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Mutations Including IS*6110* Insertion in the Gene Encoding the MPB64 Protein of Capilia TB-Negative *Mycobacterium tuberculosis* Isolates

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A simple immunochromatographic assay, Capilia TB, using anti-MPB64 monoclonal antibodies, is a kit for discriminating between the *Mycobacterium tuberculosis* complex and mycobacteria other than tubercle bacilli. The sensitivity of the kit was estimated to be 99.2% (381 of 384 samples). The sequencing analysis revealed that all of the Capilia TB-negative isolates had mutations within the *mpb64* gene, leading to the production of an incomplete protein as a result of a deletion of the C-terminal region of the protein.

Mycobacterium tuberculosis has been known to secrete more than 33 different proteins (3). One of the predominant proteins is MPB64, a 24-kDa protein initially isolated from culture filtrates of *Mycobacterium bovis* BCG Tokyo (5, 11, 20). The MPB64 antigen was found in the culture fluid of only strains of the *M. tuberculosis* complex and some substrains of *M. bovis* BCG (1, 5, 10, 11) and has shown potential for diagnostic use [1, 12, 13; T. Tomiyama, K. Matsuo, and C. Abe, abstract, Int. J. Tuberc. Lung Dis. **1**(Suppl. 1):S59, 1997]. Recently, a simple immunochromatographic assay, Capilia TB (TAUNS, Numazu, Japan), using anti-MPB64 monoclonal antibodies for rapid discrimination between the *M. tuberculosis* complex and mycobacteria other than tubercle bacilli (MOTT), was developed [1; Tomiyama et al., Int. J. Tuberc. Lung Dis. **1**(Suppl. 1)**:**S59, 1997]. The test strip consists of a sample pad, a reagent pad, a nitrocellulose membrane, and an absorbent pad. Because the test sample applied in the sample well flows laterally through the membrane, the antibody-colloidal gold conjugate binds to the MPB64 antigen in the sample. The complex then flows further and binds to the monoclonal antibodies on the solid phase in the test zone, producing a red-purple band within 15 min. Some evaluation studies have demonstrated that the Capilia TB is very useful for rapid confirmation of the *M. tuberculosis* complex in liquid cultures without any troublesome sample preparation in a laboratory [1, 2, 6, 8; Tomiyama et al., Int. J. Tuberc. Lung Dis. **1**(Suppl. 1)**:**S59, 1997]. The kit is commercially available in Japan (Nippon Becton Dickinson Co., Ltd., Tokyo, Japan) and is routinely used in most clinical laboratories in hospitals or commercial laboratory centers.

More recently, Capilia TB-negative *M. tuberculosis* strains have been isolated in some districts of Japan; therefore, the sensitivity of the Capilia TB test was reexamined by using a large number of clinical specimens in this study. A total of 13,942 clinical specimens, mostly sputum, were used for the study. Patients were admitted to Fukujuji Hospital (Japan Anti-Tuberculosis Association, Kiyose-shi, Tokyo) with symptoms of pulmonary tuberculosis from September 2001 to October 2002. Of these, 784 (5.6%) were culture positive for *Mycobacteria*. All mycobacterium-positive cultures were differentiated and identified by the Capilia TB, and the results were compared with those of the Accuprobe-*M. tuberculosis* complex culture confirmation test (Gen-Probe, San Diego, Calif.) and the DNA-DNA hybridization test (Kyokuto Pharmaceutical Co., Ltd., Tokyo, Japan) (9). A total of 498 out of 784 mycobacterium-positive cultures were identified as members of the *M. tuberculosis* complex by Capilia TB, Accuprobe, or DNA-DNA hybridization tests. Since multiple isolates per patient were included in these *M. tuberculosis* cultures, 114 cultures were removed. The resulting 384 isolates were obtained from different patients, and they were used for further analysis. The Accuprobe test detected all *M. tuberculosis* cultures, while the Capilia TB test failed to detect 3 isolates out of 384 *M. tuberculosis* cultures, as shown in Table 1. There were no Capilia TB-positive but Accuprobe-negative cultures. False-positive cultures were not observed in either Capilia TB or Accuprobe tests. The sensitivity and specificity of the Capilia TB test were 99.2 and 100%, respectively, and those of the Accuprobe test were both 100%, confirming the usefulness of the Capilia TB test for routine laboratory testing for rapid differentiation of the *M. tuberculosis* complex from MOTT bacilli.

A total of 12 Capilia TB-negative *M. tuberculosis* strains have been isolated from different hospitals and commercial laboratory centers in various districts of Japan between June 2001 and August 2002. All of the isolates were identified as members of the *M. tuberculosis* complex by the Accuprobe and DNA-DNA hybridization tests. Hasegawa et al. reported that two of the four *M. bovis* strains were negative by the Capilia TB test (6). In the present study, we determined biochemical characteristics of these negative strains in order to discriminate among *M. tuberculosis* and *M. bovis* strains. All were positive for the niacin accumulation, pyrazinamidase, nitrate reductase tests, and growth on thiophene-2-carboxylic acid hydrazide, indicating characteristics of *M. tuberculosis* but not *M. bovis*. In addition, an allele-specific PCR based on the *oxyR* sequence (4, 19) for distinguishing *M. tuberculosis* from *M. bovis* revealed

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TABLE 1. Comparison of the results obtained by Capilia TB and Accuprobe for *M. tuberculosis* complex isolates

Isolates (*n*)	No. of isolates			
	Capilia TB		Accuprobe	
	Positive	Negative	Positive	Negative
M. tuberculosis complex (384)	381	3	384	0
MOTT (161)	0	161	0	161

that amplified products were obtained only when primers specific for *M. tuberculosis* were used for the assay (data not shown). In a previous paper, we reported that some *M. bovis* BCG substrains, Glaxo, Tice, and Pasteur, showed a negative signal by the Capilia TB test (1). All four of the *M. bovis* strains used by Hasegawa et al. (6) were from stock cultures that were frozen for some time. Since many laboratories do not discriminate between *M. bovis* and *M. bovis* BCG, further study of these *M. bovis* isolates may be needed, as discussed by the authors.

All of the Capilia TB-negative isolates were sensitive to major antituberculosis drugs: for example, isoniazid, rifampin, streptomycin, and ethambutol.

To estimate whether the Capilia TB-negative strains of *M. tuberculosis* produce complete MPB64 protein, a sequencing analysis of the *mpb64* gene was carried out by using several primer sets based on the *mpb64* sequence (20). The PCR products were sequenced with a Prism 377 automated DNA sequencer (Applied Biosystems, Inc., Foster City, Calif.). As shown in Table 2, all of the Capilia TB-negative *M. tuberculosis* strains had mutations in the *mpb64* gene, and five mutations were identified. Three isolates showed a deletion of 63 bp from nucleotides 196 to 258 (amino acid positions 43 to 63): these strains were isolated in Tokyo, Japan (two isolates), and Fukuoka, Japan (one isolate). One had a single-base-pair deletion at nucleotide position 266, which resulted in a frameshift of the codons and created a stop codon at nucleotides 278 to 280 (TGA). Two strains isolated in Tokyo contained a point mutation at position 402 (G to A), which also created a stop codon at nucleotides 400 to 402 (TGA). Five strains showed a deletion of 176 bp from nucleotides 512 to 687, which resulted in a deletion of C-terminal 58 amino acids from amino acid positions 147 to 205. These five strains were isolated in various urban and rural prefectures: two isolates in Osaka, and one isolate each in Hokkaido, Kyoto, and Hiroshima. It can be speculated from these results that the mutations that easily occur may be present in the *mpb64* gene, since no epidemiological links were observed among the patients with strains having the same mutation.

TABLE 2. Mutations in the *mpb64* gene of the Capilia TB-negative *M. tuberculosis* isolates from Japan

Mutation position[a]	Mutation	No. of isolates	Place of isolation (*n*)
196	63-bp deletion	3	Tokyo (2), Fukuoka (1)
266	C deletion	1	Tokyo (1)
402	G→A mutation	2	Tokyo (2)
501	IS*6110* insertion (1,358 bp)	1	Fukuoka (1)
512	872-bp deletion	5	Hokkaido (1), Osaka (2) Kyoto (1), Hiroshima (1)

[a] The DNA sequence contained an open reading frame starting with translation initiation codon GTG at nucleotide positions 1 to 3 and ending with termination codon TAG at positions 685 to 687 (20).

One isolate from Fukuoka had the IS*6110* insertion mutation at nucleotide position 501 of the *mpb64* gene (Table 2). Sampson et al. reported that the occurrence of the unique IS*6110* insertion loci dispersed throughout the *M. tuberculosis* genome, with an unexpectedly high incidence of IS*6110* insertions occurring within the coding regions (18). However, the IS*6110*-mediated coding region disruptions identified by these investigations may only have a limited impact on the phenotype, because most of the coding regions disrupted are members of multiple gene families (3, 18). One isolate used in our study had an IS*6110* insertion mutation within the *mpb64* gene, which is not a member of the multiple gene families, indicating that a complete MPB64 protein was not produced.

The MPB64 antigen induces a strong delayed-type hypersensitivity reaction similar to the purified protein derivatives in guinea pigs sensitized with the *M. tuberculosis* complex bacilli, as well as lymphocyte proliferation responses in patients with tuberculosis (3, 5, 13, 14). Kamath et al. reported that DNA vaccines expressing *M. tuberculosis* antigen MPB64 immunization in mice resulted in a more variable level of protection against an aerosol challenge with *M. tuberculosis* (7, 15–17). These results suggest that the MPB64 protein is of importance in the bacterium, although its function is unknown.

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