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Analysis of allelic variants of *rdxA* associated with metronidazole resistance in *Helicobacter pylori*: detection of common genotypes in *rdxA* by multiplex allele-specific polymerase chain reaction

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ABSTRACT. Resistance to metronidazole (Mtz) in *Helicobacter pylori* is a major problem worldwide, especially in developing countries. Alterations in Mtz nitroreductase enzymes, such as oxygen-insensitive NADPH nitroreductase (RdxA) and NADPH flavin oxidoreductase (FrxA), are the major contributing factors for this resistance. In this study, *rdxA* and *frxA* were amplified, sequenced, and analyzed in 34 Mtz-resistant *H. pylori* isolates (MIC ≥ 8 µg/mL) using multiple allele-specific polymerase chain reaction (MAS-PCR); this method was developed to target the most common genotypes of *rdxA* in *H. pylori*. In this study, the *rdxA* and *frxA* genes in Mtz-resistant *H. pylori* strains

displayed a large number of point mutations. The *rdxA* and *frx*A genes of Mtz-resistant clinical isolates showed a higher percentage of missense mutations (97.1 and 78.6%, respectively) compared to 26695 reference strains; additionally, missense mutations were more common than frameshift (20.6 and 32.1%) and nonsense mutations (8.8 and 10.7%, respectively) in these genes. The most common missense mutations in *rdxA* were D 59 N (94.1%), T 31 E (88.2%), and R 131 K (85.3%). The most common missense mutations in *frxA* were F 72 S (57.1%), G 73 S (57.1%), and C 193 S (53.6%). The developed MAS primers, specific to position 175 and 392 of *rdxA*, successfully amplified the common alleles and distinguished the variants. MAS-PCR could be a useful tool for epidemiological studies of *H. pylori*, associated with Mtz resistance. *rdxA* variants must be screened in order to ensure the effectiveness of Mtz-based *H. pylori* therapies in developing countries.

Key words: *Helicobacter pylori*; Metronidazole; *rdxA* gene; *frxA* gene; MAS-PCR

INTRODUCTION

Helicobacter pylori has been recognized as the major cause of peptic ulcer and gastritis, and a primary risk factor for various types of gastric cancer, including gastric adenocarcinoma and mucosa-associated lymphoid tissue lymphoma (Eidt and Stolte, 1995; Wisniewski and Peura, 1997). Approximately 50% of the world population are infected with *H. pylori*; however, the rate of infection is variable among countries: for example, the infection rate is 62% in China, 27.5% in Japan, 65.6% in Vietnam, and 45.9% in Thailand (Shi et al., 2008; Nguyen et al., 2010; Hirayama et al., 2014; Uchida et al., 2015). Metronidazole (Mtz) is a standard triple therapy recommended by the 1994 National Institute of Health Consensus for *H. pylori* eradication (NIH Consensus Development Panel on *Helicobacter pylori* in Peptic Ulcer Disease, 1994). Mtz is also a critical component of hybrid, concomitant, quadruple, and sequential therapies for *H. pylori* infection (Egan et al., 2007). Reduction of the nitro group in Mtz gives rise to cytotoxic radicals that are essential for the mediation of DNA strand breakage, helix destabilization, unwinding, and ultimately, cell death (Edwards, 1993; Scarpignato, 2004). However, several countries have seen a marked increase in the prevalence of Mtz resistance, leading to treatment failure. Previous studies have demonstrated a geographic variance in the rate of Mtz resistance (44.1% in America, 92.4% in Africa, 37.1% in Asia, and 17.0% in Europe) (De Francesco et al., 2010). Mtz resistance is predominantly dependent on the mutational inactivation of *rdxA*, which encodes an oxygen-insensitive NADPH nitroreductase (Goodwin et al., 1998). Mutations in *frxA*, which encodes the NADPH flavin oxidoreductase, have also been shown to enhance Mtz resistance in the presence of *rdxA* mutations (Kwon et al., 2000; Binh et al., 2015). However, intact *rdxA* or *frxA* have also been implicated in the development of Mtz resistance in *H. pylori* strains; conversely, mutations in *rdxA* or *frxA* have been identified in both Mtz-sensitive and -resistant isolates (Matteo et al., 2006). The exact role of genomic variants in the acquisition of Mtz resistance in *H. pylori* remains controversial. A previous analysis of the *rdxA* and *frxA* nucleotide sequences of individual resistant and

sensitive strains revealed a large number of point mutations (Mirzaei et al., 2014). Frameshift and nonsense mutations in *rdxA* and *frxA* genes are believed to cause Mtz resistance, affecting the efficiency of *H. pylori* eradication (Alfizah et al., 2014). Molecular characterization of sensitive and resistant strains has yielded information that could be useful in assessing the genotypic association with Mtz resistance, phylogenetic evolution, and epidemiological study. Some of the molecular techniques used to study genotypic variations include sequencing, rapid amplified polymorphic DNA (RAPD), polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP), real-time-PCR, denaturing gel gradient electrophoresis, ribotyping, and pulse field gel electrophoresis; however, these methods are sophisticated (requiring high-end instrumentation), time-consuming, and expensive (Kansau et al., 1996; Tee, 1997; Sjunnesson et al., 2003; Rimbara et al., 2013).

Multiplex allele-specific PCR (MAS-PCR) has been previously used in the detection of point mutations, such as single nucleotide polymorphisms associated with genetic diseases and antibiotic resistance in infectious organisms, in various biomedical applications (Bagheri and Rad, 2011; Chia et al., 2012). The perfect match of a 3'-allele-specific primer with the DNA template yields an amplification product, while a mismatch inhibits DNA polymerase elongation, which in turn inhibits the development of an amplification product. In this study, the genomic sequence of *rdxA* and *frxA* was analyzed in 34 Mtz-resistant *H. pylori* clinical isolates from Thailand and the results compared to the findings of previous studies. Moreover, the nucleotide sequence of *rdxA* was determined in 30 *Campylobacter*-like organism-positive (CLO+) samples. The most common genomic variants of *rdxA* were determined by MAS-PCR.

MATERIAL AND METHODS

Bacterial strains and growth conditions

Thirty four Mtz-resistant *H. pylori* clinical isolates (MIC ≥ 8 μg/mL), provided by Dr. Ratha-Korn Vilaichone (Associate Professor, Division of Gastroenterology, Department of Medicine, Thammasat University Hospital, Pathumthani, Thailand), were incubated in 1 mL tryptic soy broth with 15% glycerol at -80°C. *H. pylori* ATCC 51932 was cultured on brain heart infusion agar supplemented with 5% (v/v) sheep blood, and incubated in a micro-aerobic atmosphere (10% CO_2, 5% O_2) at 37°C for 5 days. Thirty samples that were positive for the CLO test, and which were confirmed to be *H. pylori* by PCR (in a previous study), were obtained from the Department of Gastroenterology, Faculty of Medicine, Chulalongkorn University (Champathai et al., 2014).

DNA extraction

Genomic DNA was extracted from all *H. pylori* strains using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA) according to the manufacturer instructions. DNA from CLO+ samples was extracted using the boiling method. Briefly, agar of each CLO+ sample was boiled with 300 μL deionized water in a 1.5-mL microtube for 10 min. The suspension was centrifuged at 7000 *g* for 5 min and 200 μL the supernatant was transferred to a new tube. The supernatant was incubated with 600 μL cold absolute ethanol at -80°C for 10 min and subsequently centrifuged at 7000 *g* for 2 min. The pellet was washed with 70% ethanol, air dried prior to resuspension in 100 μL TE buffer, and stored at -20°C until further use.

PCR amplification of *rdxA* and *frxA*

The *rdxA* gene was amplified by PCR in a 50-µL mixture containing 1X PCR buffer (10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$), 0.2 mM dNTPs, 1.25 U Taq DNA polymerase (New England Biolabs, Ipswich, MA, USA), 0.5 µM each of the *rdxA* primers (5'-GCAGGAGCATCAGATAGTTCT-3' and 5'-GGGATTTTATTGTATGCTACAA-3'), and 100 ng DNA template (Yang et al., 2004). The PCR conditions were set as follows: denaturation at 92°C for 2 min; 40 cycles of denaturation at 95°C for 60 s, annealing at 50°C for 60 s, and extension at 72°C for 60 s; and a final extension at 72°C for 10 min. The 886-bp PCR product was detected by electrophoresing on a 1.5% agarose gel using the TBE buffer (45 mM Tris-borate, 1 mM EDTA; pH 8.0) at 100 V. The gel was stained with ethidium bromide and visualized under a UV transilluminator (G: BOX, SYNGENE, USA).

frxA was amplified in a 50-µL PCR mixture containing 1X PCR buffer (10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$), 0.2 mM dNTPs, 1.25 U Taq DNA polymerase (New England Biolabs), and 0.5 µM each of the *frxA* primers (5'-GGATATGGCAGCCGTTTATCATT-3' and 5'-GAATAGGCATCATTTAAGAGATTA-3') (Yang et al., 2004). The PCR conditions were set as follows: denaturation at 92°C for 2 min; 40 cycles of denaturation at 95°C for 60 s, annealing at 52°C for 60 s, and extension at 72°C for 60 s; and a final extension at 72°C for 10 min. The 780-bp PCR product was detected by electrophoresing on a 1.5% agarose gel, as described previously in this section.

DNA sequence determination, alignment, and phylogenetic analysis

The PCR fragments were purified and sequenced by Bioneer (Daejeon, Korea). The *rdxA* and *frxA* sequences were analyzed using the Basic Local Alignment Search Tool (BLAST; http://blast.ncbi.nlm.nih.gov/Blast.cgi) (Altschul et al., 1990) and aligned with the *H. pylori* 26695 reference strain using BioEdit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). The obtained sequence was phylogenetically analyzed using BioEdit (2013).

Allele-specific primer design

Allele-specific (AS) primers were designed to detect the most common genotype of *rdxA*. The 3' terminal base of each AS primer was adapted to a common allele. Amplification was performed with *rdxA* forward and reverse primers. The primers were synthesized and supplied by BioDesign Co., Ltd. (Pathumthani, Thailand). The primer sequences are listed in Table 1.

Table 1. Primers used in multiple allele-specific polymerase chain reaction (MAS-PCR) to detect the most common alleles in *rdxA*.

Primer	Sequence	Concentration (µM)	Product length (bp)
rdxA-R	5' GCAGGAGCATCAGATAGTTCT 3'	0.4	886
rdxA-F	5' GGGATTTTATTGTATGCTACAA 3'	0.2	
AS59-F	5' GCATTTTGTGATGGTTACTG 3'	0.2	681
AS131-F	5' TCAACCACAGCATGCAAAG 3'	0.1	463

MAS-PCR

Common alleles of *rdxA*, associated with Mtz resistance in *H. pylori*, were determined by MAS-PCR, in a total reaction volume of 50 µL containing 1X PCR buffer (10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$), 0.2 mM dNTPs, 1.5 U *Taq* DNA polymerase (New England Biolabs), and 100 ng DNA template. Optimized concentrations of all primers are summarized in Table 1. The reaction was performed under the following conditions: an initial denaturation at 95°C for 10 min; 10 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 45 s, extension at 72°C for 60 s; 20 cycles of denaturation at 95°C for 30 s, annealing at 64°C for 45 s, extension at 72°C for 60 s; 20 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 45 s, extension at 72°C for 60 s; and a final extension at 72°C for 10 min. The 886-, 681-, and 463-bp MAS-PCR products were analyzed by electrophoresing on a 1.5% agarose gel.

RESULTS

Effect of *rdxA* and *frxA* mutations on Mtz resistance in *H. pylori*

The effect of mutations in *rdxA* and *frxA* on Mtz resistance in *H. pylori* was analyzed in 34 *H. pylori* clinical strains and 26695 reference strains (Figures 1 and 2). Missense and frameshift mutations were observed in *rdxA* in 33 (97.1%) and 7 (20.6%) strains, respectively. On the other hand, truncated *rdxA* was detected in 3 strains (8.8%). Missense mutations at positions 1, 4, 10, 21, 37, 41, 74, 79, 91, 92, 104, 106, 113, 115, 116, 176, 183, 193, and 203 in *rdxA* have not been reported in published Mtz-resistant strains. Additionally, frameshift mutations at positions 4, 6, 9, 71, 72, 79, and 182 and nonsense mutations at positions 2 and 52 were unique to our study. Analysis of *frxA* mutations revealed 78.6% missense mutations, 32.1% frameshift mutations, and 10.7% nonsense mutations (Figure 2). Missense mutations at positions 1, 27, 35, 37, 39, 60, 65, 66, 67, 70, 94, 131, 154, 177, 200, and 206, a frameshift mutation at position 109, and a nonsense mutation at position 30 were unique to our study. The *rdxA* and *frxA* genes were non-mutated in 1 and 3 Mtz-resistant isolates, respectively.

Phylogenetic analysis of the *rdxA* and *frxA* trees (Figures 3 and 4) provided further evidence of the high genomic diversity found at the nucleotide level. The topologies of both trees were different, and the strains did not cluster significantly, based on the *rdxA* and *frxA* sequences.

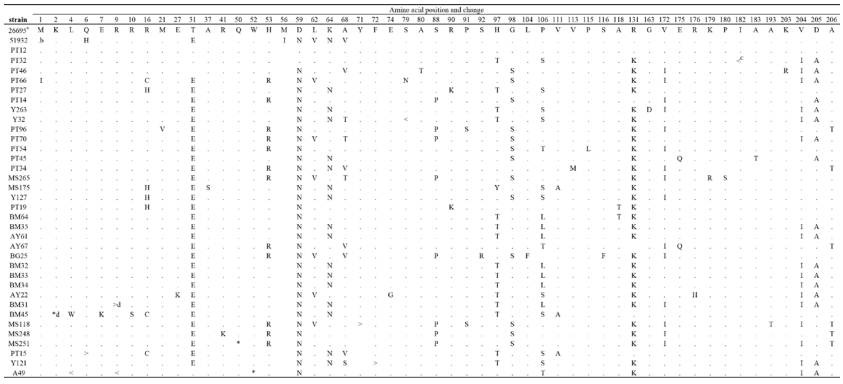

Figure 1. Alignment of RdxA from 34 Mtz-resistant *Helicobacter pylori* clinical isolates and ATCC 51932.

strain	Amino acid position and change																																										
	1	7	16	18	27	30	34	35	37	39	40	43	44	60	65	66	67	68	70	72	73	94	96	109	111	117	124	126	129	130	131	132	154	169	176	177	193	200	206	208
26695a	M	V	A	K	Q	W	V	E	G	L	A	S	I	K	P	M	A	W	A	F	G	D	V	D	N	I	N	M	N	S	E	R	A	E	E	E	C	E	R	K
PT12	.b	I	.	.	.	*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	*e	.	.	.	.	.	.	.	.
PT32	V	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	S	S	.	.	.	H	.	S	.	.	.	.	.	.	.	.	.	S	.	.	.
PT66	.	I	T	.	.	.	.	.	.	.	.	A	F	.	.	.	V	.	.	S	S	.	.	.	D	M	S	.	.	.	G	.	.	.	K	.	S	.	.	.
PT14	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	S	S	.	.	.	H	.	.	.	T	D	.	.	.	.	.	.	.	.	.	.
Y263	.	.	T	.	.	.	.	.	.	.	.	V	.	.	.	.	.	.	.	S	S	G	.	.	H	.	.	.	.	.	.	.	.	.	.	.	S	.	L	.
PT96	.	.	T	.	.	.	I	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
PT70	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
PT54	.	I	.	<c	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	H	.	.	.	.	.	.	.	.	.	.	K	.	.	.	.
PT45	.	.	.	<	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
PT34	.	I	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	>d	.	.	.	.	.	.	.	.	.	K	.	.	.	K	.	.
Y127	.	.	.	.	.	.	.	.	.	.	.	.	V	.	Q	I	.	.	V	S	S	.	.	.	.	.	S	.	.	.	.	.	.	.	K	.	S	.	.	.
PT19	.	.	.	<	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
BM64	.	.	T	.	.	.	.	.	.	.	.	.	V	.	.	.	.	.	V	S	S	.	.	.	H	.	S	.	.	.	.	.	.	.	.	.	S	.	.	.
BM35	.	I	.	.	.	.	.	.	.	.	.	.	V	.	.	I	.	.	.	S	S	.	I	>	D	.	.	.	.	.	.	.	.	.	K	.	S	.	.	.
AY61	.	I	T	.	.	.	.	.	.	.	.	A	F	.	.	.	.	.	.	S	S	.	.	.	D	M	S	.	.	N	.	.	.	.	K	.	S	.	.	.
AY67	.	I	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	S	S	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
BG25	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	V	.	.	.	S	.	.	.
BM32	.	I	.	.	.	.	.	.	.	.	.	.	V	.	.	I	.	.	.	S	S	.	I	>	D	.	.	.	.	.	.	.	.	.	K	.	S	.	.	.
BM33	.	I	.	.	.	.	.	.	.	.	.	.	V	.	.	I	.	.	.	S	S	.	I	>	D	.	.	.	.	.	.	.	.	.	K	.	S	.	.	.
BM34	.	I	.	.	.	.	.	.	.	.	.	.	V	.	.	I	.	.	.	S	S	.	I	>	D	.	.	.	.	.	.	.	.	.	K	.	S	.	.	.
AY22	.	.	T	.	.	.	.	.	.	.	.	.	.	R	S	.	.	.	.	S	S	.	.	.	H	.	S	I	.	.	.	.	.	.	K	.	S	.	.	.
BM31	.	I	.	.	.	.	.	.	.	.	.	.	V	.	.	I	.	.	.	S	S	.	I	>	D	.	.	.	.	.	.	.	.	.	K	.	S	.	.	.
BM45	.	I	T	.	.	.	.	.	.	.	.	.	F	.	.	.	.	.	.	S	S	.	.	.	D	M	S	.	.	.	.	.	.	.	K	.	S	.	.	N
PT15	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Y121	.	.	T	.	H	.	.	D	C	F	H	.	.	.	.	.	.	.	V	S	S	.	.	.	H	.	.	.	.	.	.	.	.	.	.	.	S	.	.	.
A49	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	K	.	.	.	.	.	.	.
MS251	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
MS248	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.

Figure 2. Alignment of FrxA from 28 Mtz-resistant *Helicobacter pylori* clinical isolates.

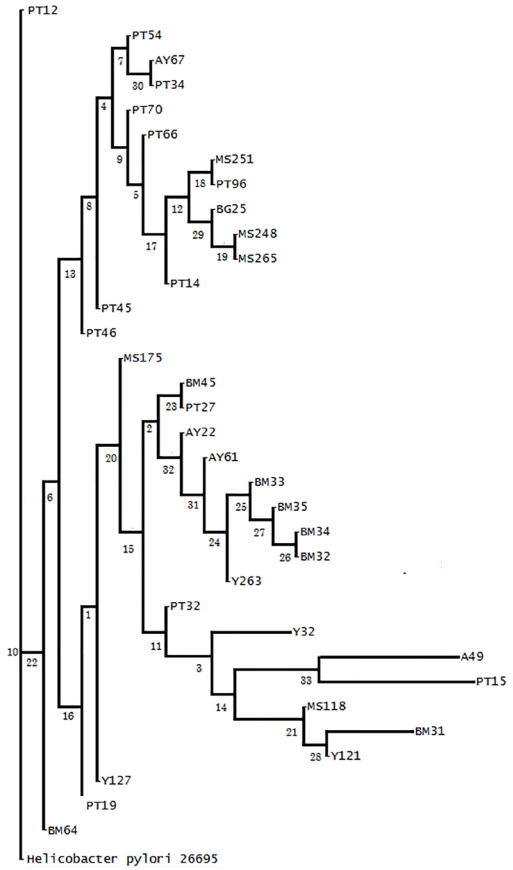

Figure 3. Phylogenetic tree generated from nucleotide sequences of *rdxA* from 34 Mtz-resistant *Helicobacter pylori.*

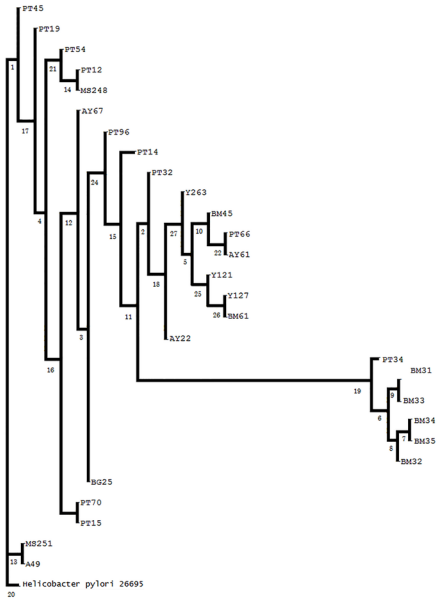

Figure 4. Phylogenetic tree generated from nucleotide sequences of *frxA* from 28 Mtz-resistant *Helicobacter pylori.*

Analysis of genomic variants of *rdxA* in Mtz-resistant *H. pylori* isolates and CLO+ samples

The frequency of *rdxA* missense mutations in the 34 Mtz-resistant *H. pylori* isolates and 30 CLO+ samples is summarized in Table 2. The most common *rdxA* alleles in Mtz-resistant *H. pylori* and CLO+ samples were A (94.1%) and A (96.7%) at position 59 [Asp → Asn, G (175) → A], GA (88.2%) and GA (80.0%) at position 31 [Thr → Glu, AC (91, 92) → GA], and A (85.3%) and A (76.7%) at position 131 [Arg → Lys, G (392) → A].

Table 2. Genetic variants of *rdxA* missense mutations in 34 Mtz-resistant *Helicobacter pylori* isolates and 30 positive CLO test samples.

Type of mutation	Nucleotide position	Mtz-resistant *H. pylori* (%)	Positive CLO test samples (%)	Type of mutation	Nucleotide position	Mtz-resistant *H. pylori* (%)	Positive CLO test samples (%)	Type of mutation	Nucleotide position	Mtz-resistant *H. pylori* (%)	Positive CLO test samples (%)
G→A	175	94.1	96.7	T→G	11	2.9	0	G→A	29	0	36.7
AC→GA	91, 92	88.2	80	A→T	30	2.9	0	A→G	106	0	36.7
G→A	392	85.3	76.7	G→A	19	2.9	10	A→G	17	0	26.7
A→C	614	50	33.3	A→G	61	2.9	0	C→T	548	0	10
G→A	610	50	23.3	G→A	79	2.9	0	C→G	60	0	6.7
A→T	192	47.1	36.7	G→T	109	2.9	0	AT→GA	106, 107	0	6.7
CAC→ACG	289, 290, 291	47.1	20	GG→AA	122, 123	2.9	0	A→C	18	0	3.3
G→A	514	44.1	33.3	G→T	202	2.9	0	A→C	21	0	3.3
G→A	292	38.2	20	A→G	221	2.9	0	A→C	24	0	3.3
A→G	158	35.3	16.7	G→A	236	2.9	0	AA→CC	22, 24	0	3.3
C→T	316	29.4	10	G→A	238	2.9	0	AAA→CTC	22, 23, 24	0	3.3
T→C	262	23.5	16.7	C→G	276	2.9	0	AG→CA	28, 29	0	3.3
CC→TT	316, 317	20.6	10	C→T	289	2.9	3.3	A→G	77	0	3.3
G→A	616	17.6	26.7	CC→TT	310, 312	2.9	0	G→A	89	0	3.3
T→G	184	17.6	6.7	G→A	337	2.9	0	C→T	117	0	3.3
C→T	203	14.7	10	C→T	344	2.9	0	CG→TA	114, 145	0	3.3
G→A	47	11.8	0	C→T	347	2.9	0	C→A	152	0	3.3
C→A	316	8.8	6.7	G→A	448	2.9	0	AT→CG	308, 309	0	3.3
T→C	332	8.8	3.3	G→A	527	2.9	0	C→G	316	0	3.3
C→T	46	8.8	0	A→G	536	2.9	0	G→A	367	0	3.3
G→A	202	8.8	0	C→T	538	2.9	0	G→T	427, 429	0	3.3
G→A	269	5.9	23.3	G→A	547	2.9	3.3	T→G	479	0	3.3
G→A	352	5.9	3.3	G→A	577	2.9	0	G→T	514, 516	0	3.3
G→C	523	5.9	3.3	AA→GG	608, 609	2.9	0	G→A	525	0	3.3
CC→TT	271, 273	5.9	3.3	G→C	42	0	46.7				
G→A	3	2.9	0	G→A	168	0	43.3				

MAS-PCR assay to detect common *rdxA* variants

The samples were subjected to MAS-PCR using *rdxA* forward and reverse primers and two AS-primers. The 3'-terminal base was designed to be guanine (G) in both forward AS-primers (AS59-F and AS131-F; at positions 175 and 392, respectively) of the *rdxA* gene. The MAS-PCR results are summarized in Figure 5. The 3'-end of the AS primer pairs (with their corresponding common alleles) yielded 886-, 681-, and 463-bp strands. The amplification products of AS-primers did not show a 3'-end mismatch. The positive 886-bp band generated by the main *rdxA* primer was used as the internal control.

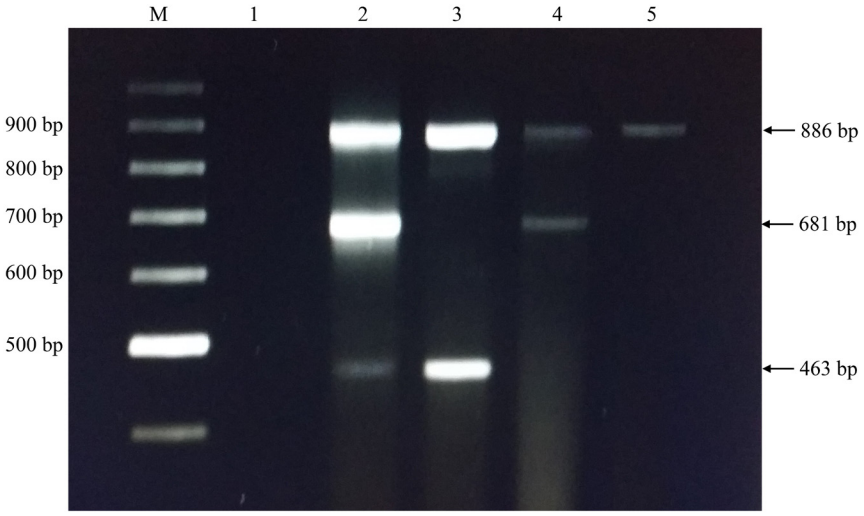

Figure 5. Agarose gel electrophoresis (1.5%) of MAS-PCR products. *Lane M* = 100-bp molecular marker, *lane 1* = negative control, *lane 2 = rdxA* without mismatch, *lane 3 = rdxA* with amino acid substitution at position 59, *lane 4 = rdxA* with amino acid substitution at position 131, *lane 5 = rdxA* with amino acid substitution at positions 59 and 131.

DISCUSSION

Antibiotic resistance is a major obstruction to successful *H. pylori* eradication. Metronidazole is broadly used in the treatment of various infections, and is known to challenge the development of resistance (Jenks and Edwards, 2002). The prevalence of Mtz resistance in *H. pylori* is higher than that of clarithromycin and amoxicillin, the drugs used to treat *H. pylori* infections, in many countries, especially developing countries. The resistance rates of *H. pylori* against antibiotics such as Mtz, clarithromycin, and amoxicillin are 26.7, 17.2, and 11.2%, respectively. On the other hand, the resistance rates of *H. pylori* against Mtz, clarithromycin, and amoxicillin are 37.1, 18.9, and 11.6%, respectively (De Francesco et al., 2010). The evolution of Mtz resistance in *H. pylori* is associated with mutational inactivation of the *rdxA* and *frxA* genes (Kwon et al., 2000). The crystal structure of RdxA revealed a homodimer that underwent domain swapping with two molecules of FMN bound at the dimer interface; moreover, a cysteine side chain close to FMN was believed to be involved in the reducing activity (Martínez-Júlvez M et al., 2012). In this study, all Mtz-resistant *H.*

pylori clinical isolates harbored several point mutations, such as missense mutation (97.1%), frameshift mutations (20.6%), and nonsense mutations (8.8%). Previous studies have reported the presence of various missense mutations in *rdxA* in both susceptible and resistant strains, via amino acid substitutions. The exact genotype patterns of missense mutations contributing to Mtz-resistance remain to be elucidated, while the three most common alleles, adenine at position 175 (Asn59), guanine and adenine at positions 91 and 92 (Glu31), and adenine at position 392 (Lys131), were identified in Mtz-resistant *H. pylori* clinical strains. These common genotypes were also observed in the CLO+ samples (96.7, 80, and 76.7% for alleles A, G and A, and A, respectively). These *rdxA* variants were also unique in susceptible and resistant *H. pylori* strains analyzed in previous studies (Tankovic et al., 2000; Mirzaei et al., 2014), in agreement with our findings. The Mtz-resistant role of these *rdxA* alleles in *H. pylori*, however, remains to be further elucidated. A phylogenetic tree constructed by Solca et al. (2000), who aligned 30 *rdxA* nucleotide sequences, could not specify the cluster associated with the resistance phenotype, as the resistant and susceptible strains were randomly distributed throughout the dendrogram. In this study, the phylogenetic analysis of 34 *rdxA* nucleotide sequences obtained from Mtz-resistant *H. pylori* and 30 CLO+ samples revealed high genetic polymorphism. However, no particular characteristic could be clustered by the phylogenetic tree topologies of four *H. pylori* housekeeping genes (*atpD*, *scoB*, *glnA*, and *recA*) that arose from frequent recombination, similar to the results obtained in a previous study (Maggi Solca et al., 2001).

Some amino acid substitutions, such as Arg16His (N = 4), His53Arg (N = 12), Gly163Asp (N = 1), Val204Ile (N = 17), Ala206Thr (N = 6), were observed only in resistant isolates, as seen in previous studies (Jeong et al., 2000; Solca et al., 2000; Yang et al., 2004; Matteo et al., 2006). In this study, three Mtz-resistant *H. pylori* isolates contained stop codons (at position 2, 50, and 52). Similarly, Yang et al. (2004) reported a stop codon at position 50 of *rdxA* in the resistant phenotype. Frameshift mutations producing a truncated RdxA protein were commonly associated with resistance worldwide, with a minimum inhibitory concentration of 64-128 mg/L (Yang et al., 2004; Matteo et al., 2006). We also identified a number of novel missense mutations at positions 1, 4, 10, 21, 37, 41, 74, 79, 91, 92, 104, 106, 113, 115, 116, 176, 183, 193, and 203. Mutations in RdxA could lead to a loss of reductase activity, which is strongly correlated with Mtz resistance. Mutations such as those affecting residues Arg16, Ser18, Lys20, Asn73, Ile142, Gly162, and Lys200 were expected to decrease the affinity of the apoprotein for the FMN cofactor. Mutations in Leu42, Ser43, Arg41, Gln50, Val55, Met56, Ile142, Gly145, Lys202, and Leu209 residues were theorized to destabilize dimer formation. Additionally, mutations in Cys19, Tyr47, and Cys159 were believed to be associated with the redox function of the enzyme and those in Gly149 and His17 were expected to cause protein destabilization not related to dimerization (Martínez-Júlvez et al., 2012).

In this study, mutations in *frxA* gene were found in 89.3% Mtz-resistant *H. pylori* clinical isolates (78.6% of missense mutations, 32.1% frameshift mutations, and 10.7% nonsense mutations). Mutations in *frxA* do not contribute significantly to Mtz resistance; however, alterations in this gene enhance the level of Mtz resistance in *rdxA* mutants (Yang et al., 2004; Binh et al., 2015). The exact role of genomic variants of *rdxA* and *frxA* in MTZ resistance in *H. pylori* remains controversial. The structure and mechanism of FrxA mutations, which are also associated with Mtz resistance, have not been elucidated in this study.

MAS-PCR is a rapid, sensitive, specific, inexpensive (using a basic thermal cycler) method, and was therefore used in this study to detect point mutations. In this study, we

successfully developed a MAS-PCR assay to detect the two most common genetic variants of *rdxA* in *H. pylori*. The 3'-terminal base of each AS primer was designed to be guanine, in order to detect the variants at positions 175 and 392. MAS-PCR conditions were optimized to distinguish the common alleles and their variants. This assay could be a useful tool for epidemiological studies and in the future development of molecular typing, which would facilitate the identification of *H. pylori* strains, prediction of antibiotic susceptibility, and public health management (prevention and surveillance).

Conflicts of interest

The authors declare no conflict of interest.

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