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Characterisation of point mutations in domain V of the 23S rRNA gene of clinical *Helicobacter pylori* strains and clarithromycin-resistant phenotype in central Vietnam

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ABSTRACT

Objectives: The prevalence of clarithromycin (CLR)-resistant *Helicobacter pylori* is increasing worldwide, including in Vietnam. The aims of this study were to determine point mutations in the 23S rRNA domain V of clinical *H. pylori* strains in central Vietnam, to estimate the prevalence of phenotypic CLR resistance and to assess the association between 23S rRNA domain V genotype and CLR-resistant phenotype.
Methods: Sequencing of the 23S rRNA domain V of *H. pylori* strains from gastric biopsy specimens was performed for 185 patients with *H. pylori*-positive chronic gastritis, of which 104 samples were subjected to susceptibility testing to determine CLR resistance.
Results: A total of 24 types of point mutation were detected. A2143G and A2142G mutations were observed in 40.5% and 4.3%, respectively. New point mutations were detected (C2041T, C2083T, C2191T, G2220A, G2225A, G2240A, C2273T, T2276C, G2287A, C2399T, A2445G and C2622T). 23S rRNA domain V genotypes were diversified, with combinations of two or more point mutations as well as single point mutations. The rate of phenotypic CLR resistance was 53.8%, increasing from 40.4% in 2012–2014 to 70.2% in 2015–2017 ($P = 0.0045$). A2143G and A2142G accounted for 89.3% of phenotypically CLR-resistant *H. pylori* isolates.
Conclusions: A diversity of point mutations in the 23S rRNA domain V was observed in clinical *H. pylori* isolates. The rate of phenotypically CLR-resistant *H. pylori* is significantly increasing in central Vietnam. Further research is necessary to clarify the role of the combination of 23S rRNA domain V mutations in the molecular mechanism of CLR resistance.

1. Introduction

Helicobacter pylori is the most common agent in chronic gastritis and has been classified as a primary cause of gastric cancer by the International Agency for Research on Cancer (IARC) since 1994 [1]. Approximately 50% of the world's population is estimated to be infected with *H. pylori* [2]. Vietnam is a region of high prevalence, with an investigation in 2005 reporting an *H. pylori* seroprevalence of 74.6% [3]. Eradication of *H. pylori* is considered to be an effective and active treatment for prevention of gastric cancer. The Maastricht V/Florence Consensus Report [4] and the Toronto Consensus statement [5] recommend to use clarithromycin (CLR) as the main antibiotic in triple therapy for *H. pylori* eradication. However, the prevalence of *H. pylori* resistance to CLR is increasing worldwide, including in Vietnam, leading to significant rates of unsuccessful treatment [6,7]. In 2006, De Francesco et al. emphasised that the rate of *H. pylori* eradication by triple therapy was reduced to mean values as low as 18–44% [8].

In 1996, Versalovic et al. detected point mutations in domain V of the 23S RNA gene that were responsible for the CLR-resistant status of *H. pylori* strains [9]. Today, these mutations are known as A2143G and A2142G. In addition, many other mutations have also been detected, such as A2115G, T2182C, C2195T, A2223G, C2244T, C2288T, T2295C, T2289C, A2302G, T2717C, etc. [10–14], but their clinical relevance has not been clearly elucidated. In our previous

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preliminary research, the rate of phenotypic CLR-resistant *H. pylori* was 42.4% in central Vietnam [13]. The aims of the current study were (i) to determine point mutations in 23S rRNA domain V of clinical *H. pylori* strains in central Vietnam, (ii) to estimate the rate of phenotypic CLR-resistant *H. pylori* and (iii) to assess the association between 23S rRNA domain V genotype and CLR-resistant phenotype.

2. Materials and methods

2.1. Study population

Gastric biopsy specimens were collected by endoscopy from patients with chronic gastritis at the Gastrointestinal Endoscopy Center, Hue University Hospital (Hue, Vietnam) between 2012 and 2017. Diagnosis of chronic gastritis was confirmed by histopathology. Screening for *H. pylori* infection was done by rapid urease test (RUT) on gastric biopsy specimens. DNA samples were extracted from RUT-positive specimens using a Wizard® Genomic DNA Purification Kit (Promega Corp., Madison, WI). *Helicobacter pylori* infection was confirmed by PCR with a primer pair for the *ureC* gene as previously described [15]. Exclusion criteria were treatment with antibiotics and/or proton pump inhibitors and/or an H_2 blocker within 4 weeks prior to endoscopy.

2.2. Genotyping the 23S rRNA domain V of H. pylori

DNA sequencing of the 23S rRNA domain V was performed for *H. pylori*-positive samples. A 699-bp fragment was amplified using the universal 23S rRNA primers previously described by Jensen and Aarestrup [16]. The conditions were as follows: initial denaturation at 95 °C for 5 min; 30 cycles of denaturation at 95 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 1 min 30 s; and a final extension cycle at 72 °C for 8 min. PCR was performed using an Applied Biosystems™ 2720 Thermal Cycler (Thermo Fisher Scientific, Singapore).

Direct DNA sequencing was performed using a BigDye® Terminator v.3.1 Kit (Applied Biosystems, Foster City, CA) on an Applied Biosystems® 3730 Genetic Analyzer (Thermo Fisher Scientific) at First BASE Laboratory (Seri Kembangan, Malaysia). A total of 185 samples with good signal peaks were obtained. Sequence alignment was analysed using Basic Local Alignment Search Tool (BLAST). The known nucleotide sequence of *H. pylori* J99 strain (GenBank accession no. **CP011330.1**; gi: 816207297) was used as the reference.

2.3. Determining the clarithromycin-resistant phenotype of H. pylori by Etest

Of 185 *H. pylori*-positive biopsy gastric specimens with DNA sequencing results, 104 clinical isolates were cultured and subjected to CLR minimum inhibitory concentration (MIC) determination by Etest as previously described [13]. Gastric biopsy specimens were preserved in saline solution and were sent for culture within 4 h. Quality control was performed with an isolated *H. pylori* strain with an MIC of 0.016 mg/L and without any point mutations in the 23S rRNA gene by DNA sequencing. CLR resistance was defined according to the Clinical and Laboratory Standards Institute (CLSI) approved breakpoint (≥1.0 mg/L) [17].

2.4. Statistical analysis

The prevalence of 23S rRNA domain V genotypes was calculated for the overall study group. The prevalence of phenotypic CLR-resistant *H. pylori* isolates by Etest was calculated by group according to age, sex, antibiotic history and year of sample collection. The association between CLR-resistant status and these characteristics was determined by χ^2 test. The association between point mutations in the 23S rRNA domain V and CLR-resistant phenotype of *H. pylori* was evaluated by logistic regression analysis. Statistical analysis was performed using MedCalc statistical software v.12.2.1.0 (MedCalc Software, Ostend, Belgium).

3. Results

A total of 185 patients with *H. pylori*-positive chronic gastritis were analysed in this study, of whom 64.3% were recruited from 2012–2014 and 35.7% from 2015–2017. The mean ± standard deviation age of enrolled patients was 42.3 ± 12.7 years (range 16–72 years) and 50.3% were male and 49.7% were female. Moreover, 27.5% of patients had a history of CLR use either in triple-therapy regimens for *H. pylori* eradication (17.8%) or in the treatment of others infections (9.7%) (Table 1).

3.1. Genotyping the 23S rRNA domain V of H. pylori

DNA sequencing of the 23S rRNA domain V was performed for 185 clinical *H. pylori* isolates. A total of 24 types of point mutation were detected (Table 2). Mutations at positions 2142 and 2143 accounted for 44.9%, with a predominance of A2143G (40.5%; 75/185); the rate of A2142G was only 4.3% (8/185). Neither A2142C nor the combination of A2142G and A2143G was found. In addition, there were 22 types of point mutation at other positions, with T2182C accounting for a high rate (89.7%; 166/185). The A2223G and A2302G mutation rates were 45.9% (85/185) and 9.2% (17/185), respectively. The rates of other point mutations were very low. Besides previously reported mutations, many new mutations were detected in the clinical *H. pylori* strains.

Of the 185 *H. pylori* strains evaluated for 23S rRNA domain V mutations, only 5 strains had no mutation. The rate of single mutation was 26.5% (49/185). Notably, the combination of two or three mutations accounted for 32.4% (60/185) and 35.1% (65/185), respectively. In addition, the combination of four mutations was detected in 3.2% (6/185). The most predominant genotypes were A2143G + T2182C + A2223G (22.7%), single T2182C (20.5%), T2182C + A2223G (12.4%) and A2143G + T2182C (9.2%) (Table 2).

Table 1
Characteristics of patients included in the study ($n = 185$).

Characteristic	*n* (%)
Age (years)	
≤29	34 (18.4)
30–39	43 (23.2)
40–49	51 (27.6)
50–59	38 (20.5)
≥60	19 (10.3)
Sex	
Male	93 (50.3)
Female	92 (49.7)
Antibiotic history	
CLR for *Helicobacter pylori*[a]	33 (17.8)
CLR for other diseases[b]	18 (9.7)
No CLR history	111 (60.0)
No reported antibiotic history	23 (12.4)
Year of sample collection	
2012–2014	119 (64.3)
2015–2017	66 (35.7)
Total	185 (100.0)

CLR, clarithromycin.
[a] This regimen contained a proton pump inhibitor, amoxicillin and CLR.
[b] Most diseases were upper respiratory tract infections. CLR was used as monotherapy or combined with other antibiotics.

Table 2
Genotypes of the 23S rRNA domain V of *Helicobacter pylori* isolates.

Genotype	*n*	Genotype	*n*
No mutation	5	A2142G + T2182C + A2223G	2
A2142G	1	A2143G + T2182C + G2212A	1
A2143G	2	A2143G + T2182C + A2223G	42
T2182C	38	A2143G + T2182C + G2225A	2
G2220A	3	A2143G + T2182C + C2244T	1
A2223G	3	A2143G + T2182C + T2295C	1
C2244T	1	A2143G + T2182C + A2302G	5
A2445G	1	T2182C + C2195T + A2223G	2
C2083T + T2182C	1	T2182C + C2195T + C2399T	1
A2142G + T2182C	3	T2182C + A2223G + G2287A	2
A2142G + A2223G	1	T2182C + A2223G + C2288T	1
A2143G + T2182C	17	T2182C + A2223G + C2622T	1
A2143G + A2223G	1	T2182C + A2302G + G2220A	1
T2182C + A2223G	23	T2182C + A2302G + T2276C	1
T2182C + C2195T	1	A2223G + A2435G + C2622T	1
T2182C + G2240A	1	C2041T + T2182C + A2223G + C2561T	1
T2182C + C2244T	1	A2143G + T2182C + C2195T + A2223G	1
T2182C + C2273T	1	A2143G + T2182C + A2223G + G2287A	1
T2182C + A2302G	9	A2143G + T2182C + A2223G + T2295C	1
T2182C + C2561T	1	T2182C + C2191T + C2195T + A2223G	1
A2142G + T2182C + G2212A	1	T2182C + A2223G + T2276C + A2302G	1

3.2. Clarithromycin-resistant phenotype of H. pylori

Overall, among 104 *H. pylori* isolates with Etest performed, 48 (46.2%) CLR-susceptible isolates (MIC = 0.016–0.75 mg/L) and 56 (53.8%) CLR-resistant isolates (MIC = 1.5–256 mg/L) were detected. The phenotypic CLR resistance rate in the group with CLR treatment history was statistically significantly higher than in the group without CLR history (75.9% vs. 44.1%; $P = 0.0081$). Resistance to CLR did not differ between males and females, but increased with age ($P = 0.0295$, <50 years vs. ≥50 years). Notably, the rate of CLR-resistant isolates in the years 2012–2014 was only 40.4% and increased up to 70.2% in years 2015–2017 ($P = 0.0045$) (Table 3).

Among the CLR-resistant isolates, the rates of isolates with MICs of 1.5 to <32 mg/L, 32 to <64 mg/L, 64 to <128 mg/L and 128–256 mg/L were 51.8%, 8.9%, 7.1% and 32.1%, respectively.

3.3. Relationship between 23S rRNA domain V genotype and clarithromycin-resistant phenotype

Thirteen types of point mutation were detected among the 104 *H. pylori* isolates with MIC determined (A2142G, A2143G, T2182C, C2195T, G2212A, G2220A, A2223G, C2244T, G2287A, C2288T, T2295C, A2302G and C2622T). Logistic regression analysis demonstrated that A2143G mutation was associated with a CLR-resistant phenotype of *H. pylori* both in the univariate and multivariate analysis, with an adjusted odds ratio (aOR) of 718.66 [95% confidence interval (CI) 59.01–8751.82] (Table 4). Of 56 phenotypically CLR-resistant *H. pylori* isolates, 5 isolates carried A2142G mutations and 45 isolates carried A2143G, accounting for 89.3% of the CLR-resistant isolates. Six phenotypically CLR-resistant isolates without the mutations A2142G and A2143G included two non-mutated isolates, two isolates with T2182C + A2223G, one isolate with T2182C + A2223G + C2195T and one isolate with a single point mutation G2220A. The genotypes of two CLR-susceptible isolates with A2143G mutation were A2143G + T2182C + A2223G and A2143G + T2182C + G2212A, respectively.

4. Discussion

The A2142G/C and A2143G mutations are currently considered the main causes of CLR resistance among *H. pylori* isolates. Some other mutations are also recognised as causes of CLR-resistant *H. pylori*, but their clinical significance remains controversial.

The first notable result of the current research was the high rate of mutations at positions 2142 and 2143, predominantly A2143G. Notably, no A2142C mutation was found. The distribution of A2142G, A2142C and A2143G mutations in the present study was similar to many others, particularly in Asia [11,18–21]. Overall, most studies showed that A2142C was a very rare mutation and was only found in Caucasian individuals [22–25]. To date, the A2142C mutation has not been found in Vietnam.

Besides A2142G and A2143G, clinical *H. pylori* strains in this study carried some previously reported mutations, including T2182C, C2195T, G2212A, A2223G, C2244T, C2288T, T2295C, A2302G, A2435G and C2561T. To the best of our knowledge, T2182C, C2195T, A2223G, C2244T and A2302G have been reported by many authors, whereas the others are rarely reported. The G2212A mutation was recently reported in 2017 by Zerbetto De Palma et al. [26], and C2288T and T2295C mutations were detected in the study by Toracchio et al. [24]. The A2435G mutation was reported by Qi et al. in 2015 in a Chinese article [27]. The C2561T mutation was detected in Slovenia by Brezovec [28]. In the current study, some new nucleotide substitutions such as C2041T, C2083T, C2191T, G2220A, G2225A, G2240A, C2273T, T2276C, G2287A, C2399T, A2445G and C2622T were also detected. However, new

Table 3
Rate of phenotypically clarithromycin (CLR)-resistant *Helicobacter pylori* isolates by patient characteristics.

Characteristic	No. of samples	No. (%) of CLR resistance	*P*-value
Age (years)			0.0295 (<50 years vs. ≥50 years)
≤29	14	6 (42.9)	
30–39	23	9 (39.1)	
40–49	33	17 (51.5)	
50–59	24	17 (70.8)	
≥60	10	7 (70.0)	
Sex			N/S
Male	51	25 (49.0)	
Female	53	31 (58.5)	
Antibiotic history			0.0081 ([a] vs. [b])
CLR history for *H. pylori* and other diseases	29	22 (75.9)[a]	
No CLR history	68	30 (44.1)[b]	
No reported antibiotic history	7	4 (57.1)	
Year of sample collection			0.0045
2012–2014	57	23 (40.4)	
2015–2017	47	33 (70.2)	
Total	104	56 (53.8)	

N/S, not significant.
[a] Refers to the rate of CLR resistance in the group having CLR history for *H. pylori* and other diseases.
[b] Refers to the rate of CLR resistance in the group having no CLR history.

Table 4
Association between point mutations in the 23S rRNA domain V and clarithromycin-resistant phenotype of *Helicobacter pylori* clinical isolates.

Mutation		Phenotype		Univariate analysis [crude OR (95% CI)]	Multivariate analysis [adjusted OR (95% CI)]
		Resistant	Susceptible		
A2142G	Yes	5	0	10.36 (0.56–192.37)	–
	No	51	48		
A2143G	Yes	45	2[a]	94.09 (19.74–448.53)	718.66 (59.01–8751.82)
	No	11[b]	46		
T2182C	Yes	51	42	1.46 (0.42–5.11)	0.12 (0.01–1.15)
	No	5	6		
C2195T	Yes	2	1	1.74 (0.15–19.81)	15.98 (0.67–381.07)
	No	54	47		
G2212A	Yes	1	1	0.85 (0.04–14.04)	–
	No	55	47		
G2220A	Yes	1	1	0.85 (0.04–14.04)	2.86 (0.10–81.15)
	No	55	47		
A2223G	Yes	33	15	3.16 (1.40–7.09)	2.20 (0.33–14.65)
	No	23	33		
C2244T	Yes	1	1	0.85 (0.04–14.04)	0.31 (0.002–43.57)
	No	55	47		
G2287A	Yes	0	1	0.28 (0.01–7.04)	–
	No	56	47		
C2288T	Yes	0	1	0.28 (0.01–7.04)	–
	No	56	47		
T2295C	Yes	1	0	2.62 (0.10–65.86)	–
	No	55	48		
A2302G	Yes	2	3	0.56 (0.09–3.47)	0.71 (0.005–107.72)
	No	54	45		
C2622T	Yes	0	1	0.28 (0.01–7.04)	–
	No	56	47		

OR, odds ratio; CI, confidence interval.
[a] The genotypes of these two isolates were A2143G + T2182C + A2223G and A2143G + T2182C + G2212A.
[b] Five isolates carried A2142G mutation and the remaining six isolates included two non-mutated isolates, two isolates with T2182C + A2223G, one isolate with T2182C + A2223G + C2195T and one isolate with a single G2220A point mutation.

point mutations accounted for only very low rates. Among the new point mutations, only G2220A and A2445G were present as single point mutations in some strains, whereas others were combined with different mutations. Four strains with G2220A were detected, three of which were single G2220A mutations, and MIC results were determined for two strains including one CLR-resistant and one CLR-susceptible isolate. Unfortunately, MIC results were not available for the *H. pylori* strain with a single A2445G mutation.

In this study only 5 strains with a 23S rRNA domain V sequence similar to the J99 reference strain (i.e. no mutation) were detected, and there were 49 strains with a single point mutation, predominantly T2182C (38/49). Over 70% of the strains in this study carried a combination of two or more mutations. Kim et al. showed that the combination of two and more mutations in 23S rRNA domain V were not found in primary CLR-resistant *H. pylori* isolates, whereas secondary resistant isolates carried many types of mutation including single or combined mutations [11]. The diversity of 23S rRNA domain V mutation types in the current study might be considered a certain consequence of uncontrolled antibiotic use, including CLR.

These results highlight a very high prevalence of phenotypic CLR resistance of clinical *H. pylori* isolates (53.8%) that increased with time from 40.4% in 2012–2014 to 70.2% in 2015–2017. Overall, most medical literature in the world has demonstrated the rapid increase and geographical variations of rates of phenotypic CLR-resistant *H. pylori* [25,29,30]. In Vietnam, CLR is prescribed not only for treating *H. pylori* infection but also upper respiratory tract infections, particularly in children. Moreover, the prevalence of *H. pylori* infection is very high in Vietnam. Therefore, many *H. pylori* strains in Vietnam might have been previously exposed to CLR in therapies for other infections. The rate of phenotypic CLR-resistant *H. pylori* was compared between two groups according to CLR history (for *H. pylori* or other diseases) and no CLR history and a statistically significantly higher rate of CLR resistance was observed in the group with a CLR history. Antibiotic use is not yet been well controlled in Vietnam. Sometimes patients can take antibiotics by themselves without a medical doctor's prescription. This problem was considered as the first cause of 23S rRNA domain V mutations conferring CLR resistance in clinical *H. pylori* strains.

A total of 104 specimens having both MIC and DNA sequencing data were analysed. Univariate analysis showed that A2143G and A2223G mutations were associated with a CLR-resistant phenotype. However, multivariate analysis demonstrated that only A2143G mutation was responsible for CLR resistance. There were only five samples with A2142G mutation in this study, which is not enough to obtain a statistically significant result, although all five A2142G-harbouring isolates had a CLR-resistant phenotype. Up to now, A2142G and A2143G mutations were considered as the predominant causes of CLR resistance without any controversy. However, in this study there were two clinical *H. pylori* isolates with A2143G mutation and a CLR-susceptible phenotype. Both isolates carried a combination of three mutations (A2143G + T2182C + A2223G and A2143G + T2182C + G2212A, respectively).

The point mutations T2182C, C2195T, A2223G and A2302G were previously considered as factors conferring a CLR-resistant status in *H. pylori*, but the current logistic analysis demonstrated the contrary. In particular, multivariate analysis showed an aOR for T2182C of only 0.12 (95% CI 0.01–1.15). A combination of this analysis and the presence of T2182C in two CLR-susceptible *H. pylori* isolates with A2143G mutation listed above indicated that we should learn more about the role of T2182C in the molecular mechanism of CLR resistance.

In addition, six CLR-resistant *H. pylori* isolates with neither A2142G/C nor A2143G were found, including two non-mutated isolates, two isolates with T2182C + A2223G, one isolate with T2182C + A2223G + C2195T and one isolate with a single G2220A point mutation. The Etest plates were checked very carefully but no colonies in the inhibition ellipse were observed, an indication of

the mixture of resistant and susceptible strains. DNA sequencing results of 23S rRNA domain V of these isolates showed no two-peak signals at each site, an indication of heterozygosis or heterogeneity. These CLR-resistant cases may be caused by changes in efflux ability or another mechanism that would not be revealed by whole-genome sequencing, which represents a limitation of this study.

5. Conclusions

The prevalence of resistance to CLR of clinical *H. pylori* isolates in central Vietnam was very high and increasing significantly. Clinicians should be aware of this important problem when deciding an *H. pylori* eradication regimen. The clinical *H. pylori* strains in this study had a diversity of point mutations in 23S rRNA domain V, which represents a very distinct phenomenon in Vietnam. Further research is necessary to clarify the role of the combination of 23S rRNA domain V mutations in the molecular mechanism of *H. pylori* CLR resistance.

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Competing interests

None declared.

Ethical approval

This study was approved by Ethics Committee of Hue University of Medicine and Pharmacy (Hue, Vietnam). Informed consent was obtained from all of the study participants.

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