KAT G MUTATIONS IN ISONIAZID RESISTANT *MYCOBACTERIUM TUBERCULOSIS* ISOLATES FROM THAI PATIENTS

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Abstract. Isoniazid resistant mechanisms in *Mycobacterium tuberculosis* have been shown to involve at least two genes, *kat* G and *inh* A. Alteration in the *kat* G gene has been found in a great number of resistant isolates. Percentage of resistant isolates harboring alteration in this gene varied among laboratories suggesting that different mutations were presented in different geographic areas. Fourteen isoniazid resistant and five multidrug resistant isolates from the Central Chest Hospital, Thailand, were examined for the *kat* G gene mutations in the region between base position 17 to 299. No different pattern of mutations were found between these two groups. Among nineteen isolates, there were nine isolates which showed point mutations and five isolates with base insertions of the *kat* G gene. The remaining five isolates revealed gene deletion. Heteroduplex formation technique also confirmed base alterations in these nine mutants.

INTRODUCTION

Tuberculosis remains a serious health problem in both developed and developing countries. A main problem in tuberculosis therapy is the rising of resistance to important first line drugs causing unsuccessful short-course treatment. Isoniazid, a very efficient bactericidal drug, is one of the main line drugs of antituberculous chemotherapeutics and chemoprophylaxis since 1952. A nationwide survey of drug resistant tuberculosis isolates revealed that 10-12% of the isolates were resistant to isoniazid and 5% of the isolates were resistant to rifampin. In Bamrasnaradura Hospital, Thailand, number of resistant tuberculosis isolates in HIV patients have increased to 17% for isoniazid and 7% for rifampin.

It was noted that some of the isoniazid resistant strains lacked or had greatly decreased catalase activity, lost of acid fastness and attenuated virulence for guinea pigs when compared with isoniazid sensitive isolates. The catalase-peroxidase enzyme of *Mycobacterium tuberculosis* was encoded by the *kat* G gene with substantial homology to other bacterial enzymes. Deletions or mutations of this gene were found in certain isoniazid resistant strains (Zhang *et al*, 1992; Stockle *et al*, 1993). Furthermore, the functional *kat* G gene restored drug susceptibility in isoniazid resistant isolates (Zhang *et al*, 1993). These findings have led to intensified investigation of the *kat* G gene. Another gene which is not only involved in isoniazid resistance but also in ethionamide resistance is the *inh* A gene (Banejee *et al*, 1994). This gene encodes *Inh* A protein which is the NADH-specific reductase enzyme in fatty acid elongation (Quemard *et al*, 1995). However, the *kat* G mutations never occurred in association with mutations in the *inh* A gene. Alteration in the *kat* G gene as found more frequent than in the *inh* A gene.

Complete deletion of the *kat* G gene was rare, only 10-24%, but mutations were found in most cases. In contrast to *rpo* B gene mutations in rifampicin resistance, the *kat* G gene mutations in isoniazid resistance were highly diversified (Ferrazoli *et al*, 1995; Musser *et al*, 1996). We have determined the *kat* G gene alteration in isoniazid resistance among sensitive and resistant *M. tuberculosis* isolates from Thai patients admitted in the Central Chest Hospital, Thailand. The *kat* G gene mutations in single-drug and multidrug resistant isolates were compared in this study. We propose

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PCR-heteroduplex formation technique as an alternative way to detect *kat* G mutations.

MATERIALS AND METHODS

Mycobacterial isolates and drug susceptibility test

Susceptible and resistant clinical isolates were obtained from the Central Chest Hospital, Thailand. The isolates were cultured on Lowenstein-Jensen medium and subjected to drugs susceptibility test using 0.2 and 10 μg/ml, of isoniazid and rifampicin, respectively.

DNA preparation

Mycobacterial cells were suspended in TE buffer (10mM Tris-HCl, 1mM EDTA pH8.0) and heat inactivated at 80°C for 20 minutes followed by overnight lysozyme treatment (1mg/ml) at 37°C. The mixture was incubated with 10% SDS and proteinase K (0.1mg/ml) at 65°C for 10 minutes followed by NaCl and CTAB treatment at 65°C for 10 minutes. The DNA was subsequently purified by phenol-chloroform and precipitated with absolute ethanol. The pellet was suspended in 20 μl TE buffer and used in PCR.

DNA amplification

Mycobacterial DNA was amplified in a thermal cycler (Perkin Elmer Cetus). Reaction mixture was composed of 10mM Tris-HCl pH9.0, 50mMKCl, 1.25mM $MgCl_2$, 0.01% gelatin, 0.1% Triton X-100, 1.25mM of each deoxynucleotide triphosphate, 10pmoles of each primer and 0.5 μl of Taq polymerase. The amplification was performed for 35 cycles at 94°C for 1 minute, at 61°C for 2 minutes and at 72°C for 1 minute. The specific primers for *kat* G gene from *Mycobacterium tuberculosis* were originally designed by Altamirano *et al* (1994). The sequences were 5'-GCCCGAGCAACACCC-3' and 5'-ATGTCCCGCGTCAGG-3'. The PCR products were examined in 2% agarose gel electrophoresis. All samples were repeated for three times.

Dot blot hybridization

Primers were labeled by digoxigenin (DIG, Boehringer Mannheim) and used as probes. Hybridization and detection was performed as described by the manufacturer. Briefly, mycobacterial DNA was heat denatured and chilled on ice. One μl (1 μg) of denature DNA was spotted on nylon modified membrane (Sigma) and baked at 80°C for 1 hour. The membrane was prehybridized in hybridization buffer with blocking solution at 42°C for 1 hour. The labeled probe was added to hybridization buffer at the final concentration of 5 ng/ml. Hybridization was performed at 42°C for at least 6 hours. The membrane filter was washed at the same temperature. Immunological detection was performed by the addition of conjugated antibody followed by X-phosphate solution and NBT to produce color.

Heteroduplex formation technique

Equal amounts of the PCR products of the H37Rv strain and isoniazid resistant isolates were mixed and denatured at 95°C for 5 minutes, and left at room temperature for renaturation. The treated mixtures were electrophored in 18 × 23 cm^2-6% polyacrylamide gel at 250 voltages for 6 hours. Gels were stained in ethidium bromide solution and visualized under UV transillumination. The H37Rv PCR product was treated and loaded onto the same gel as control.

DNA sequencing analysis

The PCR products were directly sequenced by using sequenase enzyme (United States Biochemical). They were treated with Exonuclease I and Shrimp Alkaline Phosphatase before subjected to sequencing as described by the manufacturer.

RESULTS

Nineteen isoniazid resistant isolates were examined in this study. In addition, two sensitive isolates of *M. tuberculosis* were used as controls. PCR products of these sensitive isolates were the same size of 280 bp as that of H37Rv (lanes 17, 18 in Fig 1). Five of the nineteen (25%) isoniazid resistant isolates lacked the *kat* G sequence as detected by PCR method (lanes 3, 7, 8,16, 22 in Fig 1) whereas the amplification in other region of these samples were detectable. These results were confirmed by dot blot hybridization (data not shown) indicating

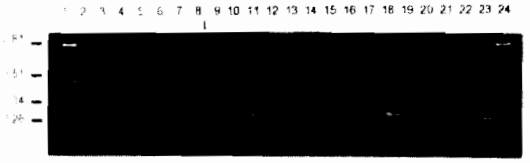

Fig 1–Gel electrophoresis of *kat* G PCR product : lane 1 and 24 are DNA molecular weight marker (λ/PstI). Numbers in the left side are DNA size in base pair, lane 2 is the product of H37Rv, lanes 3-16 are the products of isoniazid resistant isolates, lanes 17 and 18 are the products of sensitive strains, lanes 19-23 are the products of multidrug resistant isolates.

that sequence of the *kat* G of the isolates were deleted. The PCR products of five resistant isolates had insertions as they migrated slower than that of the sensitive strain H37Rv in 2% agarose gel electrophoresis (lanes 12, 14, 15, 19, 20 in Fig 1).

The PCR products of the remaining nine isolates could not be distinguishable from the products of the sensitive strains by the agarose gel electrophoresis. However, mutations in their *kat* G genes were detected by heteroduplex formation technique. Fig 2 showed different migrations of heteroduplex and homoduplex DNA. Sequence of the *kat* G mutants were analysed and compared to the *kat* G sequence of *M. tuberculosis* ATCC 27294 (Genbank accession : U06259) revealing missense mutations, base deletions and base insertions (Fig 3). The common mutation in isolates 1, 5 and 6 was found at base

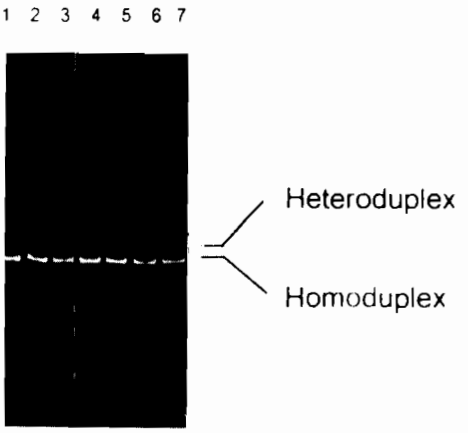

Fig 2–PCR-heteroduplex formation of H37Rv and isoniazid resistant isolates which have the same size of PCR products. The electrophoresis was performed in 6% polyacrylamide gel. The mutated sequences formed heteroduplex when annealed to the product of H37Rv. Lane 1 is the product of H37Rv. Lanes 2-7 are mixed PCR products of H37Rv and resistant isolates.

position 48 (G*A*C to G*G*C) causing an amino acid change from Asp to Gly. Stop codons in the sequence were found in isolates 1, 2 and 4 resulting in the immature protein.

DISCUSSION

Catalase-peroxidase gene, *kat* G, is believed to play a crucial role in mediating the antituberculous

	A	A	N	N	D	C	P	V	V	G	H	M	K	Y	P	V	E	G	G	G
H37Rv	aac	gac	tgt	ccc	gtc	gtg	ggt	cat	atg	aaa	tac	ccc	ntc	gag	ggc	ggn	gga	aac	cag	gac
Isolate 1		g		*		*t* a														
Isolate 2										*										
Isolate 3																				
Isolate 4																				
Isolate 5		g											*agc*							
Isolate 6		g																		

	N	Q	D	W	X	P	N	R	L	N	L	K	V	L	H	Q	N	P	A	V	A
H37Rv	tgg	tgn	ncc	aac	cgn	ctc	aat	ctg	aag	gta	ctg	cac	caa	aac	cgc	ccg	tcg	ctg	anc	cga	tgg	gt
Isolate 1																						
Isolate 2													*t*									
Isolate 3		*	*										*gt*									
Isolate 4	a																					
Isolate 5																						
Isolate 6					*g*										*c*							

Fig 3–Comparison of *kat* G nucleotide sequences between H37Rv and isoniazid resistant isolates (base positions 44 to 170). Asterisks indicate base deletion. Italicized letters show base insertion. The *taa, tga, tag* are stop codons. Capital letters represent amino acid sequences.

effect of isoniazid. Alteration in this gene is related to mechanism of isoniazid resistance. Deletions, insertions and mutations have been detected in *kat* G gene in isoniazid resistant *M. tuberculosis*. Complete deletion of *kat* G was first reported by Zhang *et al* in 1992. Later, it was found that complete deletion did not occur frequently. Percent finding of *kat* G mutation in isoniazid resistant isolates varied between reports suggesting that different mutations were presented in different regions.

In this study, we demonstrated that *kat* G alteration was the major mechanism of isoniazid resistant in the Thai patients. There was no obvious difference of *kat* G alteration among the isoniazid resistant and multidrug resistant isolates (Table 1). We were successfully using the PCR-heteroduplex formation method to detect *kat* G mutations which were found in almost 50% of the resistant isolates. Combining of the PCR and heteroduplex formation technique, we could detect mutations in all of the isoniazid resistant isolates. These results suggest that the PCR-heteroduplex formation method may be useful for rapid detection of isoniazid resistant tuberculous bacilli in Thailand.

Table 1

Number of *M. tuberculosis* clinical isolates according to type of *kat* G alterations.

Type of *kat* G gene alteration	No. of resistant isolates	
	Isoniazid	Multidrug
Deletion	4	1
Insertion	3	2
Mutation	7	2
Total	14	5

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