Hiroshima J. Med. Sci.
Vol.42, No.1, 21~31, March, 1993
HIJM 42-3

Rapid Detection and Identification of Mycobacteria in Sputum Samples by Nested Polymerase Chain Reaction and Restriction Fragment Length Polymorphisms of *dnaJ* heat shock protein gene

Kyosuke INYAKU, Keiko HIYAMA, Shinichi ISHIOKA, Tsutomu INAMIZU and Michio YAMAKIDO

The Second Department of Internal Medicine, Hiroshima University School of Medicine, 1-2-3, Kasumi, Minami-ku, Hiroshima 734, Japan

ABSTRACT

In the diagnosis of mycobacterial infection, more than 4-8 weeks is required to identify the species of mycobacterium responsible for an infection. Therefore, the development of a method for the rapid detection and identification of mycobacteria is necessary for selecting an optimal therapeutic plan early in the patient's course. For this purpose, we developed a method combining a nested polymerase chain reaction (nested PCR) procedure and a restriction fragment length polymorphisms (RFLP) analysis of the *dnaJ* gene of mycobacteria, which codes for a heat shock protein. The PCR procedure allowed the sensitive detection of mycobacterial DNA in clinical samples. Using only 10 femtograms of mycobacterial DNA as a reaction mixture, a detectable band of target DNA segments could be yielded on an agarose gel. This indicates that even with a single genome amount, the PCR is able to detect mycobacteria. The RFLP analysis of the PCR products allowed us rapidly to distinguish the strains belonging to the *M.tuberculosis* complex from 11 different strains of nontuberculous mycobacteria. Within 2 days, the method is able to identify the mycobacterial species present in the sputum. Moreover, it has the advantage of not requiring the use of radioisotopes, which strongly enhances its clinical usefulness.

Key words: *Mycobacteria, dnaJ gene, PCR, RFLP*

Mycobacterial infections have decreased considerably, following advances in anti-tuberculosis therapy. In developing countries, however, this disease still accounts for a high percentage of respiratory diseases. Tuberculosis is estimated to occur in about 10 million people worldwide each year[16]. In developed countries, mycobacterial infections are now complicating the course of compromised patients, such as those with AIDS[4,26,30].

While the diagnosis of a mycobacterial infection is usually based on microscopic examination and culture of clinical samples, this approach has disadvantages[3,8]. Although microscopic examination of the sputum can provide a rapid diagnosis, a relatively large number of bacteria (1,000-5,000/ml) must be present in the sample[14,27]. Moreover, this approach does not allow the identification of mycobacteria. Because of the slow growth of mycobacteria, culture techniques require more than 4-8 weeks before identification can be made. Furthermore, multiple cultures are required to ensure positive results[15,18]. Therefore, a sensitive and rapid method for the diagnosis of mycobacterial infections has been sought. Of the newer methods reported, the polymerase chain reaction (PCR) is noteworthy because this molecular technique allows the rapid amplification of minute amounts of gene fragments[28]. Attempts have been made to employ this rapid and sensitive technique in the diagnosis of mycobacterial infections since the first report published by Hance and colleagues[12]. According to these reports, PCR is both sensitive and specific for detecting *M.tuberculosis* complex and/or nontuberculous mycobacteria from clinical specimens. However, few studies have applied PCR to the identification of mycobacteria. In Japan, about 10% of all pulmonary mycobacterial infections are attributed to nontuberculous mycobacteria, and the incidence is reported to be increasing[36]. Some nontuberculous mycobacteria show patterns of drug sensitivity different from those of *M.tuberculosis*. Therefore, a technique for the rapid and easy identification of mycobacteria is required for the most effective therapeutic approach.

We recently developed a nested PCR procedure

for the *dnaJ* gene of mycobacteria to detect the mycobacteria contained in sputum with high sensitivity. This gene codes for a heat shock protein, and its sequence was reported by Lathigra and colleagues[19]. We applied RFLP analysis to the PCR products to distinguish the strains belonging to the *M.tuberculosis* complex from the 11 different strains of nontuberculous mycobacteria whose *dnaJ* gene was amplified by the nested PCR.

MATERIALS AND METHODS

Bacterial strains and sputum samples

The strains of mycobacteria studied (Table 1) were provided by the Research Institute of Tuberculosis of the Japan Anti-Tuberculosis Association. Clinical isolates of mycobacteria and other bacteria, as well as sputum samples from patients were provided by Yoshijima Hospital, National Hiroshima Hospital, National Yanai Hospital, and by the Central Clinical Laboratory of Hiroshima University Hospital.

Sputum samples were collected from 67 patients with verified pulmonary tuberculosis. All patients were receiving anti-tuberculous therapy as inpatients in tuberculosis wards. Serving as controls were 17 non-tuberculous inpatients observed in general wards, whose sputum was confirmed to be free of mycobacteria by examination of smear and culture.

Table 1. Mycobacterial strains used in this study

Species	Strain	
Mycobacterium tuberculosis H37Rv	KK11-20	
Mycobacterium tuberculosis H37Ra	KK11-05	
Mycobacterium tuberculosis Aoyama B	KK11-19	
Mycobacterium bovis	JATA12-01	ATCC19210
Mycobacterium bovis BCG (Tokyo strain)	KK12-02	
Mycobacterium africanum	KK13-01	
Mycobacterium microti	KK14-01	ATCC19422
Mycobacterium kansasii	KK21-01	ATCC12478
Mycobacterium marinum	KK22-01	ATCC927
Mycobacterium simiae	KK23-01	ATCC25275
Mycobacterium scrofulaceum	JATA31-01	ATCC19981
Mycobacterium szulgai	JATA32-01	ATCC35799
Mycobacterium gordonae	KK33-02	ATCC14470
Mycobacterium avium	JATA51-01	ATCC25291
Mycobacterium intracellulare	JATA52-01	ATCC13950
Mycobacterium xenopi	KK42-02	ATCC19250
Mycobacterium haemophilum	KK49-01	ATCC29548
Mycobacterium shimoidei	JATA54-01	ATCC27962
Mycobacterium gastri	KK44-01	ATCC15754
Mycobacterium nonchromogenicum	JATA45-01	ATCC19530
Mycobacterium fortuitum	KK61-01	ATCC6841
Mycobacterium chelonae subsp. *chelonae*	JATA62-01	ATCC35752
Mycobacterium chelonae subsp. *abscessus*	JATA63-01	ATCC19977

KK,JATA: Japan Anti-Tuberculosis Association.
ATCC: American Type Culture Collection.

Table 2. Sequences and locations of oligonucleotide primers

Name	Sequence	Location*
M5	5'-GGG-TGA-CGC-GAC-ATG-GCC-CA-3'	1394-1413
M2	5'-CGG-GTT-TCG-TCG-TAC-TCC-TT-3'	1610-1629
J1'	5'-TGA-AGA-GAT-CAA-ACG-TGC-CTA-TCG-GAA-3'	1474-1500
J1K	5'-TGA-AGA-AAT-CAA-GCG-AGT-GGC-TCG-AAA-3'	1474-1500

* : The numbering of the residues of *dnaJ* gene is as in reference 19.

Bacteriological examination

Of each sputum sample, 0.5 ml was used for DNA extraction and the remainder was used for bacteriological examination. Microscopic examination was performed after Ziehl-Neelsen staining. For the isolation of mycobacteria, two volumes of 4% NaOH were mixed with the sputum specimens for digestion, and the mixtures were allowed to stand for 15 min at room temperature. From these alkali-treated samples, 0.1 ml was

directly inoculated on 3% Ogawa egg medium[1,22] (Kyokuto Pharmaceutical Co., Ltd., Tokyo, Japan.) without neutralization. All media were incubated at 37°C and checked once a week for up to 8 weeks. All primary isolates were confirmed by Ziehl-Neelsen staining and niacin tests were performed after secondary culture using N.T. paper (Kyokuto Pharmaceutical Co., Ltd., Tokyo, Japan.) according to the manufacturer's instructions. Niacin test positive mycobacteria were regarded as *M.tuberculosis*.

Oligonucleotide primers

Table 2 shows the nucleotide sequences and locations of the primers used in this study. They were synthesized using the β-cyanoethyl phosphoramidite method with a Model 394 DNA Synthesizer (Applied Biosystems, Inc., Foster City, CA) by Bex Co., Ltd., (Tokyo, Japan). The sequences of primers M5, M2, and J1' were complementary to the *dnaJ* gene of *M.tuberculosis*[19]. The sequence of primer JlK was complementary to the J1'-equivalent site of the *dnaJ* gene of *M.kansasii*[21,34]. Primers M5 and M2 were used in the first PCR, and primers J1'(or JlK) and M2 were used in the second PCR.

Extraction of DNA from sputum samples and bacterial strains

DNA was extracted from sputum using the procedure reported by Hermans and colleagues[13] with some modification. In brief, the sputum specimen (0.5 ml) was mixed with 0.5 ml of 1N NaOH, and incubated at room temperature for 30 min. The mixture was then neutralized with 620 μl of 1M NaH_2PO_4 and centrifuged at 7,000×*g* for 10 min. The sediment was suspended in 400 μl of 50 mM Tris-HCl and 5 mM EDTA (pH8.0), mixed with lysozyme in a concentration of 1 mg/ml, and incubated at 37°C for 90 min. Proteinase K and sodium dodecyl sulfate (SDS) were added at final concentrations of 1 mg/ml and 1%, respectively, incubated at 55°C for 60 min, extracted by phenol/chloroform treatment and precipitated with ethanol[29]. The DNA thus obtained was dissolved in 50 μl of distilled water to serve as the test sample.

To extract DNA from the bacterial strains, several loops of bacteria were subjected to the above procedures, excluding alkali treatment.

PCR

Specific sequences of mycobacterial DNA were amplified using DNA polymerase obtained from *Thermus aquaticus* YT1 (Wako Pure Chemical Industries, Ltd., Osaka, Japan). A volume of 50 μl of the reaction mixture, containing 200 μmol each of the deoxyribonucleoside triphosphates, 50 pmol each of primers M5 and M2, 1.25 units of DNA polymerase, 10 mM Tris-HCl (pH8.8), 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100 and 2 μl of the sample, was subjected to 3 min of heat denaturing at 95°C, followed by 30 thermal cycles using a programmable temperature control system (PC-700, ASTEC Co., Ltd., Fukuoka, Japan). Each thermal cycle consisted of 95°C for 1 min, 63°C for 1 min and 74°C for 1 min. After the first PCR, 2 μl of the amplified product was mixed with the reaction mixture described above, except for the replacement of primer M5 with primer J1' or JlK, and was then subjected to 30 cycles of amplification under the same conditions (second PCR). Purified *M.tuberculosis* DNA serving as a positive control, and the reaction mixture without any DNA serving as a negative control were included in each experiment. One-tenth of the final PCR products was electrophoresed on a 2% agarose gel. DNA was stained with ethidium bromide and visualized by an ultraviolet transilluminator. Samples showing a band of the expected size (156-bp) were regarded as positive. The degree of positivity of the nested PCR and the bacteriological test were then compared using McNemar's test[20].

Sequencing of amplified DNA

The nucleotide sequences of the PCR products in a region between primers M5 and M2 for the seven strains of *M.tuberculosis* complex and the 11 strains of nontuberculous mycobacteria, whose *dnaJ* gene could be amplified by the nested PCR, were determined by direct sequencing. In brief, the PCR products from the first PCR were electrophoresed on a 2% agarose gel, the target band was excised, and the DNA was extracted using Geneclean II (BIO 101, Inc., La Jolla, CA). The nucleotide sequences were then determined using a dsDNA cycle sequencing system (BRL, Gaithersburg, MD), according to the manufacturer's instructions.

Restriction Fragment Length Polymorphism (RFLP) analysis of PCR products

To determine whether rapid identification of mycobacteria would be possible, RFLP analyses were performed on the 156-bp DNA fragments amplified by the second PCR. Seven restriction enzymes (*Sma*I, *Nae*I, *Alu*I, *Bam*HI, *Hin*fI, *Hae*III and *Stu*I) were selected based on the nucleotide sequences determined above.

After 5-15 μl of the PCR products had been adjusted to the optimum conditions for each restriction enzyme, using Tris-HCl (pH7.5), NaCl, $MgCl_2$, DTT solution, each restriction enzyme was added. The mixture was then incubated at an optimum temperature for 2 hours, followed by 3% agarose gel electrophoresis (SepRate-SDF, Amersham International PLC., Buckinghamshire, England) to check for specific cleavage.

RESULTS

The PCR amplification for the DNA extracted from the sputum samples of the tuberculous patients resulted in a band of the expected size (Fig. 1). The amplified fragments were also shown to correspond to the expected region of the *dnaJ* gene by their cleavage patterns with restriction enzymes (*Sma*I, *Taq*I and *Hin*fI) and sequences (data not shown).

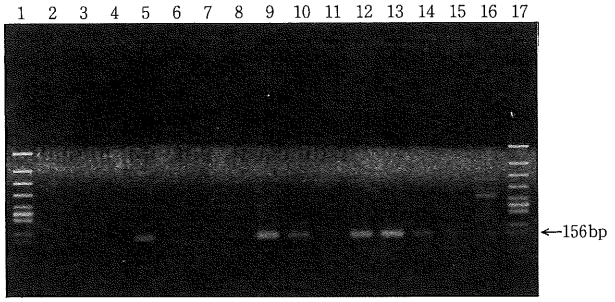

Fig. 1. Detection of *M.tuberculosis dnaJ* fragment by the nested PCR.
DNA extracted from sputum samples of tuberculous patients was subjected to the nested PCR, electrophoresed on a 2% agarose gel, and stained with ethidium bromide. The arrow indicates the amplified fragments correspond to the *dnaJ* gene. Lanes 1 and 17: ϕX174 digested with *Hin*cII, as DNA size standards.(1,057, 770, 612, 495, 392, 340, 294, 210, 162, and 79 bp.) ; Lanes 2–15: sputum samples; Lane 16: 250 ng of human genome, as negative control. Lanes 5, 9, 10, 12, 13, and 14 were regarded as positive.

Sensitivity

To test the sensitivity of our PCR procedure, 10-fold serial dilutions of DNA solution extracted from clinical isolates of *M.tuberculosis* were subjected to our PCR procedure (Fig. 2). With a minimum of 10 fg of mycobacterial DNA in one reaction, the PCR procedure efficiently yielded a result. The value corresponded to the amount of DNA contained in only two mycobacteria[2]. Furthermore, the intensity of the bands was similar in the range from 1 ng to 10 fg, indicating that even the minimal value produces a sufficient amount of DNA for the band formation to reach a plateau under the experimental conditions. When 1 fg of DNA was used as the template, some experimental cases exhibited no band, but others exhibited a band similar in intensity to that obtained from a larger amount of template DNA (data not shown). This indicates that only a single mycobacterium in one reaction may be sufficient for this procedure.

Specificity

The specificity of the PCR procedure was assessed using seven strains belonging to the *M.tuberculosis* complex and 16 strains of nontuberculous mycobacteria (Fig. 3). The fragments of *dnaJ* gene from all strains belonging to the *M.tuberculosis* complex and 12 of the strains of nontuberculous mycobacteria were amplified by 30 cycles of PCR using first PCR primers. Using the second PCR primers J1' and M2, the fragments of *dnaJ* gene from all strains of the *M.tuberculosis* complex and 9 of the strains of nontuberculous mycobacteria were amplified. When primer J1' was replaced with primer JlK, the fragments of *dnaJ* gene from *M.kansasii* and *M.gastri* were amplified (data not shown). Similar amplification was observed for all of the clinical isolates of the mycobacterial strains examined (*M.tuberculosis*, *M.avium*, *M.intracellulare* and *M.kansasii*). Thus, amplification of *dnaJ* gene was observed for all strains of the *M.tuberculosis* complex and for 11 of the 16 strains of nontuberculous mycobacteria using both PCR primer pairs, and we concluded that these strains were detectable by our nested PCR procedure. In contrast, none of the bacteria other than the mycobacteria examined was negative by PCR (*Streptococcus pneumoniae, Streptococcus pyogenes, Enterococcus faecalis, Staphylococcus aureus, Klebsiella pneumoniae, Haemophilus influenzae, Escherichia coli, Pseudomonas aeruginosa, Branhamella catarrhalis,* and others).

Sequencing of PCR products

For the mycobacterial strains whose *dnaJ* gene could be amplified by the nested PCR, we examined the nucleotide sequences in a region between primers M5 and M2 (Fig. 4). The seven strains belonging to the *M.tuberculosis* complex had an identical nucleotide sequence. The nucleotide sequences of the nontuberculous mycobacterial strains showed 73–90% homology to the sequence of *M.tuberculosis*.

RFLP analysis of PCR products

RFLP analyses were performed using seven restriction enzymes that were expected to be useful in bacterial distinction based on the nucleotide sequences determined. Whether or not the PCR products were cleaved by these enzymes was assessed by electrophoresis on a 3% agarose gel (Fig. 5). The results, shown in Table 3, agreed with those expected from the nucleotide sequences. Using the seven restriction enzymes, distinction was possible for *M.tuberculosis* complex and for the 11 nested PCR-positive nontuberculous mycobacteria. Based on these results, we established a model of a decision tree (Table 4). The test mycobacteria were first divided into four groups based on whether their PCR products were cleaved by *Sma*I and *Nae*I. Cleavage of the PCR products by both enzymes was observed only for the strains belonging to the *M.tuberculosis* complex. The other three groups included clinical-

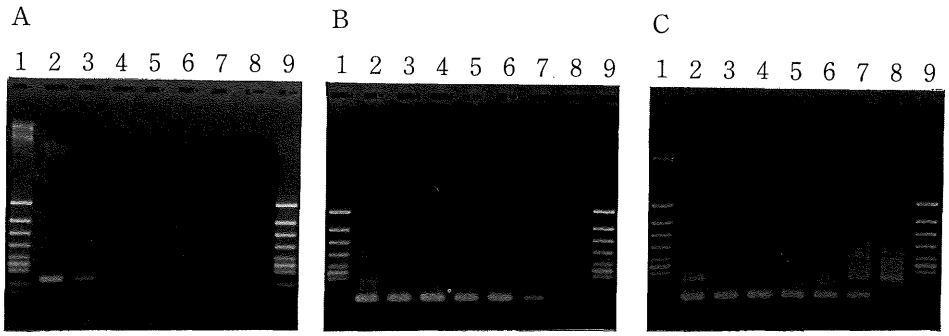

Fig. 2. Sensitivity of detection of mycobacterial DNA by the nested PCR.
Ten-fold serial dilutions of DNA extracted from a clinical isolate of *M.tuberculosis* were amplified by (A) first PCR, (B) nested PCR, and (C) nested PCR with addition of 250 ng of human genome, and electrophoresed on 2% agarose gels. Lanes 1 and 9: ϕX174 digested with *Hin*cII, as DNA size standards. ; Lanes 2-8: 1 ng, 100 pg, 10 pg, 1 pg, 100 fg, 10 fg, and 1 fg of *M.tuberculosis* DNA were subjected to PCR. Sensitivity of detection of first PCR was 100 pg per reaction, and that of nested PCR was 10 fg per reaction. Similar result was obtained in the presence of human genome.

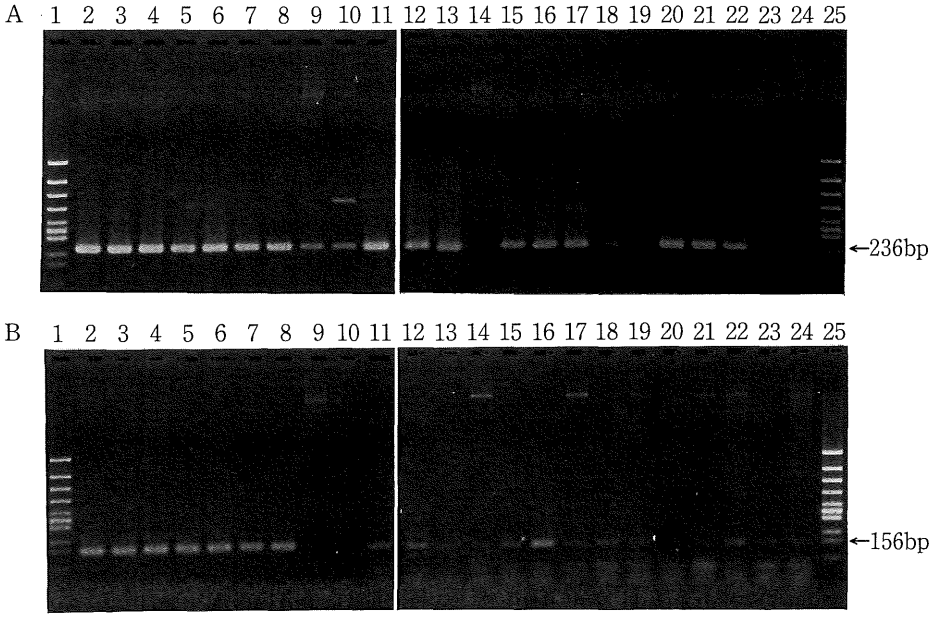

Fig. 3. Specificity of the nested PCR.
DNA extracted from seven strains of *M.tuberculosis* complex and 16 strains of nontuberculous mycobacteria were subjected to 30 cycles of PCR using (A) the outer primers M5 and M2, and (B) the inner primers J1' and M2. Lanes 1 and 25: ϕX174 digested with *Hin*cII, as DNA size standards. ; Lane 2: *M.tuberculosis* H37Rv; Lane 3: *M.tuberculosis* H37Ra; Lane 4: *M.tuberculosis* Aoyama B; Lane 5: *M.bovis*; Lane 6: *M.bovis* BCG; Lane 7: *M.africanum*; Lane 8: *M.microti*; Lane 9: *M.kansasii*; Lane 10: *M.marinum*; Lane 11: *M.simiae*; Lane 12: *M.scroflaceum*; Lane 13: *M.szulgai*; Lanes 14: *M.gordonae*; Lane 15: *M.avium*; Lane 16: *M.intracellulare*; Lane 17: *M.xenopi*; Lane 18: *M.haemophilum*; Lane 19: *M.shimoidei*; Lane 20: *M.gastri*; Lane 21: *M.nonchromogenicum*; Lane 22: *M.fortuitum*; Lane 23: *M.chelonae* subsp. *chelonae*; Lane 24: *M.chelonae* subsp. *abscessus*; Lanes 2-8, 11-13, 15-18, and 21-22 were regarded as positive for both primer pairs.

ly significant *M.avium*, *M.intracellulare*, and *M.kansasii*, respectively. These three mycobacteria could be distinguished from the other mycobacteria in each group by using a single restriction enzyme (*Bam*HI, *Hin*fI, and *Alu*I, respectively).

Comparison of bacteriological findings and PCR results

The results of the nested PCR were compared with the results of the bacteriological test (Table 5). Of the 67 tuberculous patients, the positive rate for the microscopic test and culture were 31.3%(21/67) and 32.8%(22/67), respectively, and 28 were bacteriologically classified as positive for *M.tuberculosis*. Of those 28 patients, 26 (92.9%) were positive by the nested PCR. Of the 39 bacteriologically negative patients with tuberculosis, 19 (48.7%) were positive by the nested PCR. The

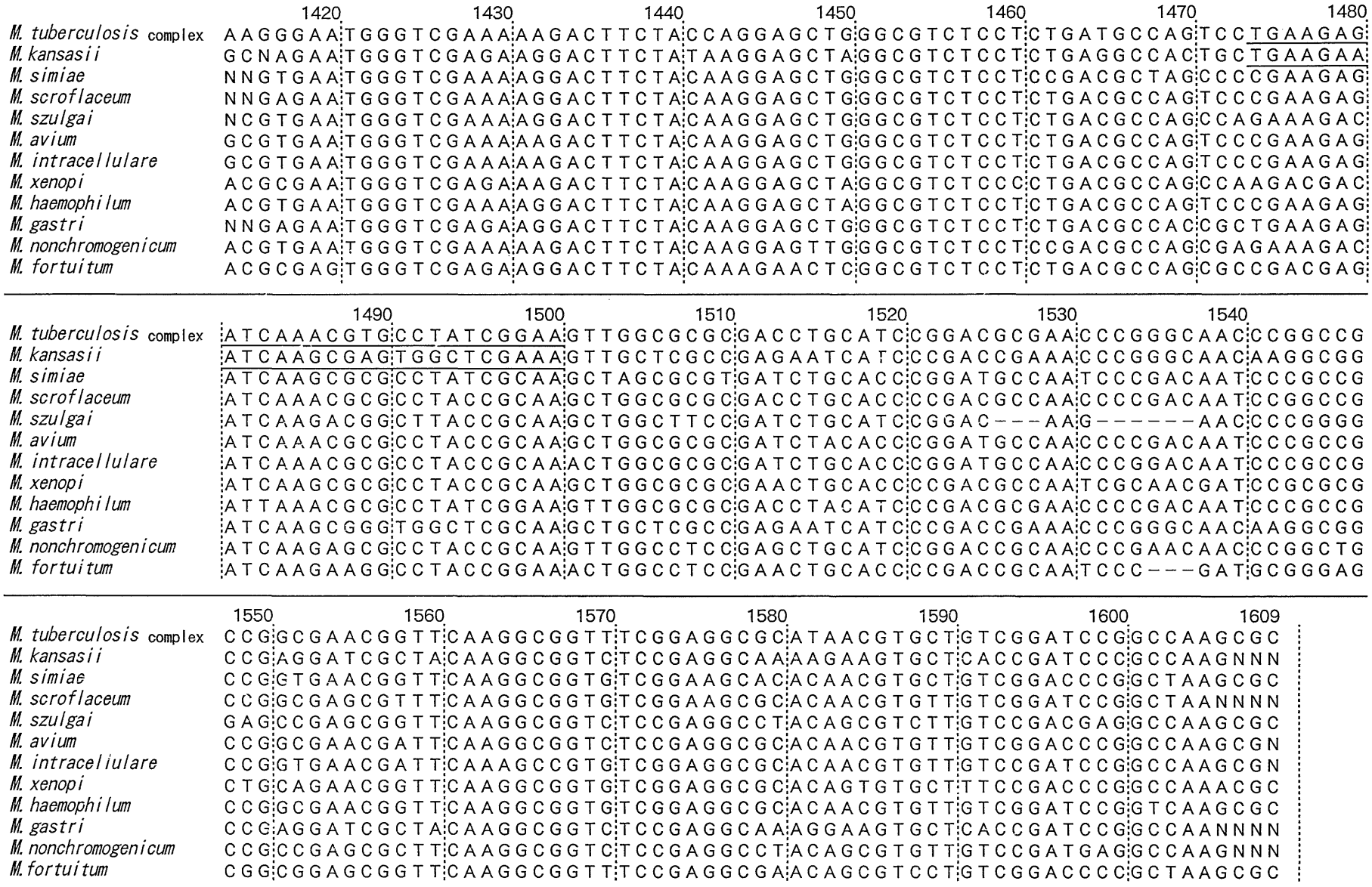

Fig. 4. Nucleotide sequences of the fragments of *dnaJ* gene between primers M5 and M2.
Primers J1' and J1K used for the second PCR are underlined. The numbering of the residues of *dnaJ* gene is as in reference 19. The strains examined are as in table 1.
– : deletion, N : not determined

PCR-positive rate was 67.2% (45/67) for the group with tuberculosis, which was significantly higher than the bacteriologically positive rate of 41.8% (28/67) in the same group ($p<0.01$). All 17 patients in the non-tuberculosis group were negative by the nested PCR, indicating that none was a false positive.

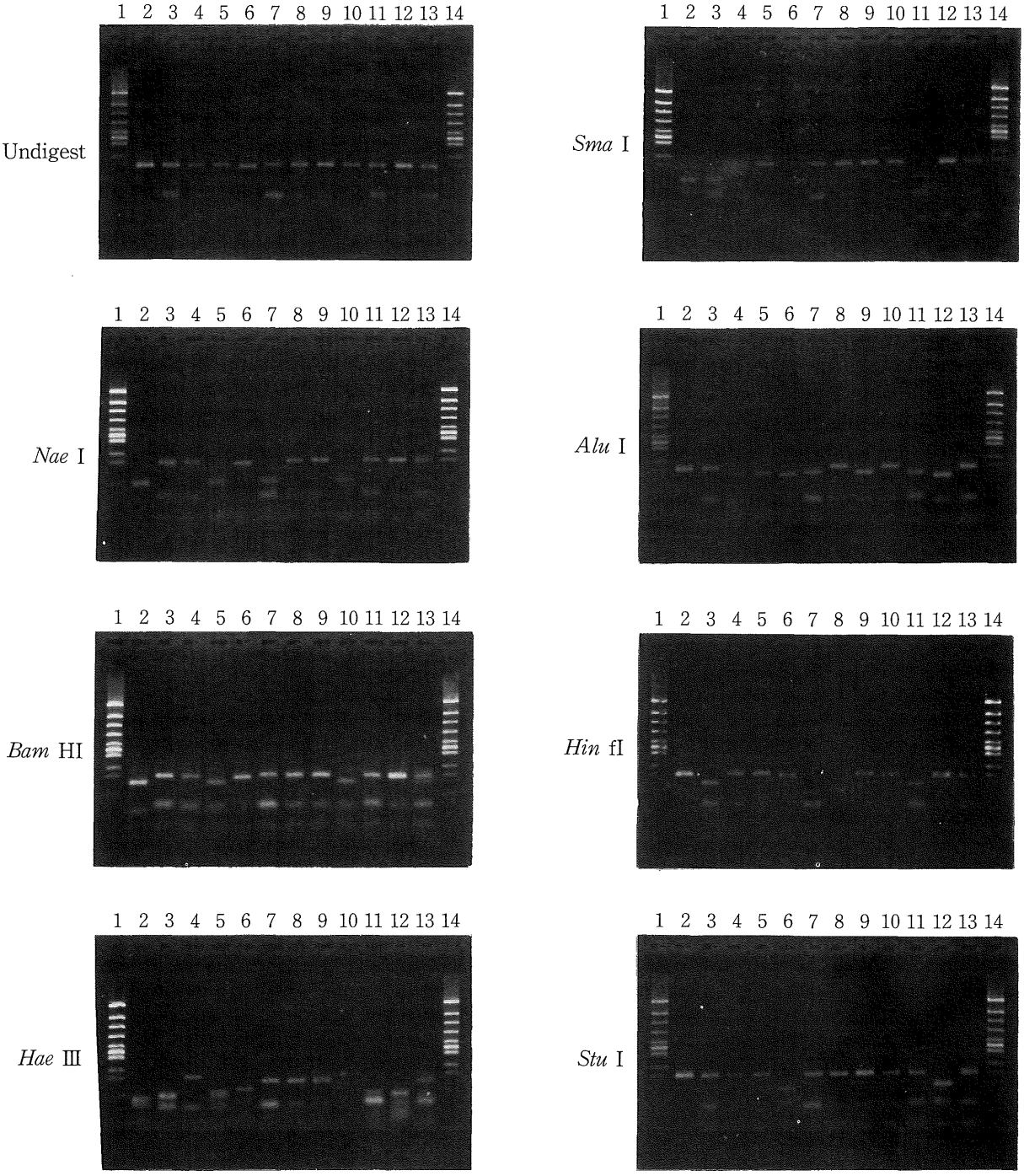

Fig. 5. RFLP analysis of PCR products.
PCR products (156-bp) were subjected to RFLP analysis using seven restriction enzymes (*Sma*I, *Nae*I, *Alu*I, *Bam*HI, *Hin*fI, *Hae*III, and *Stu*I, respectively). The digested fragments were electrophoresed on 3% agarose gels. Lanes 1 and 14: $\phi \times 174$ digested with *Hin*cII, as DNA size standards ; Lane 2: *M.tuberculosis* H37Rv; Lane 3: *M.kansasii*; Lane 4: *M.simiae*; Lane 5: *M.scrofulaceum*; Lane 6: *M.szulgai*; Lane 7: *M.avium*; Lane 8: *M.intracellulare*; Lane 9: *M.xenopi*; Lane 10: *M.haemophilum*; Lane 11: *M.gastri*; Lane 12: *M.nonchromogenicum*; Lane 13: *M.fortuitum*

Table 3. RFLP analysis of PCR products*

Species and strain		*Sma*I	*Nae*I	*Alu*I	*Bam*HI	*Hin*fI	*Hae*III	*Stu*I
M. tuberculosis complex		+	+	−	+	−	+	−
M. kansasii	ATCC12478	+	−	−	−	+	+	−
M. simiae	ATCC25275	−	−	+	−	−	−	−
M. scroflaceum	ATCC19981	−	+	+	+	−	+	−
M. szulgai	ATCC35799	+	−	+	−	−	+	+
M. avium	ATCC25291	−	+	+	−	+	+	−
M. intracellulare	ATCC13950	−	−	−	−	+	+	−
M. xenopi	ATCC19250	−	−	+	−	−	+	−
M. haemophilum	ATCC29548	−	+	−	+	−	−	−
M. gastri	ATCC15754	+	−	+	−	+	+	−
M. nonchromogenicum	ATCC19530	−	−	+	−	−	+	+
M. fortuitum	ATCC6841	−	−	−	−	−	+	−

* : 156-bp fragment amplified by the nested PCR
(+) : Restriction site (+)
(−) : Restriction site (−)

Table 4. Identification of mycobacterial species by RFLP analysis of PCR products*

Restriction site	Species and strain		Restriction site for further identification			
*Sma*I(+) *Nae*I(+)	*M. tuberculosis* complex					
*Sma*I(+) *Nae*I(−)	*M. kansasii*	ATCC12478	*Alu*I(−)			
	M. gastri	ATCC15754	*Alu*I(+)	*Hin*fI(+)		
	M. szulgai	ATCC35799	*Alu*I(+)	*Hin*fI(−)		
*Sma*I(−) *Nae*I(+)	*M. avium*	ATCC25291	*Bam*HI(−)			
	M. scroflaceum	ATCC19981	*Bam*HI(+)	*Alu*I(+)		
	M. haemophilum	ATCC29548	*Bam*HI(+)	*Alu*I(−)		
*Sma*I(−) *Nae*I(−)	*M. intracellulare*	ATCC13950	*Hin*fI(+)			
	M. fortuitum	ATCC6841	*Hin*fI(−)	*Alu*I(−)		
	M. simiae	ATCC25275	*Hin*fI(−)	*Alu*I(+)	*Hae*III(−)	
	M. xenopi	ATCC19250	*Hin*fI(−)	*Alu*I(+)	*Hae*III(+)	*Stu*I(−)
	M. nonchromogenicum	ATCC19530	*Hin*fI(−)	*Alu*I(+)	*Hae*III(+)	*Stu*I(−)

* : 156-bp fragment amplified by the nested PCR
(+) : Restriction site (+)
(−) : Restriction site (−)

Table 5. Comparison between bacteriological findings and PCR results

	Bacteriological examination	Nested PCR positive	
Tubercular patients (n=67)	microscopic exam. (+) and culture (+)	14/15 (93.3%)	
	microscopic exam. (+) and culture (−)	6/6 (100%)	26/28 (92.9%)
	microscopic exam. (−) and culture (+)	6/7 (85.7%)	
	microscopic exam. (−) and culture (−)		19/39 (48.7%)
Nontubercular patient (n=17)			0/17 (0%)

DISCUSSION

The application of PCR for diagnosing mycobacterial infections has been reported for the 65 kDa antigen gene[6,7,12,23,24], repetitive DNA element IS*6110*[6,9-11,25,35], MPB64 protein gene[17,31,32], β-galactoside fusion protein gene[13], ribosomal RNA[5], antigen b gene[33], *dnaJ* gene[21,34], and others. These PCR protocols can be roughly divided into two groups based on the procedure used to amplify specifically the *M.tuberculosis* complex alone, and the procedure used for amplifying both the *M.tuberculosis* complex and nontuberculous mycobacteria. However, few studies have used PCR to distinguish among mycobacterial species. Some nontuberculous mycobacteria have patterns of drug sensitivity that differ considerably from those of *M.tuberculosis*. If such mycobacteria can be directly detected from clini-

cal samples and be distinguished by a PCR procedure, it will contribute to the selection of an optimal therapeutic plan. Thus, we attempted to establish a method which could sensitively detect the mycobacterial gene in clinical samples and allow the identification of mycobacteria.

Hance and colleagues reported that the combination of a PCR procedure and the Southern blotting for the 65 kDa antigen gene allowed one to distinguish among three groups of mycobacteria (*M.tuberculosis*, *M.avium*/*M.paratuberculosis*, and *M.fortuitum*)[12]. However, that technique appears to have little clinical utility because it can identify only a small number of species. Also, it requires the use of radioisotopes and involves the problem of cross hybridization. Nagai and colleagues devised a PCR procedure for the *dnaJ* gene of *M.tuberculosis*, and reported that RFLP analysis of the products of this PCR allowed the identification of four clinically important species of mycobacteria (*M.tuberculosis*, *M.avium*, *M.intracellulare*, and *M.kansasii*)[21,34]. However, its sensitivity does not seem adequate for detecting mycobacteria in clinical samples. Although *dnaJ* gene is a single copy gene, as it contains polymorphisms between various species of mycobacteria, we targeted this gene for detecting and identifying the mycobacteria.

In assessing the sensitivity of the amplification procedure for the detection of mycobacteria, we found that only 10 fg of mycobacterial DNA for one reaction was detectable by our nested PCR procedure. Considering that a single *M.tuberculosis* organism contains 5 fg of DNA[2], this indicates that a single mycobacterium organism may be detected approximately by this method. The mean amplifying efficiency of the nested PCR was about 1.6:1, and 10^{12}-to 10^{13}-fold amplification was achieved by 60 cycles of PCR (30 cycles in the first and 30 cycles in the second PCR). As suggested by Pierre and colleagues[24], the sensitivity of a nested PCR procedure for the detection of mycobacteria appears comparable to that of PCR-Southern blotting.

To assess the specificity of this procedure, DNAs extracted from 23 mycobacterial strains were subjected to the nested PCR. Specific amplification was observed in all strains belonging to *M.tuberculosis* complex and in 11 of the 16 strains of nontuberculous mycobacteria. These 11 strains included *M.avium*, *M.intracellulare* and *M.kansasii*, which are clinically important as pathogens in humans. On the other hand, PCR did not amplify the DNA of any clinically isolated bacteria other than the mycobacteria we examined. Although the *dnaJ* gene of *M.tuberculosis* has a 48.9% homology in nucleotide sequence to that of *Escherichia coli*, the primers used in the present study showed no significant homology and the *dnaJ* gene from *E.coli* was not amplified by the nested PCR.

Analysis of the nucleotide sequences of the PCR products revealed polymorphisms among different species of mycobacteria, endorsing the report of Nagai and colleagues[21]. Although we tested a single strain for each non-tuberculous mycobacterial species in the present study, the nucleotide sequences of the fragments from *M.avium*, *M.intracellulare*, and *M.kansasii* agreed with those reported by Nagai and colleagues[21]. Therefore, we concluded that the *dnaJ* gene was applicable in the distinction of mycobacterial species.

The RFLP analysis using seven restriction enzymes allowed us to distinguish the strains belonging to the *M.tuberculosis* complex from the 11 nontuberculous mycobacterial strains whose *dnaJ* gene could be amplified by the nested PCR. The *M.tuberculosis* complex was distinguished from other mycobacteria by using two restriction enzymes (*Sma*I and *Nae*I). When two or more species coexist in one sample, the RFLP pattern will be complicated and it is impossible to identify plural species. But the coexistence of plural species may be expected from the complicated RFLP pattern.

To assess the clinical utility of our PCR procedure, we compared the results of the bacteriological test with those of nested PCR. In the tuberculosis group, the positive rate of the nested PCR (67.2%, 45/67) was significantly higher than that of the bacteriological examination (41.8%, 28/67).(McNemar's test, $p<0.01$). These percentages were comparable to those reported by Pierre and colleagues[24], but lower than the rate reported by Brisson-Noël and colleagues[6]. This difference may be explained by the following factors: some patients in our study appeared to have become negative for *M.tuberculosis* following effective chemotherapy, and only one sputum sample was examined for each patient. Of the 28 patients who were bacteriologically classified as having *M.tuberculosis*, 26 (92.9%) were PCR-positive. Two patients were false-negative by the nested PCR, probably because the amount of sputum examined was not large enough. Therefore, the heterogeneity of sputum was reflected in the difference between the bacteriological and PCR judgments. We cannot exclude the possibility that some inhibitors were present in the sample and hampered PCR, as suggested by Brisson-Noël and colleagues[6]. However, considering that PCR showed a high positive rate (48.7%) in the 39 patients classified as bacteriologically negative, we conclude that our nested PCR procedure is more sensitive than conventional bacteriological methods.

A combination of nested PCR and RFLP analysis seems to be superior to a combination of PCR and Southern blotting in that it does not involve the problem of cross hybridization, it is simpler,

does not require radioisotopes, and does not require numerous probes. Thus, our nested PCR-RFLP procedure is clinically promising.

We examined only sputum samples in this study. However, this examination procedure seems also to be applicable to gastric juice, pleural effusion, pericardial effusion, cerebrospinal fluid, urine, blood, abscess aspirate, and other specimens as previously reported. This procedure would be particularly useful in cases in which the diagnosis of mycobacterial infections and identification of the mycobacterial species responsible are urgently required.

ACKNOWLEDGEMENTS

We thank Dr.C.Abe (Research Institute of Tuberculosis, Japan Anti-Tuberculosis Association. Tokyo, Japan) for the supply of mycobacterial strains. We are grateful to Dr.C.Satoh (Radiation Effects Research Foundation. Hiroshima, Japan) for generous support. This study was supported in part by a Grant-in-Aid for Scientific Research (No.04670467) from the Ministry of Education, Science and Culture of Japan. A preliminary report of this work was presented at the annual meeting of the American Thoracic Society, Florida, USA, May 17-20, 1992.

(Received September 10, 1992)
(Accepted December 22, 1992)

REFERENCES

1. **Abe, C., Hosojima, S., Fukasawa, Y., Kazumi, Y., Takahashi, M., Hirano, K. and Mori, T.** 1992. Comparison of MB-check, BACTEC, and egg-based media for recovery of mycobacteria. J. Clin. Microbiol. **30:** 878-881.
2. **Baess, I.** 1984. Determination and re-examination of genome sizes and base ratios in deoxyribonucleic acid from mycobacteria. Acta Pathol. Microbiol. Immunol. Scand. Sect. **B92:** 209-211.
3. **Bates, J.H.** 1979. Diagnosis of tuberculosis. Chest **76(Suppl.):** 757-763.
4. **Blaser, M.J. and Cohn, D.L.** 1986. Opportunistic infections in patients with AIDS: Clues to the epidemiology of AIDS and the relative virulence of pathogens. Rev. Infect. Dis. **8:** 21-30.
5. **Böddinghaus, B., Rogall, T., Flohr, T., Blöcker, H. and Böttger, E.C.** 1990. Detection and identification of mycobacteria by amplification of rRNA. J. Clin. Microbiol. **28:** 1751-1759.
6. **Brisson-Noël, A., Aznar, C., Chureau, C., Nguyen, S., Pierre, C., Bartoli, M., Bonete, R., Pialoux, G., Gicquel, B. and Garrigue, G.** 1991. Diagnosis of tuberculosis by DNA amplification in clinical practice evaluation. Lancet **ii:** 364-366.
7. **Brisson-Noël, A., Gicquel, B., Lecossier, D., Lévy-Frébault, V., Nassif, X. and Hance, A.J.** 1989. Rapid diagnosis of tuberculosis by amplification of mycobacterial DNA in clinical samples. Lancet **ii:** 1069-1071.
8. **Daniel, T.M.** 1990. The rapid diagnosis of tuberculosis: A selective review. J. Lab. Clin. Med. **116:** 277-282.
9. **Eisenach, K.D., Cave, M.D., Bates, J.H. and Crawford, J.T.** 1990. Polymerase chain reaction amplification of a repetitive DNA sequence specific for *Mycobacterium tuberculosis*. J. Infect. Dis. **161:** 977-981.
10. **Eisenach, K.D., Sifford, M.D., Cave, M.D., Bates, J.H. and Crawford, J.T.** 1991. Detection of *Mycobacterium tuberculosis* in sputum samples using a polymerase chain reaction. Am. Rev. Respir. Dis. **144:** 1160-1163.
11. **Godfrey-Faussett, P., Wilkins, E.G.L., Khoo, S. and Stoker, N.** 1991. Tuberculous pericarditis confirmed by DNA amplification. Lancet **i:** 176-177.
12. **Hance, A.J., Grandchamp, B., Lévy-Frébault, V., Lecossier, D., Rauzier, J., Bocart, D. and Gicquel, B.** 1989. Detection and identification of mycobacteria by amplification of mycobacterial DNA. Mol. Microbiol. **3:** 843-849.
13. **Hermans, P.W.M., Schuitema, A.R.J., Soolingen, D.V., Verstynen, C.P.H.J., Bik, E.M., Thole, J.E.R., Kolk, A.H.J. and Embden, J.D.A.V.** 1990. Specific detection of *Mycobacterium tuberculosis* complex strains by polymerase chain reaction. J. Clin. Microbiol. **28:** 1204-1213.
14. **Hobby, G.L., Holman, A.P., Iseman, M.D. and Jones, J.M.** 1973. Enumeration of tubercle bacilli in sputum of patients with pulmonary tuberculosis. Antimicrob. Agents Chemother. **4:** 94-104.
15. **Hong Kong Chest Service / Tuberculosis Research Centre, Madras / British Medical Research Council.** 1981. A Study of the characteristics and course of sputum smear-negative pulmonary tuberculosis. Tubercle **62:** 155-167.
16. **Joint International Union Against Tuberculosis and World Health Organization.** 1982. Tuberculosis control. Tubercle **63:** 157-169.
17. **Kaneko, K., Onodera, O., Miyatake, T. and Tsuji, S.** 1990. Rapid diagnosis of tuberculous meningitis by polymerase chain reaction (PCR). Neurology **40:** 1617-1618.
18. **Krasnow, I. and Wayne, L.G.** 1969. Comparison of methods for tuberculosis bacteriology. Appl. Microbiol. **18:** 915-917.
19. **Lathigra, R.B., Young, D.B., Sweetser, D. and Young, R.A.** 1988. A gene from *Mycobacterium tuberculosis* which is homologous to the DnaJ heat shock protein of *E.coli*. Nucleic Acids Res. **16:** 1636.
20. **McNemar, Q.** 1947. Note on the sampling error of the differences between corrected proportions or percentages. Psychometrika. **12:** 153-157.
21. **Nagai, R., Takewaki, S., Wada, A., Okuzumi, K., Tobita, A. and Ohkubo, A.** 1990. Rapid detection and identification of mycobacterial DNA by PCR. Jpn. J. Clin. Pathol. **38:** 1247-1253. (In Japanese with English summary)
22. **Ogawa, T., Saba, K. and Suzuki, T.** 1950. Quantitative measurement of culture of tubercle bacilli. Report **VII.** The quantitative measurement of tubercle bacilli in sputum. Kekkaku **25:** 207-220. (In Japanese with English summary)
23. **Pao, C.C., Yen, T.S.B., You, J., Maa, J., Fiss,**

E.H. and Chang, C. 1990. Detection and identification of *Mycobacterium tuberculosis* by DNA amplification. J. Clin. Microbiol. **28:** 1877–1880.

24. **Pierre, C., Lecossier, D., Boussougant, Y., Bocart, D., Joly, V., Yeni, P. and Hance, A.J.** 1991. Use of a reamplification protocol improves sensitivity of detection of *Mycobacterium tuberculosis* in clinical samples by amplification of DNA. J. Clin. Microbiol. **29:** 712–717.
25. **Plikaytis, B.B., Eisenach, K.D., Crawford, J.T. and Shinnick, T.M.** 1991. Differentiation of *Mycobacterium tuberculosis* and *Mycobacterium bovis* BCG by a polymerase chain reaction assay. Mol. Cell. Probes **5:** 215–219.
26. **Report of a National Heart, Lung, and Blood Institute Workshop.** 1984. Pulmonary complications of the acquired immunodeficiency syndrome. N. Engl. J. Med. **310:** 1682–1688.
27. **Rouillon, A., Perdrizet, S. and Parrot, R.** 1976. Transmission of tubercle bacilli: The effects of chemotherapy. Tubercle **57:** 275–299.
28. **Saiki, R.K., Gelfand, D.H., Stoffel, S., Scharf, S.J., Higuchi, R., Horn, G.T., Mullis, K.B. and Erlich, H.A.** 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science **239:** 487–491.
29. **Sambrook, J., Fritsch, E.F. and Maniatis, T.** 1989. Molecular Cloning: A Laboratory Manual–2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
30. **Selik, R.M., Starcher, E.T. and Curran, J.W.** 1987. Opportunistic diseases reported in AIDS patients: Frequencies, associations, and trends. AIDS **1:** 175–182.
31. **Shankar, P., Manjunath, N., Lakshmi, R., Aditi, B., Seth, P. and Shriniwas.** 1990. Identification of *Mycobacterium tuberculosis* by polymerase chain reaction. Lancet **i:** 423.
32. **Shankar, P., Manjunath, N., Mohan, K.K., Prasad, K., Behari, M., Shriniwas and Ahuja, G.K.** 1991. Rapid diagnosis of tuberculous meningitis by polymerase chain reaction. Lancet **i:** 5–7.
33. **Sjöbring, U., Mecklenburg, M., Andersen, Å.B. and Miörner, H.** 1990. Polymerase chain reaction for detection of *Mycobacterium tuberculosis*. J. Clin. Microbiol. **28:** 2200–2204.
34. **Takewaki, S. and Nagai, R.** 1991. Detection and identification of mycobacterial DNA by PCR. J. Clin. Exp. Med. **158:** 773–778. (In Japanese)
35. **Thierry, D., Brisson-Noël, A., Vincent-Lévy-Frébault, V., Nguyen, S., Guesdon, J. and Gicquel, B.** 1990. Characterization of a *Mycobacterium tuberculosis* insertion sequence, IS*6110*, and its application in diagnosis. J. Clin. Microbiol. **28:** 2668–2673.
36. **Tsukamura, M., Kita, N., Shimoide, H., Arakawa, H. and Kuze, A.** 1988. Studies on the epidemiology of nontuberculous mycobacteriosis in Japan. Am. Rev. Respir. Dis. **137:** 1280–1284.