCTGTTCTGTGTGGAAGGGCC	490			
Sbjct 2812	ACAAGTCAAGCAGAGACGAAAGTCCGGTATAGTATCGGCTGTTCTGTGTGGAAGGGCC	2872			
Query 491	ATCGCTCAAAGGATAAAAAGTACCCCGGGGATAACAGGCTGATCTCCCCAAGAGCTCAC	550			
Sbjct 2872	ATCGCTCAAAGGATAAAAAGTACCCCGGGGATAACAGGCTGATCTCCCCAAGAGCTCAC	2932			
Query 551	ATCGACGGGGAGGTTGGCACCTCGATGTCGGCTCATCCGACCTCGGGGCTGGAGCAGGT	610			
Sbjct 2932	ATCGACGGGGAGGTTGGCACCTCGATGTCGGCTCATCCGACCTCGGGGCTGGAGCAGGT	2992			
Query 611	CCCAAGGGTATGGCTGTTCCGCATTTAAAGCGGTACCGAGCTGGGTTGAGAACCTCGTG	670			
Sbjct 2993	CCCAAGGGTATGGCTGTTCCGCATTTAAAGCGGTACCGAGCTGGGTTGAGAACCTCGTG	3052			

corresponding to, sterile water and DNA extracted from previously *H. pylori ureC* gene positive gastric biopsies, respectively.

One hundred and sixty-nine *H. pylori*-positive samples were confirmed by PCR amplification of *ureC* gene.

Detection of mutations at domain V of 23S rRNA by DNA sequencing

DNA sequencing of domain V of 23S rRNA gene corresponding to nucleotides 2103 to 2709 (GenBank U27270.1) was performed on *H. pylori*-positive DNA samples. A fragment corresponding to nucleotides 1980 to 2692 was amplified using the universal 23S rRNA primers DoV-F:5'-GTAAACGGCGGCCGTA ACTA-3' and DoV-R:5'-GACCGA ACTGTCTCACGACG-3' previously described by Jensen *et al.* [13]. The conditions were following: an initial denaturation step of 95°C for 5 minutes, then 30 cycles of 95°C for 1 minute, 55°C for 1 minute, 72°C for 1 minute 30 seconds, and a final cycle at 72°C for 8 minutes. All amplicons were sequenced with forward primer (DoV-F) by Sanger method to assess the validity of the RFLP results and to characterize sequence variation. Sequencing was performed at First BASE Laboratory (Selangor, Malaysia), using BigDye Terminator v3.1 kit with standard protocol in ABI PRISM 3730xl Genetic Analyser developed by Applied Biosystems. Sequence alignment analyses were performed by using Basic Local Alignment Search Tool (BLAST). The nucleotide sequence of *H. pylori* 23S rRNA gene, publicly available on GenBank database with accession number U27270.1, was used as reference sequence (Figure 1).

Determination of A2142C, A2142G and A2143G mutations at domain V of 23S rRNA by PCR-RFLP

H. pylori-positive DNA samples were subjected to PCR-RFLP to detect A2142G, A2143G and A2142C nucleotide mutations with the modified Menard's protocol. A 267-bp fragment corresponding to nucleotides 1931 to 2197 of 23S rRNA (GenBank U27270.1) was amplified using primers HPY-S: 5'-AGGTTAAGAGGATGCGTCAGTC-3' and HPY-A: 5'-CGCATGATATTC CATTAGCAGT-3' [14]. PCR conditions were following: an initial denaturation of 95°C for 5 min followed by 30 cycles of 94°C for 1 minute, 52°C for 1 minute, 72°C for 1 minute 30 seconds; and the final cycle at 72°C for 8 minutes.

5 µL of each amplified DNA fragment (267-bp) was digested overnight in 20 µL with the restriction enzymes *BpiI* (10 U, Thermo Fisher Scientific, Vilnius,

Lithuania), *Eco31I* (10 U, Thermo Fisher Scientific, Vilnius, Lithuania), and *BceAI* (1 U, New England Biolabs, Ipswich, Massachusetts, USA) for identifying A2142G, A2143G and A2142C mutations in the domain V of 23S rDNA, respectively.

The digested products were analyzed by electrophoresis on a 2.5% agarose gel stained with RedView, 100-bp DNA Molecular ruler (BioRad, Hercules, California, USA) was used as reference size marker. The sizes of digested products were previously described by Menard *et al* (2002) [14] as follows: A2142G mutation generates a *BpiI* recognition site on the 267-bp PCR products, yielding two fragments of 219 bp and 48 bp, while A2143G mutation generates a *Eco31I* recognition site on the 267-bp PCR products, yielding two fragments of 207 bp and 60 bp. The enzyme *BceAI* recognizes two restriction sites 5'-ACGGC(N)₁₂↓N-3' and 5'-N↓(N)₁₄GCCGT-3' on the 267-bp products of *H. pylori* strains without A2142C mutation, yielding fragments of 195 bp, 48 bp, 24 bp; this enzyme also recognizes one more restriction site corresponding to A2142C mutation and digests the 195-bp fragment into 153-bp and 42-bp products [14].

Determination of the heterogeneity of mutation (wild-type/mutant mixture)

The heterogeneity of mutation (wild-type/mutation mixture) was detected by two different genotypes of *H. pylori* populations within a gastric biopsy specimen. It was indicated by two peaks of fluorescent signal at the same site in the sequencing trace, and by the combination of DNA bands corresponding to both wild-type and mutant strains after electrophoresis on 2.5% agarose gel.

Statistical analysis

Statistical analysis was performed using Medcalc 12.0 and VassarStats (Website for Statistical Computation). The prevalence of 23S rRNA clarithromycin-resistant mutations was calculated for overall study group, by age categories (< 40 years old; ≥ 40 years old), and by gender. The mean age was calculated for all types of mutation. The associations between mutations and gender or age were determined by comparing rates using the Chi square test or Fisher's exact test. The Student's t test was used for comparison between means.

Results

Among 180 RUT-positive samples, 169 samples were confirmed *H. pylori* infection by PCR assay with *ureC*-specific primers. These 169 samples were

performed the sequence analysis of 23S rRNA domain V and the PCR-RFLP assay on DNA extracted from gastric biopsy specimens in order to detect mutations associated with clarithromycin resistance (Figure 2). The BLAST with 23S rRNA sequence of *H. pylori* strain U27270.1 and the analysis of peaks of fluorescent signal in Sanger sequencing traces were done to identify mutations. Simultaneously, the analysis of DNA bands on 2.5% agarose gel after electrophoresis of digested products was done to identify A2142G, A2143G and A2142C mutations. Samples with single genotype at 2142 and 2143 positions of 23S rRNA domain V were recognized by images of one peak at these positions in sequencing traces. Samples with the heterogeneity were recognized by images of two peaks (A and G) at the same position (2142 or 2143). The PCR-RFLP yielded suitable DNA bands after electrophoresis on 2.5% agarose gel stained RedView. The wild type was identified by the presence of 267-bp fragments undigested by *BpiI* and *Eco31I*; and the presence of 195-bp fragment created from digested reaction by *BceAI*. The A2142G mutation was identified by the presence of 219-bp fragment created from digested reaction by *BpiI*. The A2143G mutation was identified

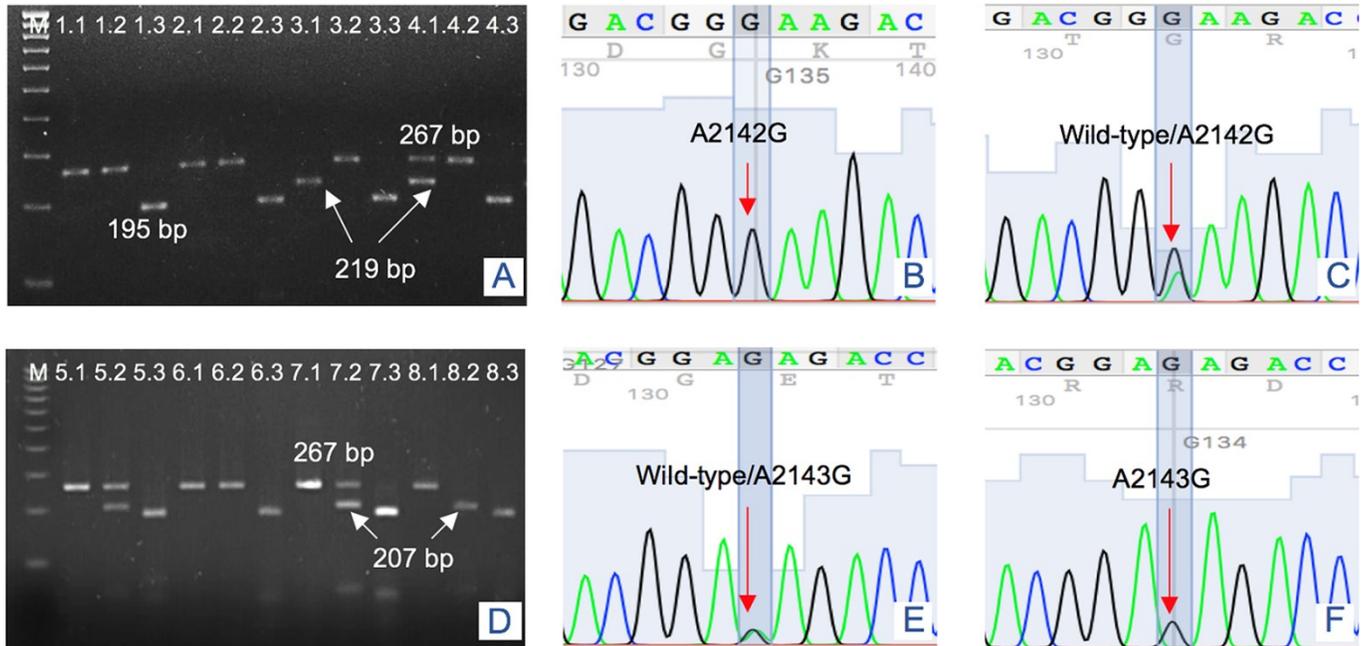
by the presence of 207-bp fragment created from digested reaction by *Eco31I*. The heterogeneity was identified by the combination of digested and undigested DNA fragments. The A2142G and A2143G mutations as well as the heterogeneity were detected by both above methods with an absolute concordance in all 169 samples ($\kappa = 1$, 95% CI: 0.9723 – 1).

Overall, A2142G and A2143G mutations were found in 3.6% (6 out of 169) and 36.1% (61 out of 169), respectively. On the contrary, neither A2142C mutation nor a combination of A2142G and A2143G were detected in our study group.

Both PCR-RFLP and sequence analysis revealed the presence of mixed samples of wild type and mutant type with an overall rate of 25% (17 out of 67). The rate of wild-type/A2142G mutant mixture was 50% (3 out of 6) of samples with A2142G mutation. The rate of wild-type/A2143G mutant mixture was found in 23% (14 out of 61) of samples with A2143G mutation.

The mean age of study cases was 43.8 ± 13.0 years, with 34.3% under 40 years and 65.7% of 40 years or older. 52.7% were men and 47.3% were women. Mean ages of A2142G mutation, A2143G mutation and wild-

Figure 2. Diagnosis of A2142G and A2143G mutations by PCR-RFLP and DNA sequencing.



Images A and D: The images of DNA bands observed after electrophoresis of digested products; Images B, C, E and F: Sequencing traces with peaks of fluorescent signal; Each sample was done three digested reactions with *BpiI*, *Eco31I* and *BceAI* enzymes corresponding to three lanes of electrophoresis x.1, x.2 and x.3, respectively. M lane was Molecular ruler 100 bp; Sample 3 (lanes 3.1, 3.2 and 3.3) had pure A2142G mutation: lane 3.1 showed a band of 219 bp. It was corresponding to one black peak (G) at 2142 position (image B); Sample 4 (lanes 4.1, 4.2 and 4.3) had mixture of wild type and A2142G mutation: lane 4.1 showed both bands of 267 bp and 219 bp. They were corresponding to two peaks (green for A and black for G) at 2142 position (image C); Sample 7 (lanes 7.1, 7.2 and 7.3) had mixture of wild type and A2143G mutation: lane 7.2 showed both bands of 267 bp and 207 bp. They were corresponding to two peaks (green for A and black for G) at 2143 position (image E); Sample 8 (lanes 8.1, 8.2 and 8.3) had pure A2143G mutation: lane 8.2 showed a band of 207 bp. It was corresponding to one black peak (G) at 2143 position (image F).

Table 1. The prevalence of A2142C, A2142G, and A2143G mutations of 23S rRNA gene among *H. pylori* strains in Vietnam in 2015 – 2016.

Patients characteristics	Total	Mutations		Wild-type (%)	p value
		A2142G (%)	A2143G (%)		
Total	169	6 (3.6)	61 (36.1)	102 (60.3)	
Age categories					
< 40 years	58	1 (1.7)	18 (31.0)	39 (67.2)	0.3903
≥ 40 years	111	5 (4.5)	43 (38.7)	63 (56.8)	
Gender					
Men	89	2 (2.2)	31 (34.8)	56 (62.9)	0.2422
Women	80	4 (5.0)	30 (37.5)	46 (57.5)	
Mean age ± SD, years	43.8 ± 13.0	51.8 ± 13.9 ^(a)	44.9 ± 13.4 ^(b)	42.7 ± 12.5 ^(c)	0.2345 ^[a-b] 0.0877 ^[a-c] 0.2915 ^[b-c]

(a): refer to group A2142G; (b): refer to group A2143G; (c): refer to group wild-type; [a-b]: comparing between 2 mean values corresponding to group (a) and group (b); [a-c]: comparing between 2 mean values corresponding to group (a) and group (c); [b-c]: comparing between 2 mean values corresponding to group (b) and group (c).

type groups were 51.8 ± 13.9 years, 44.9 ± 13.4 years and 42.7 ± 12.5 years, respectively. There was no significant difference in the mean age of patients by mutation status (A2142G, A2143G, and wild type). In the group of patients under 40 years of age, the rate of A2142G or A2143G was found in 1.7% and 31.0%, respectively, and in patients 40 years or older, 4.5% and 38.7%, respectively. The rate of A2142G or A2143G was found in 2.2% and 34.8% among men, respectively, and in 5.0% and 37.5% among women, respectively. There was no significant difference in the rate of each mutation by age or gender (Table 1). There was no association between the ages of patients with the heterogeneity of A2142G mutation, but there was an association between the ages of patients with the heterogeneity of A2143G mutation. The mean age of patients with the wild-type/A2143G mutant mixture was only 38 ± 12.3 years, while the mean age of patients with pure A2143G mutation was 46.9 ± 13.2 years, p = 0,0282. The rate of wild-type/A2143G mixture was higher in patients under 40 years. There was no

association between the heterogeneity of mutations with gender (Table 2).

Discussion

Chronic gastritis associated with *H. pylori* is very common in Vietnam, a country with a medium-high gastric cancer risk. The strategy of diagnosis and eradication of *H. pylori* started officially nearly 25 years ago, and is now facing a big challenge due to an increasing antibiotic resistance, in particularly resistance to clarithromycin. In the last five years, the *H. pylori* eradication rate based on standard triple therapies, has markedly decreased in Vietnam. The *H. pylori* resistance to clarithromycin is probably the main reason for this decrease, but data from adequately designed population-based studies are still limited.

The bacteriostatic activity of clarithromycin depends on its capacity to inhibit the protein synthesis by binding to the 50S bacterial ribosomal subunit [6]. *H. pylori* resistance to clarithromycin has been associated to point mutations in 2142 and 2143 positions in the domain V of 23S rRNA gene [4,5,15].

Table 2. The heterogeneity of A2142G and A2143G mutations of 23S rRNA gene among *H. pylori* strains in Vietnam in 2015 – 2016.

Patient characteristics	A2142G mutation		A2143G mutation	
	Pure n (%)	WT*/A2142G n (%)	Pure n (%)	WT*/A2143G n (%)
Total	3 (50.0)	3 (50.0)	47 (77.0)	14 (23.0)
Mean age ± SD, years	53 ± 11.4	50.7 ± 18.7	46.9 ± 13.2	38 ± 12.3
p value		0.8625		0.0282
Age categories				
< 40 years	0 (0.0)	1 (100.0)	10 (55.6)	8 (44.4)
≥ 40 years	3 (60.0)	2 (40.0)	37 (86.0)	6 (14.0)
p value		0.9999		0.0177
Gender				
Men	1 (50.0)	1 (50.0)	21 (67.7)	10 (32.3)
Women	2 (50.0)	2 (50.0)	26 (86.7)	4 (13.3)
p value		1		0.1271

*WT: wild-type.

In this study, the point mutations of 23S rRNA gene were determined by both DNA sequencing and PCR-RFLP. We found concordance in the results obtained from these two techniques in all samples analysed. This result is important since PCR-RFLP is a rapid and accurate genotypic test for the diagnosis of clarithromycin-resistant *H. pylori* strains in gastric biopsy specimens. Thus, this method appears a good alternative to other more expensive and time-consuming methods, such as DNA sequencing in our country.

In this study, the mutations of 23S rRNA gene were found in 39.7%, mainly present at position 2143 (36.1%) with substitution A2143G and at a lesser extent at position 2142 (3.6%) with substitution A2142G. These results were consistent with data obtained from other authors from studies conducted in Iran in 2011 (the rate of clarithromycin resistance was 31.7%, all of them had A2143G mutation detected using PCR-RFLP) [16] and more recently in USA in 2016 (clarithromycin-resistant *H. pylori* was common and observed in 32.3% of cases; the specific variants A2142G and A2143G were detected using DNA sequencing) [17]. However, the rates of A2142G and A2143G mutation in our study were significantly higher than those reported from a study conducted in Malaysia in 2010 (only one sample with A2142G mutation and two samples with A2143G mutation were found among 105 samples, accounted for only 2.9%) [18], but significantly lower than the one in Yamade's study conducted in Japan (the mutation rate was 55.6% including 82 samples with A2143G mutation and 3 samples with A2142G) [19].

The A2142C mutation was not found in our study, and it has not yet been found in Vietnam. In particular, in our previous study, among 35 clarithromycin-resistant *H. pylori* strains, A2143G mutation was predominant, and neither A2142G nor A2142C were detected [10]. Our result was consistent with data from reports from many other Asian countries including Korea, China and Japan [20-22]. A multi-center study might be necessary to clarify the presence of A2142C mutation among *H. pylori* strains in this large continent.

Clarithromycin has been available in Vietnam since the end of the 1990's, and it has become increasingly popular in subsequent years, firstly for the treatment of upper respiratory infections and then for *H. pylori* eradication. This widespread use of clarithromycin and probably a suboptimal compliance from patients for many reasons (side effects, high price, and patient education), might explain the increasing resistance rate to clarithromycin in our country. This scenario warrants a suitable modification of the strategy for *H. pylori*

eradication in Vietnam, which should include reducing or even stopping triple therapy, and encouraging quadruple bismuth containing therapy and concomitant therapy, in accordance with the recent guidelines from Maastricht 2016 [23] and from Toronto 2016 [24].

In our study, we detected quite high rates of mixed template of wild-type and mutation, 25% overall, 50% in A2142G group and 23% in A2143G group. Presently, there is limited data about the mixture of mutant and wild-type strains published in the medical literature. Similar results have been reported from other studies from France (25% of A2142G; 18.6% of A2143G) [25], and from Portugal (32.3% overall) [26].

Notably, mean age of patients having pure A2143G was significantly older than mean age of patients having a mixture of A2143G mutant and wild-type *H. pylori* strains. This may be partially explained by a long history of antibiotic consumption in the elder group resulting in extremely high rate of mutations. Further studies about this mixture of wild-type/mutant strains, and particularly its clinical impact in *H. pylori* management, are warranted.

Conclusion

In conclusion, the rate of clarithromycin-resistant *H. pylori* in Vietnam is high and mainly associated with A2143G mutation. No A2142C mutation was detected. PCR-RFLP assay provides a rapid and cost-effective detection of clarithromycin resistance. The mixture of wild-type and mutation strains was found in 50% of specimens with A2142G mutation, in 23% of specimens with A2143G mutation. These data support further modification of *H. pylori* eradication strategy in our country to achieve optimal outcome.

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References

1. Malfertheiner P, Megraud F, O'Morain CA, Atherton J, Axon AT, Bazzoli F, Gensini GF, Gisbert JP, Graham DY, Rokkas T (2012) Management of *Helicobacter pylori* infection - the Maastricht IV/Florence consensus report. *Gut* 61: 646-664.
2. De Francesco V, Margiotta M, Zullo A, Hassan C, Troiani L, Burattini O, Stella F, Di Leo A, Russo F, Marangi S (2006) Clarithromycin-resistant genotypes and eradication of *Helicobacter pylori*. *Ann Intern Med* 144: 94-100.
3. Rüssmann H, Adler K, Haas R, Gebert B, Koletzko S, Heesemann J (2001) Rapid and accurate determination of genotypic clarithromycin resistance in cultured *Helicobacter*

- pylori* by fluorescent in situ hybridization. J Clin Microbiol 39: 4142-4144.
4. Versalovic J, Shortridge D, Kibler K, Griffy MV, Beyer J, Flamm RK, Tanaka SK, Graham DY, Go MF (1996) Mutations in 23S rRNA are associated with clarithromycin resistance in *Helicobacter pylori*. Antimicrob Agents Chemother 40: 477-480.
 5. Taylor DE, Ge Z, Purych D, Lo T, Hiratsuka K (1997) Cloning and sequence analysis of two copies of a 23S rRNA gene from *Helicobacter pylori* and association of clarithromycin resistance with 23S rRNA mutations. Antimicrob Agents Chemother 41: 2621-2628.
 6. De Francesco V, Zullo A, Hassan C, Giorgio F, Rosania R, Ierardi E (2011) Mechanisms of *Helicobacter pylori* antibiotic resistance: An updated appraisal. World J Gastrointest Pathophysiol 2: 35-41.
 7. Hoang TTH, Bengtsson C, Phung DC, Sörberg M, Granström M (2005) Seroprevalence of *Helicobacter pylori* infection in urban and rural Vietnam. Clin Diagn Lab Immunol 12: 81-85.
 8. Yamaoka Y (2010) Mechanisms of disease: *Helicobacter pylori* virulence factors. Nat Rev Gastroenterol Hepatol 7: 629-641.
 9. Binh TT, Shiota S, Nguyen LT, Ho DD, Hoang HH, Ta L, Trinh DT, Fujioka T, Yamaoka Y (2013) The incidence of primary antibiotic resistance of *Helicobacter pylori* in Vietnam. J Clin Gastroenterol 47: 233.
 10. Phan TN, Santona A, Tran VH, Tran TNH, Cappuccinelli P, Rubino S, Paglietti B (2015) High rate of levofloxacin resistance in a background of clarithromycin-and metronidazole-resistant *Helicobacter pylori* in Vietnam. Int J Antimicrob Agents 45: 244-248.
 11. Phan TN, Tran VH, Tran TNH, Santona A, Rubino S, Paglietti B (2015) Antimicrobial resistance in *Helicobacter pylori*: current situation and management strategy in Vietnam. J Infect Dev Ctries 9: 609-613. doi: 10.3855/jidc.6942.
 12. Bickley J, Owen R, Fraser A, Pounder R (1993) Evaluation of the polymerase chain reaction for detecting the urease C gene of *Helicobacter pylori* in gastric biopsy samples and dental plaque. J Med Microbiol 39: 338-344.
 13. Jensen LB, Aarestrup FM (2001) Macrolide resistance in *Campylobacter coli* of animal origin in Denmark. Antimicrob Agents Chemother 45: 371-372.
 14. Ménard A, Santos A, Mégraud F, Oleastro M (2002) PCR-restriction fragment length polymorphism can also detect point mutation A2142C in the 23S rRNA gene, associated with *Helicobacter pylori* resistance to clarithromycin. Antimicrob Agents Chemother 46: 1156-1157.
 15. Occhialini A, Urdaci M, Doucet-Populaire F, Bébéar CM, Lamouliatte H, Mégraud F (1997) Macrolide resistance in *Helicobacter pylori*: rapid detection of point mutations and assays of macrolide binding to ribosomes. Antimicrob Agents Chemother 41: 2724-2728.
 16. Abdollahi H, Savari M, Zahedi MJ, Moghadam SD, Abasi MH (2011) Detection of A2142C, A2142G, and A2143G mutations in 23s rRNA gene conferring resistance to clarithromycin among *Helicobacter pylori* isolates in Kerman, Iran. Iran J Med Sci 36: 104.
 17. Park JY, Dunbar KB, Mitui M, Arnold CA, Lam-Himlin DM, Valasek MA, Thung I, Okwara C, Coss E, Cryer B (2016) *Helicobacter pylori* clarithromycin resistance and treatment failure are common in the USA. Dig Dis Sci 61: 2373-2380.
 18. HO SL, Tan EL, Sam CK, GOH KL (2010) Clarithromycin resistance and point mutations in the 23S rRNA gene in *Helicobacter pylori* isolates from Malaysia. J Dig Dis 11: 101-105.
 19. Yamade M, Sugimoto M, Uotani T, Nishino M, Kodaira C, Furuta T (2011) Resistance of *Helicobacter pylori* to quinolones and clarithromycin assessed by genetic testing in Japan. J Gastroenterol Hepatol 26: 1457-1461.
 20. Kim JM, Kim JS, Kim N, Kim Y-J, Kim IY, Chee YJ, Lee C-H, Jung HC (2008) Gene mutations of 23S rRNA associated with clarithromycin resistance in *Helicobacter pylori* strains isolated from Korean patients. J Microbiol Biotechnol 18: 1584-1589.
 21. Zhu Z-H, Huang D-Q, Xie Y, Liu L, Lu N-H (2013) Characterization of 23S rRNA gene mutation in primary and secondary clarithromycin-resistant *Helicobacter pylori* strains from East China. Turk J Gastroenterol 24: 5-9.
 22. Matsuoka M, Yoshida Y, Hayakawa K, Fukuchi S, Sugano K (1999) Simultaneous colonisation of *Helicobacter pylori* with and without mutations in the 23S rRNA gene in patients with no history of clarithromycin exposure. Gut 45: 503-507.
 23. Malfertheiner P, Megraud F, O'Morain C, Gisbert J, Kuipers E, Axon A, Bazzoli F, Gasbarrini A, Atherton J, Graham DY, Hunt R, Moayyedi P, Rokkas T, Rugge M, Selgrad M, Suerbaum S, Sugano K, El-Omar EM (2017) Management of *Helicobacter pylori* infection - the Maastricht V/Florence consensus report. Gut 66: 6-30.
 24. Fallone CA, Chiba N, van Zanten SV, Fischbach L, Gisbert JP, Hunt RH, Jones NL, Render C, Leontiadis GI, Moayyedi P (2016) The Toronto consensus for the treatment of *Helicobacter pylori* infection in adults. Gastroenterology 151: 51-69. e14.
 25. Raymond J, Lamarque D, Kalach N, Chaussade S, Burucoa C (2010) High level of antimicrobial resistance in French *Helicobacter pylori* isolates. Helicobacter 15: 21-27.
 26. Oleastro M, Ménard A, Santos A, Lamouliatte H, Monteiro L, Barthélémy P, Mégraud F (2003) Real-time PCR assay for rapid and accurate detection of point mutations conferring resistance to clarithromycin in *Helicobacter pylori*. J Clin Microbiol 41: 397-402.

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