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rdxA Gene is an Unlikely Marker for Metronidazole Resistance in the Asian *Helicobacter pylori* Isolates

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Abstract Mutations in the *rdxA* gene had been reported to be associated with metronidazole resistance in *Helicobacter pylori*. In this study, sensitivity to metronidazole, RAPD profiles, and DNA sequences of the *rdxA* gene of 32 local *H. pylori* isolates were analyzed. Of these, 13 were found to be resistant, while 19 were sensitive to metronidazole. Among the 32 isolates, 10 were paired isolates from the antrum and body of the stomach of individual patients. Interestingly, the RAPD profiles of isolates from individual patients were distinctly different from each other, whereas paired isolates from the same patient were identical regardless of their sensitivities to metronidazole. DNA sequences of the *rdxA* gene of all 32 isolates showed 95% to 97% homology when compared with the HP0954 locus of *H. pylori* 26695 genome. From the 19 metronidazole-sensitive strains, 10 (with MIC≤ 0.5 μg/ml metronidazole) were selected and induced to become metronidazole resistant by sequentially passaging through serial 2-fold increasing concentrations of metronidazole. Nine of the 10 induced paired isolates showed mutations in the *rdxA* sequences which resulted in truncated protein or changes in the translated amino acid sequences. However, the changes did not occur at any specific site in the DNA or amino acid sequences of the *rdxA* gene of all the isolates analyzed. The results show that the *rdxA* gene cannot be a definitive marker for metronidazole resistance in *H. pylori* isolates of an Asian population, and that other factors may contribute to resistance to metronidazole.

Key words: *Helicobacter pylori*, metronidazole, *rdxA* gene, RAPD

The increasing trend of antibiotic resistant *Helicobacter pylori* was often suggested as the main cause of treatment failure [18, 19, 21]. Metronidazole, which is commonly used as part of the triple therapy, showed an increasing rate of resistance from 20% in late 1995 to 62% in early 1997 in clinical isolates from Singapore [7]. Several mechanisms have been suggested to cause metronidazole resistance, including the decrease in metronidazole uptake [23], increased DNA repair [3], decreased oxygen scavenging capabilities [2, 24], and deficiency in metronidazole activation (unable to reduce nitro- group) [4, 5, 10, 27].

Recent studies have demonstrated the presence of mutations in the *rdxA* gene that encodes an oxygen-insensitive NADPH nitroreductase in the metronidazole-resistant *H. pylori* [4, 5, 10, 27]. The mutation impaired the ability of metronidazole-resistant *H. pylori* to reduce the nitro group of the drug to form hydroxylamine derivative, which damages the bacterial DNA, and hence resulting in cell death [5]. In the study by Goodwin *et al.* [5], 1–3 bp point mutation at random was observed in the *rdxA* gene of paired metronidazole-resistant and metronidazole-sensitive isolates. In contrast, frameshift, insertion, deletion, and missense point mutations of the *rdxA* gene were found in various other studies [4, 10, 27]. Tankovic *et al.* [27] suggested that a variety of genetic alterations in *rdxA* are associated with metronidazole-resistant *H. pylori*.

In this study, we examined the DNA sequence of the *rdxA* gene of local individual and paired *H. pylori* isolates with respect to their susceptibility to metronidazole.

Materials and Methods

Isolation of Clinical Strains

The bacterial strains were isolated on chocolate blood agar supplemented with antibiotics [9]. *H. pylori* isolates were confirmed by Gram staining, urease, oxidase, and catalase tests. A total of 32 strains isolated from patients with gastroduodenal disease were included in the study. Of these, 10 were paired isolates from the antrum and body of the stomach of the respective patient, abbreviated as 'a' and 'b,' respectively. These paired isolates were termed the

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Table 1. Metronidazole susceptibility of *H. pylori* isolates from the re-visit patients.

Patient number	First visit Strain no.	Re-visit Strain no.	Metronidazole susceptibility
1	#53a		R
		#65a	S
		#65b	S
2	#85a		R
	#85b		S
		#117a	R
		#117b	R
3	#115a		R
	#115b		R
		#164a	R
		#164b	R

a: antrum; b: body.
R: resistant; S: sensitive.

'clinical paired isolates.' The remaining 22 isolates were individual strains isolated from the gastric antrum of individual patients, from which 10 metronidazole-sensitive strains were induced to be metronidazole resistant, and these 10 pairs were termed as the 'induced paired isolates.'

The clinical paired isolates were obtained from 3 re-visit patients (following treatment failure). The metronidazole sensitivity of the isolates and the strain numbers of isolates from the respective patients are as shown in Table 1. There were 5 pairs of antrum and body isolates. *H. pylori* was isolated only from the antrum (#53a) of Patient 1 during the first visit. However, #65a & b were obtained from the antrum and body of Patient 1 during the re-visit. The isolates obtained from the antrum and body during the first visit of Patient 2 (#85a & b) and Patient 3 (#115a & b) as well as from the re-visit of the respective patients (#117a & b and #164a & b) are as tabulated in Table 1.

Induction of Metronidazole Resistance

A total of 10 metronidazole-sensitive strains were induced to become metronidazole resistant by sequentially passaging through serial 2-fold increasing concentrations of metronidazole as illustrated in Fig. 1. The metronidazole-sensitive *H. pylori* isolate was first cultured in the presence of metronidazole at a concentration just above its minimum inhibition concentration (MIC) (e.g., 0.1 μg/ml was used for a strain with MIC of 0.094 μg/ml). After incubation under microaerobic conditions at 37°C for 3 days, the clone was then subcultured into brain heart infusion (BHI) broth containing 2-fold higher concentration of metronidazole. At each subsequent 2-fold higher concentrations of metronidazole, the strain was subcultured for 3 generations before the MIC of the 'induced' strains was measured using the E-test. The chromosomal DNA of the original metronidazole sensitive and resulting 'induced resistant strains' were extracted for RAPD profiling and DNA sequencing.

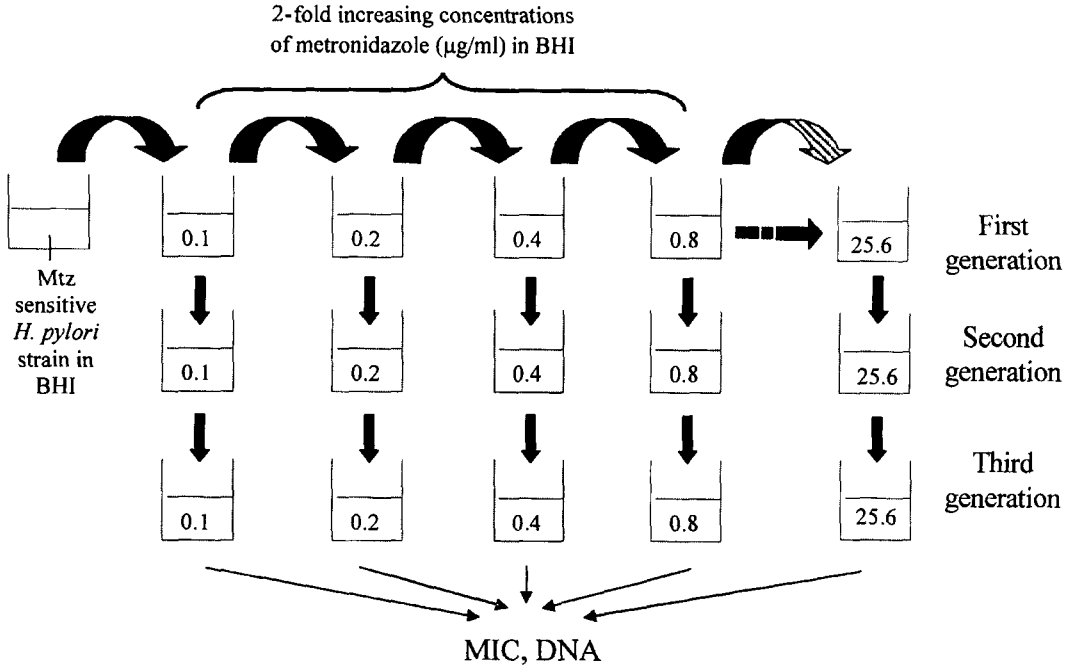

Fig. 1. Induction of metronidazole resistance.
H. pylori strains with an initial metronidazole MIC of ≤0.016 to 0.5 μg/ml in brain heart infusion (BHI) broth was sequentially passaged through serial 2-fold increasing concentrations of metronidazole.

Antibiotic Sensitivity Test

E-test was used to determine the antibiotic susceptibility of the *H. pylori* isolates [13]. In brief, the E-test strip (AB Biodisk, Solna, Sweden) was placed on the surface of a Mueller Hinton blood agar plated with an inoculum of *H. pylori* adjusted to a density of number 3 McFarland standard [7]. *H. pylori* strain was considered to be resistant to metronidazole if the MIC was ≥8 μg/ml [13, 27].

Chromosomal DNA Extraction

The chromosomal DNA was extracted from *H. pylori* by the phenol-chloroform extraction method as described by Hua *et al.* [8].

Polymerase Chain Reaction-Based Random Amplification of Polymorphic DNA (PCR-RAPD)

The PCR-RAPD was performed using an universal primer (5'-AACGCGCAAC-3') on 50 ng of DNA template. The amplification reaction included an initial step of denaturation of target DNA at 94°C for 5 min, followed by 39 cycles of denaturation at 94°C for 1 min, annealing at 360°C for 1 min, and elongation at 72°C for 2 min.

Polymerase Chain Reaction (PCR)

The PCR of *rdxA* gene was performed with primers modified from those used by Jenks *et al.* [10] (forward: 5'-CGT-TAGGGATTTTATTGTATGCTAC-3' and reverse: 5'-CAC-CCCTAAAAGAGCGATTAAAACC-3'). The amplification reaction included an initial step of denaturation of target DNA at 94°C for 5 min, followed by 39 cycles of denaturation at 94°C for 1 min, annealing at 57°C for 1 min, and elongation at 72°C for 1 min. The final cycle consisted of elongation at 72°C for 5 min.

DNA Sequencing

The *rdxA* PCR product obtained was purified using the QIAquick purification kit (Qiagen, Hilden, Germany). The purified PCR product was used as the template for sequencing using the Big-Dye Terminator sequencing kits (Applied Biosystems). The primers used were described as above. Sequencing was performed on the ABI 377 DNA sequencer (Applied Biosystems, Seoul, Korea).

RESULTS

Antibiotic Sensitivity Test

Table 1 shows that the single *H. pylori* isolate from the gastric antrum of Patient 1 during the first visit (#53a) was resistant to metronidazole, while the subsequent isolates (after treatment) from the antrum and body of the stomach of the same patient during the re-visit (#65a & b, respectively) were found to be metronidazole sensitive. Interestingly, the *H. pylori* isolate from the antrum (#85a) of Patient 2 was resistant to metronidazole, while the isolate from the body (#85b) was shown to be sensitive to metronidazole. However, both strains (#117a & b) isolated from the antrum and body of this patient on re-visit were tested to be metronidazole resistant. In contrast, the two pairs (#115a & b and #164a & b) obtained from the antrum and body of Patient 3 during the two separate visits were all resistant to metronidazole.

Of the remaining 21 *H. pylori* isolates from individual patients, 5 were shown to be resistant to metronidazole and 16 were sensitive to metronidazole. In all, there were 13 metronidazole-resistant and 19 metronidazole-sensitive strains of *H. pylori*.

All 10 out of the 16 sensitive strains that were exposed in a stepwise manner to increasing concentrations of metronidazole showed a gradual increase in their MIC values of metronidazole sensitivity to become metronidazole resistant. The MIC values of the induced resistant strains ranged from 24 to >256 μg/ml after exposing to a metronidazole concentration of 3.2 μg/ml (Table 2). All the strains when exposured further to higher concentrations

Table 2. MIC values (μg/ml) of the original parent and induced resistant strains (after induction with 3.2 μg/ml of metronidazole) based on E-test.

Strain number	Original MIC (μg/ml)	MIC after induction at 3.2 μg/ml (μg/ml)
966	0.047	48
993	0.094	>256
957	<0.016	48
1223	0.094	24
428	0.25	>256
SS1	0.032	96
905	<0.016	>256
965	0.19	>256
848	0.25	256
920	0.5	256

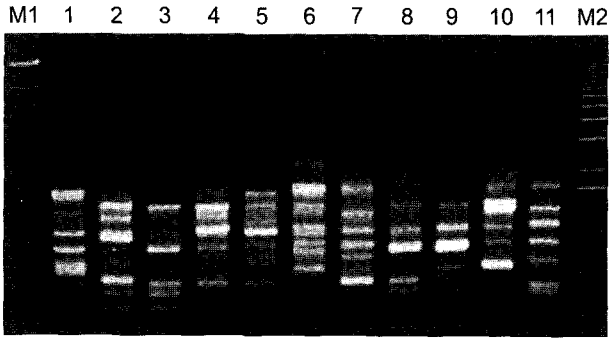

Fig. 2. RAPD profiles of individual strains.
M1: λ DNA digested with *Hin*dIII; M2: 1 kb DNA ladder (Gibco); Lane 1: 694R; Lane 2: 871R; Lane 3: 888R; Lane 4: 903R; Lane 5: 925R; Lane 6: 695R; Lane 7: 836R; Lane 8: 852S; Lane 9: 861S; Lane 10: 892S; Lane 11: 897S. (a: antrum; S: sensitive; R: resistant).

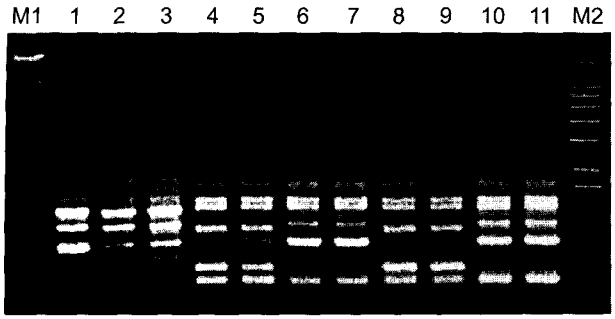

Fig. 3. RAPD profiles of clinical paired isolates.
M1: λ DNA digested with *Hin*dIII; M2: 1 kb DNA ladder (Gibco); Lane 1: 53aR; Lane 2: 65aS; Lane 3: 65bS; Lane 4: 85aR; Lane 5: 85bS; Lane 6: 115aR; Lane 7: 115bR; Lane 8: 117aR; Lane 9: 117bR; Lane 10: 164aR; Lane 11: 164bR. (a: antrum; b: body; S: sensitive; R: resistant).

of metronidazole gave an MIC value of greater than 256 μg/ml.

RAPD

The RAPD profiles of each *H. pylori* strain showed a total of 7 to 11 bands ranging from approximately 300 bp to 2.5 kb (Figs. 2 and 3). As shown in Fig. 2, the DNA fingerprints of isolates from all individual patients demonstrated different DNA patterns. However, the DNA profiles of the clinical paired isolates from the respective patients were all identical (Fig. 3) regardless of the differences in susceptibilities to metronidazole (for #85a & b obtained from Patient 2 during first visit; Fig. 3, Lanes 4 & 5) or similar metronidazole susceptibility in strains #117a & b (Fig. 3, Lanes 8 & 9) obtained during the re-visit. Similarly, the RAPD profiles of the 4 isolates from Patient 3 obtained during the two separate visits also showed identical profiles (Fig. 3, Lanes 6, 7, 10, &11).

The RAPD profiles of the metronidazole-sensitive #1223 and their induced strains at the various concentrations of metronidazole showed similar DNA fingerprints (Fig. 4). The other 9 induced paired isolates also demonstrated

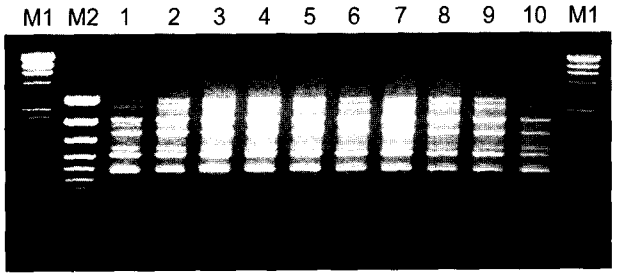

Fig. 4. RAPD profiles of *H. pylori* #1223 and their induced strains at various concentrations of metronidazole.
M1: λ DNA digested with *Hin*dIII; M2: 100 bp DNA ladder Plus (Fermentas); Lane 1: metronidazole sensitive #1223; Lanes 2- 10: #1223 induced at 0.1, 0.2, 0.4, 0.8, 1.6, 3.2, 6.4, 12.8, 25.6 μg/ml of metronidazole, respectively.

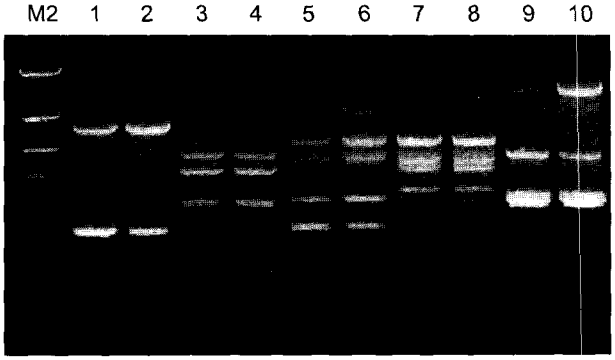

Fig. 5. RAPD profiles of 5 of the induced paired isolates.
M2: 100 bp DNA ladder Plus (Fermentas). Lanes 1 & 2: induced pair of #SS1; Lanes 3 & 4: induced pair of #957; Lanes 5 & 6: induced pair of #965; Lanes 7 & 8: induced pair of #966; Lanes 9 & 10: induced pair of #993.

identical DNA fingerprints before (metronidazole sensitive) and after (metronidazole resistant) metronidazole induction (Fig. 5).

DNA and Amino Acids Sequences

DNA sequences of the *rdxA* gene of all the 32 *H. pylori* isolates showed 95%- 97% homology when compared with the HP0954 sequence of *H. pylori* 26695 genome [28]. The number of nucleotide changes ranged from 20 to 35 bp, corresponding to 3.1% to 5.5% out of the total 633 bp of the *rdxA* gene. Majority of the nucleotide changes were G↔A and C↔T base substitutions. However, no specific definitive mutation sites were found among all the 13 metronidazole-resistant strains.

The translated amino acid sequences of the 32 strains shared 92% to 97% homology with that of HP0954. There were between 6 to 16 amino acids substitutions, corresponding to 2.9% to 7.6% of the total of 210 amino acids. No specific changes in the amino acid sequences in all the 13 metronidazole-resistant strains were observed.

Among the clinical paired isolates that had the same metronidazole susceptibility (#65a & b, #115a & b, #117a & b, #164a & b), the nucleotide and amino acid changes in the *rdxA* gene sequences were the same in both isolates of each respective pair when compared with the HP0954 locus. It is interesting to note that all the 4 metronidazole-resistant isolates (#115a & b and #164a & b) from Patient 3 isolated during the two different visits showed similar nucleotide and amino acids sequences.

Three (#85a, #117a & b) out of the 4 isolates from Patient 2 showed 28 nucleotide differences, while the last one (#85b) showed 25 nucleotide differences, when compared with HP0954. These 3 nucleotide differences were at the base positions 37, 84, and 357 (Table 3). Interestingly, the 3 metronidazole-resistant strains (#85a, #117a & b) showed nucleotides C, C, and G at positions 37, 84, and 357, respectively, while #85b (metronidazole sensitive) shared

Table 3. Comparison of the nucleotide and translated amino acid sequences of the paired isolates from Patient 2.

	Nucleotide base positions[1]			Corresponding amino acid residue positions[2]		
Strain no.	37	84	357	13	28	119
HP0954	T	T	A	L	F	Q
#85aR	C	C	G	L	F	Q
#85bS	T	T	A	L	F	Q
#117aR	C	C	G	L	F	Q
#117bR	C	C	G	L	F	Q

[1]Positions refer to the *rdxA* coding region in *H. pylori* 26695 [28].

[2]Positions refer to the RdxA protein of *H. pylori* 26695 [28].

a: antrum; b: body.

R: resistant; S: sensitive.

A: adenine; T: thymidine; C: cytosine; G: guanine.

L: leucine; F: phenylalanine; Q: glutamine.

the same base sequence as that of HP0954 at these 3 base positions with nucleotides T, T, and A, respectively. It was noted that the respective amino acid residues at these positions of all the 4 isolates were identical despite changes in the nucleotide sequences in 3 of the strains (Table 3).

The nucleotide sequences of the *rdxA* gene of the induced paired isolates were compared, and the results were as tabulated in Table 4. Of the 10 induced paired isolates, 9 pairs showed mutations in the *rdxA* gene. However, the 10th pair (#920) did not show any changes in their *rdxA* gene sequence despite their different metronidazole susceptibility (Table 4).

Interestingly, there were 3 induced pairs (#957, #966, #993) that showed insertion of a nucleotide A at the base position 193 of the *rdxA* gene which resulted in a frameshift, giving rise to a premature stop codon downstream at the 73rd amino acid. Four out of the 9 induced pairs had base substitutions of which two pairs resulted in a premature stop codon at the 32nd and 35th amino acids, respectively, while the remaining two pairs gave rise to amino acids substitutions. Deletion of a 2 bp and a 9 bp fragment was observed in the other 2 induced pairs, respectively. The 2 bp deletion led to a frameshift, which eventually resulted in a premature stop codon at the 157th amino acids. The 9 bp deletion brought about the deletion of 3 amino acids from positions 36 to 38, resulting in the loss of isoleucine, alanine, and glutamic acid, respectively.

DISCUSSION

The DNA fingerprints observed in all the 32 individual *H. pylori* isolates showed different profiles (Fig. 2) indicating inter-strain differences, thereby highlighting that different *H. pylori* strains exist in each of these patients reported earlier [7, 8, 20]. It was therefore not surprising to observe similar RAPD profiles between clinically paired isolates from the same individual patients, as it has been reported that single strain of *H. pylori* predominates in both antrum and body of the stomach of individual patients [7]. RAPD profiles observed in isolates from all the 3 re-visit patients obtained before and after the treatment were identical (Fig. 3), indicating the strong likelihood of recrudescence of *H. pylori* infection.

Of interest in this study are the clinically paired isolates that had different sensitivities to metronidazole but with identical DNA fingerprinting patterns (Fig. 3, #85a & b). This suggests the involvement of probably only a short segment of gene sequence in the mutation of metronidazole sensitive *H. pylori* to metronidazole resistant. The high genetic relatedness of this clinical pair as revealed by the RAPD also implies that the metronidazole resistance had arisen essentially by *de novo* mutation, rather than as a result of the coexistence of metronidazole-sensitive and

Table 4. Comparison of the nucleotide and translated amino acid sequences of the induced paired isolates.

Strain number	Type of mutation	Position[1]	Consequence
957	Insertion of A	193	aa changed at 65th with stop at 73rd aa
966	Insertion of A	193	aa changed at 65th with stop at 73rd aa
993	Insertion of A	193	aa changed at 65th with stop at 73rd aa
428	G→T	94	Premature stop at 32nd aa
1223	G→T	103	Premature stop at 35th aa
881	C→T	200	Ala→Val at 67th aa
905	G→A	469, 485	Asp→Asn at 157th aa, Gly→Glu at 162nd aa
965	2 bp deletion	438–439	aa changed at 146th with stop at 157th aa
848	9 bp deletion	109–117	3 aa missing (36th to 38th)
920	No change	NA	NA

Positions refer to the *rdxA* coding region in *H. pylori* 26695 [28].

NA: Not applicable.

aa: amino acid.

A: adenine; T: thymidine; C: cytosine; G: guanine.

resistant unrelated strains or horizontal gene transfer between unrelated strains [5, 27].

Furthermore, in the DNA sequence of the *rdxA* gene of the clinical pair with the metronidazole-sensitive body (#85b) and metronidazole-resistant antral isolate (#85a), despite showing nucleotide substitutions at positions 37 (T→C), 84 (T→C), and 357 (A→G), the respective amino acids at these positions remained unchanged. Thus, there was no evidence at the amino acid level that point mutations at these 3 positions were the cause of the generation of metronidazole resistance. Similarly, identical *rdxA* gene sequences had been observed in such clinical pairs in other studies [1, 17, 27]. The results suggest that the *rdxA* gene may not be the sole marker for metronidazole resistance.

The results from the induction of metronidazole resistance show that it is possible to convert metronidazole-sensitive *H. pylori* to a metronidazole resistant clone, when it was exposed to suboptimal concentration of the drug. In the present study, the method of induction of metronidazole resistance was modified slightly by carrying out in liquid medium instead of solid medium as reported earlier [6, 29]. Jenks *et al.* [11] suggested that *H. pylori* readily acquire resistance to metronidazole *in vivo* and that prior exposure of the organism to metronidazole is associated with failure of the eradication therapy. It is therefore crucial to use the appropriate dosage of metronidazole when it is used as part of *H. pylori* eradication therapy.

DNA sequences of the *rdxA* gene of *H. pylori* isolates studied in this study revealed the presence of base substitutions when compared to the DNA sequence of HP0954 of *H. pylori* 26695. The *rdxA* gene sequences of all the 32 isolates showed that the induction of AT-to-GC transition occurred more frequently than the other base substitutions, correlating with the study by Sisson *et al.* [22]. The approximately 5% (ranged from 3.1% to 5.5%) nucleotide sequence difference among the *rdxA* sequences of the 32 strains and HP0954 of *H. pylori* 26695 [28] is possibly the result of strain variation, as reported earlier [5, 16]. Furthermore, the sequencing results did not reveal change at any fixed position in both the nucleotide and amino acid sequences. This is in agreement with a phylogenetic analysis that demonstrated the absence of specific clusters that is associated with the resistant phenotype [25].

Due to the high genetic diversity of *H. pylori* genome, it was inconclusive to compare nucleotide sequences among different strains in an attempt to identify the resistance-associated nucleotide transitions [1]. It is therefore difficult to establish a specific DNA or amino acids patterns for metronidazole resistant strains as was reported for clarithromycin resistance [26, 30]. It was thus necessary to compare paired isolates, which are genetically similar but differ in their metronidazole sensitivities. Such paired isolates obtained clinically were rare (about 1%) [1], and as such, only one pair (#85a & b) was described in this study. The use of induced paired isolates in this study thus facilitated the comparison of nucleotide sequences between metronidazole sensitive and resistant strains (Table 4).

The *rdxA* gene sequences of the induced paired isolates showed a variety of mutations when comparison was made between each pair. Four out of the 10 pairs showed base substitutions, 2 pairs had deletions, while another 3 pairs demonstrated the insertion of a nucleotide A. The base substitutions and deletions of the *rdxA* gene occurred at various base positions, bringing about different consequences in the translated amino acids sequences. However, there is no correlation of the type or position of mutations in the *rdxA* gene with either the MIC values of the parental metronidazole-sensitive strains or the level of resistance of the 10 induced paired metronidazole-resistant strains.

Interestingly, the poly(A) segment from nucleotide positions 186 to 192 appeared as a hotspot for mutation in 3 of the induced pairs with an insertion of a nucleotide A. Similar mutation was observed in 2 matched metronidazole sensitive and resistant pairs in another study, suggesting that slipped-strand mispairing may be an important mechanism in the regulation of the expression of *rdxA* gene [27]. In the current study, there was however one induced pair (#920) that did not show any changes in their *rdxA* sequences. Together with similar results obtained from the clinical (#85a & b) isolates which showed differing nucleotide sequences at 3 positions but no change in amino acids sequence (Table 3), it is suggested that mutation in the *rdxA* gene is not the only factor that gives rise to metronidazole resistance. In these paired isolates that show no changes in the *rdxA* gene sequence and/or the translated amino acid sequence, the metronidazole-resistant phenotype is probably associated with the transcriptional and/or translational alterations of the *rdxA* gene [15] or the alterations of other genes [12, 14, 17].

Based on the sequences of *rdxA* gene, RAPD profiles and metronidazole sensitivity in the 5 clinical pairs, 10 induced pairs, and 12 individual strains of *H. pylori* studied, there was no evidence that mutations solely in the *rdxA* gene is responsible for metronidazole resistance. Thus, the study suggests that there are other factors playing a role in metronidazole resistance and the *rdxA* gene cannot be a definitive diagnostic marker for *H. pylori* resistance to metronidazole in an Asian population.

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