Evaluation of *katG* Codon 315 Mutations Among Isoniazid Sensitive and Resistant *Mycobacterium tuberculosis* Isolates from Thailand

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Abstract

Nine isoniazid (INH)-susceptible and 11 INH-resistant *Mycobacterium tuberculosis* clinical isolates were analyzed for *katG* codon 315 mutations by polymerase chain reaction (PCR) assay using primers MYC-32 and MYC-33, followed by restriction fragment length polymorphism. After *AciI* digestion of PCR products, all 9 INH-susceptible isolates and 5 out of 11 (45%) INH-resistant isolates showed 0.12 kb band, which was previously reported to indicate wild type, whereas, 6 of 11 (55%) INH-resistant isolates lacked this band.

Key word : *M. tuberculosis*, *katG* Codon, INH Susceptibility

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Tuberculosis is one of the leading causes of death in humans worldwide. Although *Mycobacterium tuberculosis*, an acid-fast bacillus was recognized as the causative agent more than one century ago(1), the disease remains a major health threat. Recent data has shown that many new cases are infections with bacilli resistant to at least one first-line drug(2). In Thailand, isoniazid (INH), a synthetic, bactericidal agent is used as a first-line drug against tuberculosis(3). The rate of primary resistance to INH was 22 per cent in 1992(4). Approximately 50 per cent of INH resistances are linked to mutations at a single genetic locus such as codon 315 of the catalase-peroxidase gene (*katG*), *inhA*,

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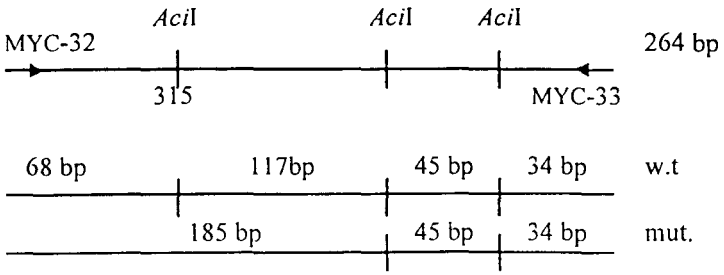

Fig. 1. The *katG* PCR amplification product (above). Given below are the expected *AciI* restriction fragment sizes from wild-type (w.t.) products and those with codon 315 mutations (mut.) affecting the overlapping *AciI* restriction site.

ahpC and *kasA*[5,6]. *KatG* substitution is the most frequent mutation found in INH-resistant isolates[7-10] and has lead to a high level of resistance to INH at the concentration of 1 μg/ml or higher[5,6]. We conducted this study to determine the frequency of *katG* mutation in INH-susceptible and INH-resistant *M. tuberculosis* isolates from Thailand.

MATERIAL AND METHOD

Mycobacterial isolates

Nine INH-susceptible and 11 resistant *M. tuberculosis* clinical isolates were obtained from the Tuberculosis Division, Bangkok, Thailand during June-July 1999. Mycobacterial species were identified by biochemical and conventional methods. Drug-susceptibility testing was done by the proportional method[11]. All INH-resistant isolates were resistant to INH at a concentration of at least 0.2 μg/mL.

DNA extraction

A loop-full isolate, grown on Loewenstein-Jensen media, was dispensed in 10mM Tris-HCl pH 7.5 and 1 per cent Triton-X. 50 μl lysis buffer (Amplicor PCR Diagnostics, Roche Diagnostic System, Branchburg, NJ) was added. After incubating at 60°C for 45 minutes, 50 μl neutralizing solution (Amplicor PCR Diagnostics, Roche Diagnostic Systems, Branchburg, NJ) was added. The DNA was stored at -20°C until used.

Polymerase Chain Reaction (PCR)

The primers MYC-32 (5′-TGG AGC AGA TGG GCT TGG-3′) and MYC-33 (5′-CAG TGG CCA GCA TCG TCG-3′) were constructed to asymmetrically encompass codon 315 (AGC) with its overlapping *AciI* restriction enzyme site (GCGG) as described previously[12]. Further *AciI* sites were located 117 bp and 162 bp downstream (Fig. 1). These sites served the purpose of internal controls for the subsequent *AciI* digestion because they yielded invariant digestion products of 45 bp and 34 bp for both wild-type and mutant genotypes. In addition to these, the wild-type sequence will give two more products of 68 bp and 117 bp, whereas, the INH resistant genotype with a codon 315 substitution affecting the first *AciI* site will result in an undigested product of 185 bp. The PCR reactions were done by using a "hot start" technique in which 3 μl of the DNA eluate with 36.5 μl water were denatured at 96°C for 2 minutes after the addition of 2 μl each of 50 μM solutions of the primers, 1.5 μl of 50 mM $MgCl_2$, 1 μl dimethyl sulfoxide and two drops of mineral oil. After cooling to 85°C, 6 μl of a freshly prepared mixture of 5 μl buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl), 0.5 μl of dNTP-mix (25 mM each) and 0.5 μl (5 u/μl) of *Taq* DNA polymerase was added.

Restriction Endonuclease Digestion

For each amplification 8 μl of the PCR reaction, 8 μl water, 2 μl of digestion buffer (Nr. 3; New England Biolabs, Beverly, MA) and 2 μl (5u/μl) of *AciI* (New England Biolabs, Beverly, MA) were incubated at 37°C for 90 minutes. Restriction products were visualized in a 3 per cent agarose gel containing 0.2 μg/ml ethidium bromide. The best diagnostic aid is the absence of a 117 bp band in mutated sequences, which is independent of possi-

Table 1. Characterization of *Mycobacterium tuberculosis.*

No.	INH Susceptibility (by Culture)	Other Resistances* (by Culture)	*katG* Genotype by PCR-RFLP
1	res	-	w.t.
2	res	-	mut.
3	res	-	mut.
4	res	-	w.t.
5	res	-	w.t.
6	res	-	mut.
7	res	-	mut.
8	sens	R	w.t.
9	sens	R	w.t.
10	sens	R	w.t.
11	sens	R	w.t.
12	sens	R	w.t.
13	sens	R	w.t.
14	sens	R	w.t.
15	sens	R	w.t.
16	sens	R	w.t.
17	res	R,S,E	w.t.
18	res	R,S,E	mut.
19	res	R,S,E	mut.
20	res	R,S,E	w.t.

* Resistances to rifampicin (R), streptomycin (S), and ethambutol (E) were tested.
Abbreviations : res., resistant; sens., sensitive; mut., mutation; w.t., wild type.

ble partial digestion products at 185 bp and which separates well from smaller digestion products, even in an agarose gel[12].

RESULTS

0.12 kb band, which indicates wild type *katG*, was found in all 9 INH-susceptible isolates and in 5 of the 11 (45%) of INH-resistant compared to 6 of 11 (55%) INH-resistant *M. tuberculosis* isolates in lane 2, 3, 6, 7, 18, and 19 (Table 1, Fig. 2) (RR=2.8, p=0.01).

DISCUSSION

Failure to recognize (multi-) drug resistance is a major threat not only for the affected patients, but also for their contacts. While the result of susceptibility testing using conventional, culture-based techniques may take several weeks before the results are available. Furthermore, patients may transmit the disease to others. Detection of mutations associated to drug resistance may offer an alternative. However, techniques such as DNA sequencing or single strand conformation analysis may not be feasible in many diagnostic laboratories. We used a relatively simple approach to detect mutation in codon 315 of the *katG* gene, associated with INH resistance. This RFLP-based technique requires little more than a PCR machine and a mini-gel chamber. It proved easy to perform and gave results within one working day.

In our study, this technique identified *katG* mutation in 55 per cent of INH-resistant isolates and none of the INH-susceptible isolates. This finding was within the range previously reported (Table 2). Correctly providing the result in a very short time, this technique could be used to identify INH resistance in clinical specimens. However, this technique identifies approximately half of the INH resistant isolates when time is critical for early diagnosis and treatment of (multi-) drug resistance. It may also be valuable for epidemiologic surveys in the absence of the possibility to culture mycobacteria as well as for sentinel studies aiming to quickly detect possible changes in resistance situations. Nonetheless, there is still need for conventional culture-based susceptibility test to further identify the rest of the INH resistant isolates.

In conclusion, our study provided proof of validity of PCR-based assay to identify INH resistant *M. tuberculosis* isolates from Thailand. Although

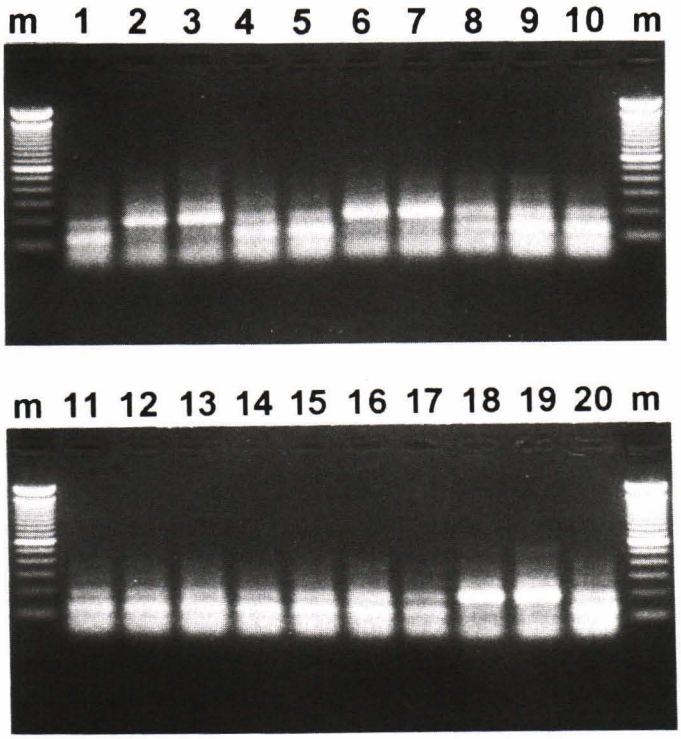

Fig. 2. ***Aci*I restriction products of *katG* PCR products of INH-sensitive and INH-resistant *M. tuberculosis* from clinical isolates. The absence of a 0.12 kb digestion product is indicative of INH resistance (see discussion). M: DNA size marker with bands at 100 bp intervals starting at 100 bp.**

Table 2. Previous studies of *katG* mutations in isoniazid-resistant strains of *Mycobacterium tuberculosis*.

Authors	Year	Countries	%	Results
Musser JM, et al(6)	1996	Various countries	55	(47 of 85)
Haas WH, et al(8)	1997	South Africa	68	(59 of 87)
Dobner P, et al(10)	1997	Germany, Sierra Leone	54	(27 of 50)
Rinder H, et al(12)	1999	Germany, Italy, France	50	(5 of 10)

a small sample size was used, the study gave a statistically significant result.

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REFERENCES

1. Koch R. Die Aetiologie der Tuberkulose. Berl Klin Wochenschr 1882; 9: 221-3.
2. Moore M, Onorato IM, McCray E, Castro KG. Trends in drug-resistant tuberculosis in the United States, 1993-1996. JAMA 1997; 278: 833-7.
3. Imwidthaya P, Suthiravitayavaniz K. Primary drug resistance to *Mycobacterium tuberculosis* in Siriraj Hospital. Siriraj Hosp Gaz 1992; 44: 957-62.
4. Ramaswamy S, Musser JM. Molecular genetic basis of antimicrobiol agent resistance in *Mycobacterium tuberculosis* : 1998 Update. Tuber Lung Dis 1998; 79: 3-29.
5. Heym B, Alzari PM, Honore N, Cole ST. Missense mutations in the catalase-peroxidase gene, *katG*, are associated with isoniazid resistance in *Mycobacterium tuberculosis*. Mol Microbiol 1995; 15: 235-45.
6. Musser JM, Kapur V, Williams DL, Kreiswirth BN, van Soolingen D, van Embden JDA. Characterization of the catalase-peroxidase gene (*katG*) and *inhA* locus in isoniazid-resistant and susceptible strains of *Mycobacterium tuberculosis* by automated DNA sequencing: restricted array of mutations associated with drug resistance. J Infect Dis 1996; 173: 196-02.
7. Rinder H, Thomschke A, Ruesch-Gerdes S, et al. Significance of *ahpC* promotor mutations for the prediction of isoniazid resistance in *Mycobacterium tuberculosis*. Eur J Clin Microbiol Infect Dis 1998; 17: 508-11.
8. Haas WH, Schilke K, Brand J, et al. Molecular analysis of *katG* gene mutations in strains of *Mycobacterium tuberculosis* complex from Africa. Antimicrob Agents Chemother 1997; 41: 1601-3.
9. Zhang Y, Heym B, Allen B, Young D, Cole S. The catalase-peroxidase gene and isoniazid resistance of *Mycobacterium tuberculosis*. Nature 1992; 358: 591-3.
10. Dobner P, Ruesch-Gerdes S, Bretzel G, et al. Usefulness of *Mycobacterium tuberculosis* genomic mutations in the genes *katG* and *inhA* for the prediction of isoniazid resistance. Int J Tuberc Lung Dis 1997; 1: 365-9.
11. Kent PT, Kubica GP. Public Health Mycobacteriology. A guide for the level III laboratory. U.S. Department of health and human service, Public health service, Centers for Disease control, Atlanta, Georgia 30333 1985: 165-84.
12. Rinder H, Feldmann K, Tortoli E, et al. Culture-independent prediction of isoniazid resistance in *Mycobacterium tuberculosis* by *katG* analysis directly from sputum samples. Mol Diagn 1999; 4: 145-52.

การศึกษายีน*แคท* จี ในเชื้อวัณโรคซึ่งแยกได้จากผู้ป่วยที่ไวและดื้อต่อยาไอโส-ไนอะซิด จากประเทศไทย

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นำเชื้อวัณโรคที่แยกได้จากผู้ป่วย 20 สายพันธุ์ ซึ่งประกอบด้วยเชื้อวัณโรคที่ไวต่อยาไอโสไนอะซิด 9 สายพันธุ์ และเชื้อวัณโรคที่ดื้อต่อยาไอโสไนอะซิด 11 สายพันธุ์ มาศึกษายีน*แคท* จี ว่ามีการเปลี่ยนแปลงที่โคดอน 315 หรือไม่โดยวิธีปฏิกิริยาลูกโซ่โพลีเมอเรส ใช้ไพรเมอร์ MYC-32 และ MYC-33 หลังจากย่อยดีเอ็นเอที่ได้จากปฏิกิริยาด้วย *Aci* I พบว่าเชื้อวัณโรคที่ไวต่อยาไอโสไนอะซิด 9 สายพันธุ์ (ร้อยละ 100) และ 5 สายพันธุ์จากจำนวนทั้งหมด 11 สายพันธุ์ (ร้อยละ 45) ของเชื้อวัณโรคที่ดื้อยาไอโสไนอะซิดมีดีเอ็นเอขนาดประมาณ 0.12 กิโลเบส ซึ่งแสดงว่ายีน*แคท* จี ไม่มีการเปลี่ยนแปลงที่โคดอน 315 ส่วน 6 จาก 11 สายพันธุ์ (ร้อยละ 55) ของเชื้อที่ดื้อต่อยาไอโสไนอะซิดขาดดีเอ็นเอที่มีขนาดนี้ การศึกษานี้แสดงว่าการตรวจหาการเปลี่ยนแปลงที่โคดอน 315 ของยีน*แคท* จี ในเชื้อวัณโรคเพื่อแสดงว่าเชื้อวัณโรคดื้อต่อยาไอโส-ไนอะซิด ได้ผลร้อยละ 55 ซึ่งผลที่ได้นี้ใกล้เคียงกับการศึกษาจากรายงานอื่น ๆ

คำสำคัญ : *มัยโคแบคทีเรียม ทูเบอร์คูโลสิส, แคท* จี โคดอน, ความไวต่อไอโสไนอะซิด

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