原　著

Self Ligation Mediated Polymerase Chain Reactionの開発とその有用性：結核の分子疫学における菌株のDNA指紋の迅速識別法

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USEFULNESS OF SELF LIGATION MEDIATED POLYMERASE CHAIN REACTION : A RAPID METHOD FOR FINGERPRINTING IN MOLECULAR EPIDEMIOLOGY OF TUBERCULOSIS

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Restriction fragment length polymorphism (RFLP) analysis based on the insertion sequence IS*6110* has been used as one of the powerful tools for epidemiological study of tuberculosis. However this technique requires more than 1 micro-gram of DNA and two days for completion. To overcome these inconvenience, we have modified a PCR-based method, self ligation mediated PCR (SL-PCR) on the molecular epidemiological study. This method uses a pair of primers whose orientations are from inside to outside of IS*6110*. The DNA fragments flanking IS*6110* are amplified by the PCR by using the *Sau* 3A I digested and ligated chromosomal DNA of *Mycobacterium tuberculosis* strains. By using this method, *M. tuberculosis* strains can be differentiated within 8 hours.

結核菌の中に挿入されているDNA断片IS*6110*をプローブとしたRFLP分析が結核の疫学や診断の強力な手段の1つとして利用されている。しかし，RFLP分析を行うにはDNA量が1 micro-gram以上必要であり，検査結果が出るまでに2日を要する難点がある。そこで，われわれはこの不便さを克服するためにPCRの技術を利用した新たな検査方法であるSelf Ligation Mediated Polymerase Chain Reaction (SL-PCR) 法を開発した。この方法は，IS*6110*の内側から外側向きに配列した1対のプライマーを用いるものである。DNA断片のIS*6110*を制限酵素*Sau* 3A Iを使って分解し，PCR法を使って増幅させ，結核菌の染色体のDNAと結合させる方法である。この方法を用いることにより，結核菌の株を8時間以内で識別することが可能となる。今後，結核患者の接触者調査や結核蔓延状況の解明などのスクリーニング検査

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(Received 26 Jul. 2000／Accepted 30 Oct. 2000)

法としても活用しうる方法である。

Key words: *Mycobacterium tuberculosis*, RFLP, SL-PCR, Molecular epidemiology

キーワーズ：結核菌，RFLP，SL-PCR，分子疫学

Introduction

Tuberculosis remains one of the most serious distresses, leading to 3 million deaths annually all over the world. The major portion of these deaths is in the developing country. However, tuberculosis is thought to be one of the biggest reemerging diseases even in the highly industrialized country. One of the reasons for this is the occurrence of many outbreaks of tuberculosis in the hospitals, schools, offices and families. Understanding the transmission of tuberculosis will be of great value in optimizing the strategies to control and prevent its development and transmission. For the aim of help to understand the transmission, molecular epidemiological procedures were established.

One of them, the restriction fragment length polymorphism (RFLP) analysis based on the IS *6110* DNA has become the standard method for epidemiological studies of tuberculosis[1)~4)]. IS *6110*, an insertion sequence belonging to the IS3 family[5)], is found in almost all members of the *Mycobacterium tuberculosis* complex and not in other members of mycobacteria[2)6)]. The polymorphism of the IS*6110* RFLP patterns among clinically unrelated strain is very high, whereas the epidemiologically related ones show identical or similar fingerprint patterns[4)]. Hence, *M. tuberculosis* strains with identical fingerprint patterns represent strains that have possibly been recently transmitted. In this context, RFLP analysis seemed to be a good tool for epidemiological study of tuberculosis. However, as this method require more than one micro-gram of DNA from large number of bacilli for one assay and the procedure is laborious, rapid and sensitive technique for molecular epidemiology of tuberculosis was eagerly awaited.

As alternatives of RFLP, pulse field gel electrophoresis, Mixed-linker PCR (ML-PCR)[7)], spoligotyping[8)] and inverse-PCR[9)] were applied for this field. Among them, mixed-linker PCR and inverse-PCR have an advantage that these methods based on the IS*6110* and can inherit vast amount of the data of RFLP analysis. In this paper, we will discuss about the usefulness of self ligation mediated PCR, an improved procedure of inverse-PCR, for molecular epidemiology of tuberculosis.

Materials and Methods

Patient population

This study included 13 clinical cases from 5 outbreaks and 21 clinically independent cases, of all being treated for tuberculosis.

Bacterial strains

Clinical isolates and the type strain H37Ra of *M.tuberculosis*, have been maintained on Ogawa egg medium (Nissui Seiyaku Co., Ltd., Tokyo, Japan) in our laboratory.

DNA preparation

DNAs for PCR were prepared by mechanical disruption as described previously[10)]. Briefly, a single colony on Ogawa medium was picked and suspended in 0.5 m*l* of lysis buffer consisting of 0.3 M of Tris-HCl pH 8.0, 0.1 M of NaCl, and 6 mM of EDTA in a conical 2 m*l* screw-cap vial which is one-fourth filled with 0.17 mm acid-washed sterile glass beads. Mycobacterial cells were disrupted by vigorous shaking with 0.5 m*l* of chloroform on a Mini-Bead Beater cell disrupter (Biospec Products, Bartlesville, UK) for 5 min. DNAs in the upper layer after centrifugation were further purified by phenol-chloroform extraction, concentrated by ethanol precipitation and dissolved in 300 μl of TE buffer consisting of 10 mM Tris-HCl pH 8.0, 1 mM EDTA.

RFLP Analysis

Table Oligonucleotides used in this study

Method	Primer name	Sequence	Length	Reference
SL-PCR	TBIS6110-SauN1	GAGGTACCCGCCGGAGCTGC	20bp	this work
	TBIS6110-SauN2	GACCCCATCCTTTCCAAGAA	20bp	
	TBIS6110-Sau1	GGCGGTGCGGATGGTCGCAG	20bp	
	TBIS6110-Sau2	AGTCTCCGGACATGCCGGGG	20bp	
ML-PCR	IS04-92	UGCGAGUCGAGGUCAGUUCT	20bp	7
	IS08-92	AGAACTGACCTCGACTCGCACG	22bp	
	IS09-92	AGAACTGACCTCGACTCGCA	20bp	
	IS54-92	TCGACTGGTTCAACCATCGCCG	22bp	
	IS62-92	ACCAGTACTGCGGCGACGTC	20bp	
	IS55-92	TCTGATCTGAGACCTCAGC	19bp	

RFLP analysis was performed according to van Embden et al.[11] Briefly, the genomic DNA from *M. tuberculosis* was digested with *Pvu* II, subjected to agarose gel electrophoresis, followed by blotting onto a nylon membrane. After hybridization with a digoxigenin (DIG)-labelled IS*6110* DNA probe, the hybridization bands were detected by the DIG detection procedure according to the manufacturer's manual (Boehringer Mannheim GmbH, Mannheim, Germany). *Pvu* II-digested supercoiled DNA ladder (Gibco-BRL, Life Technologies Ltd., Paisley, UK) and X174-*Hae* III DNA (Advanced Biotechnologies, London, UK) were DIG labelled with a random-primed DNA-labelling method. Pair wise similarities of IS*6110* fingerprint patterns were calculated by the Dice coefficient of similarity with GelCompar software (version 4.0; Applied Maths, Kortrijk, Belgium).

Mixed-linker PCR (ML-PCR)

Mixed-linker PCR was performed as described by Haas et al.[7] Briefly, genomic DNA was digested with *Hha* I and the restriction product was ligated with 1000 × molar excess of mixed linker (Table; IS04-92 and IS08-92) by T4 DNA ligase (BRL Life Technologies Ltd., Paisley, UK) overnight at 16℃. Then the samples were treated with uracil N-glycosylase (UNG) (Perkin-Elmer, Tenessee, USA). UNG treated samples were used as template of PCR. The PCR reaction mix contained 0.5U of *Taq* DNA polymerase (Takara Shuzo, CO., Ltd., Shiga, Japan), deoxynucleotide triphosphates (200mM each), and 1.0mM each of oligonucleotide primers IS 54-92 and IS09-92 (Table) and UNG treated samples. PCR reactions were performed 35 cycles in Thermal cycler personal (Takara Shuzo, Co., Ltd., Shiga, Japan) with each cycle consisting of denaturation for 30 sec at 94℃, annealing for 30 sec at 55℃, and extension for 1 min at 72℃. For nested amplification, 1μl of the PCR product was diluted 1/100, and 10μl was amplified for with IS62-92 and IS09-92 as primers (Table). The PCR products were separated by polyacrylamide gel electrophoresis (15-25%, Multigel; Daiichi pure chemicals Co., Ltd., Japan) at 20mA for two hours using TG buffer (25mM Tris-base, 192mM glycin, pH8.4) followed by SYBR green staining (Molecular Probes, Inc, Oregon, USA) according to manufacturer's manual.

Self ligation mediated PCR (SL-PCR)

The oligonucleotides primers used are shown in Table. Primers TBIS*6110-Sau*1, TBIS*6110-Sau*2, TBIS*6110-Sau*N1, TBIS*6110-Sau*N2 are located in the IS*6110* element as in Fig. 2. The strategy for self ligation mediated PCR is schematically displayed in Fig. 1. The genomic DNA (50ng/μl) was digested for 2 hours at 37℃ with excess amount of *Sau*3A I, followed by heating at 65℃ for 20 min to inactivate enzyme. The restriction fragments were then ligated at 16℃ for 2 hours by mixing with same volume of ligation solution I (Takara Shuzo, Co., Ltd.),

followed by first PCR. The first PCR reaction mixture (50 μl) consisted of PCR ; 2.5 mM MgC 12 ; 200 mM each dATP, dCTP, dGTP, and dTTP ; ligated TB DNA ; 1.25 units of Takara *Taq* DNA polymerase and 2 μM each primer TBIS*6110-Sau*N1 and TBIS*6110-Sau*N2. PCR was carried out for 35 cycles in Thermal cycler Personal, with each cycle consisting of denaturation for 30 sec at 94℃, annealing for 30 sec at 55℃, and extension for 1 min at 72℃. Secondary PCR were performed using 1 μl of first PCR products as templates. The condition of PCR was same as first PCR with the exception of primers used (TBIS*6110-Sau*1 and TBIS *6110-Sau*2). The PCR products were analyzed by polyacrylamide gel electrophoresis (15-25%, Multigel) as described above. Every solution used in this study was prepared at the DNA-free area separated from template preparation and amplification. Templates were added with positive-displacement pipettes to further decrease the risk of carryover contamination.

Results

Amplification of flanking DNA sequences

The general strategy of the self ligation mediated PCR approach is outlined in (Fig. 1). The genomic DNA is digested to completion with restriction endonuclease *Sau*3A I , and the fragments were ligated by itself. This step is followed by PCR with an IS*6110*-specific oligonucleotide primers (Fig. 2). In the first cycle of amplification, only the self ligated restriction fragments containing the IS*6110* sequence can serve as templates for the PCR. The PCR products after the nested amplification were analyzed directly by polyacrylamide gel electrophoresis. Between 5 and 15 bands were observed by the SL-PCR with the size distribution between 50 and 400 bp (Fig. 3). The reproducibility of this method was confirmed by the repeated experiments (Data not shown).

SL-PCR fingerprinting analysis of randomly chosen strains

Twenty-one clinically independent *M. tuberculosis* isolates were analyzed by SL-PCR and compared with the results obtained by both RFLP analysis and ML-PCR. Fig. 3A and B shows the fingerprint pattern obtained by SL-PCR and ML-PCR, respectively. We obtained independent patterns with all isolates. This result possessed compatibility with the fingerprint pattern obtained by RFLP analysis (Fig. 3C).

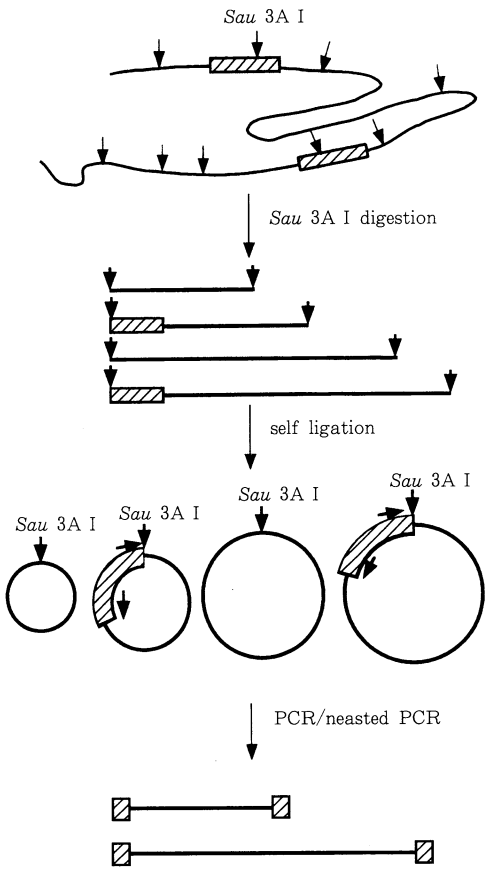

Fig. 1 The general strategy of the SL-PCR. The genomic DNA is digested with *Sau*3A I and the restriction fragments (hached boxes) were ligated by itself, followed by PCR with an IS6110 specific oligonucleotide primers.

SL-PCR fingerprinting analysis of strains from outbreak

Thirteen *M. tuberculosis* strains isolated from five outbreaks were analyzed by SL-PCR (Fig. 4A) and confirmation were done with the results obtained by both ML-PCR (Fig. 4B) and traditional RFLP analysis (Fig. 4C). The fingerprint-

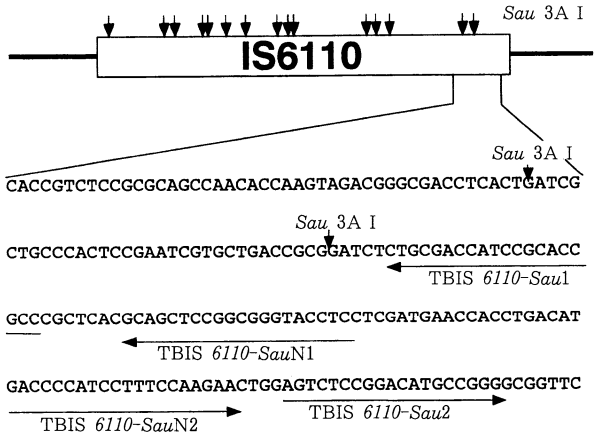

Fig. 2 Position and orientation of primers used for SL-PCR. All the primers were designated in the 150 bp *Sau*3A I fragment at the end of IS*6110*. TBIS*6110-Sau*1 and TBIS*6110-Sau*N1 were oriented inward to IS*6110*. TBIS*6110-Sau*2 and TBIS*6110-Sau*N2 were oriented outward to IS*6110*.

ing patterns by SL-PCR were well correlated to RFLP and ML-PCR fingerprinting results in four of five outbreak cases. In cases a, b and c, identical fingerprint patterns were obtained. In case d, one additional band was observed in RFLP of isolate 2 when comparing to isolate 1 (Fig. 4C, series d). Correlating to this, changes in the fingerprint patterns were observed in both SL-PCR and ML-PCR. In SL-PCR, one band in isolate 1 (asterisk) was not observed and two new bands (arrow) were observed in isolate 2 (Fig. 4A, series d). Similar changes were observed in the fingerprint pattern of ML-PCR (Fig. 4B, series d). In contrast to these, the change in RFLP analysis correlated only to SL-PCR in case e. When the intensity of two bands in RFLP analysis (asterisk and arrow) were compared, the band of asterisk was more intense than that of arrow in isolate 1 and 2 (Fig. 4C, series e, lane 1 and 2). On the contrary, the intensity of the band of arrow was higher than the band of asterisk in isolate 3 (Fig. 4C, series e, lane 3). The bands with higher intensity might be double bands. Changes correlated to this were found in the fingerprint pattern of SL-PCR. Two bands in isolate 1 (asterisk) were not and a new band (arrow) was observed in isolate 3 (Fig. 4A, series e) in it. In contrast, no difference was observed in the fingerprint pattern among other three outbreak of isolates (Fig. 4B series a, b, e).

Discussion

We have been interested in molecular epidemiological study of tuberculosis. The Restriction fragment length polymorphism (RFLP) analysis is extensively used all over the world for this study. Analysis of vast number of clinical isolates by this method resulted in the accumulation of large number of RFLP data together with the clinical data. So, the RFLP analysis on the molecular epidemiological study in recognized as standard procedure. However, this method has shortcomings of the requirement of subculture and its complicated steps. Hence, the rapid and simple methods posessing compatibility with RFLP have been looked for.

Standard RFLP method is based on the polymorphism of the DNA fragment flanking the insertion sequence IS*6110*. Thus, amplification

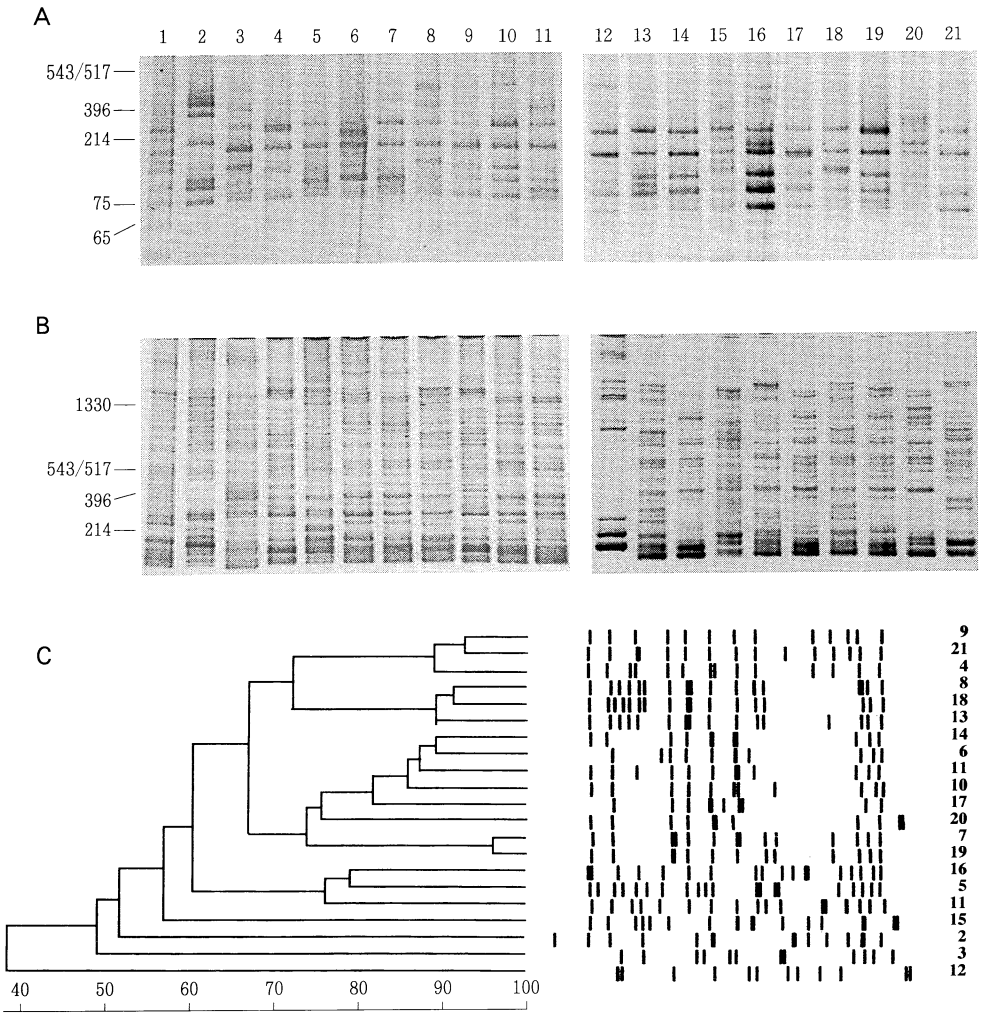

Fig. 3 Molecular epidemiological analysis of 21 independent isolates of *M. tuberculosis*.
A. SL-PCR analysis, B. ML-PCR analysis, and C. The dendrogram and the fingerprint pattern of 21 independent isolates of *M. tuberculosis*. Molecular weight markers were shown at left side.

of the IS*6110* flanking sequences by PCR will give the data compatible with RFLP. However, typical PCR utilize oligonucleotide primers that are oriented inwards across the region between the two primers. By the conventional PCR procedure, DNA sequences that lie outside the primers can not be amplified because oligonucleotides that prime DNA synthesis into flanking regions permit only a linear increase in the number of copies.

For the amplification of the flanking sequences by PCR, three basic methods have been reported. Ochaman et al. described inverse PCR[12]. Frohman et al. demonstrated the usefulness of anchor PCR for the rapid production of full-length cDNA's[13]. In addition to these, single-site PCR was established by Riley et al. for the isolation of terminal sequences from yeast

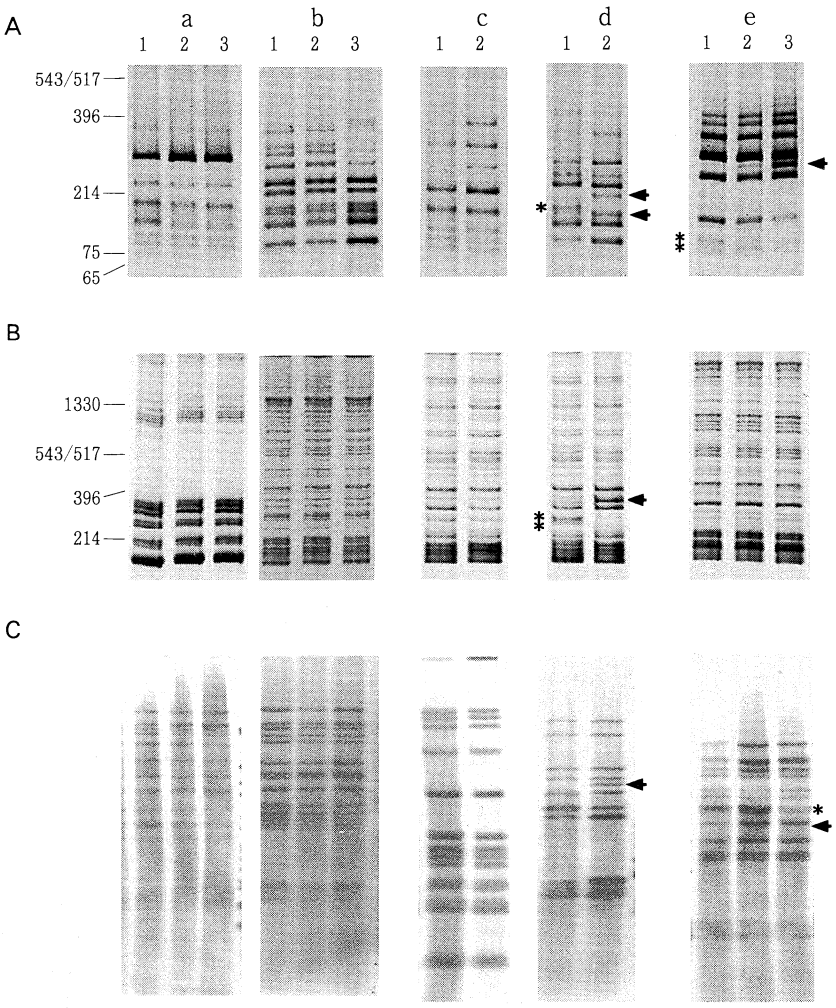

Fig. 4 Analysis of 13 outbreak isolates of *M. tuberculosis* by SL-PCR, ML-PCR and RFLP analysis. A. SL-PCR analysis, B. ML-PCR analysis, and C. RFLP analysis of 13 isolates from five outbreaks. Series a to e are outbreak cases. Lanes 1 to 3 are number of isolates. Molecular weight markers were shown at left side.

artificial chromosome (YAC) clones[14].

We used an inverse PCR related method termed self ligation mediated PCR (SL-PCR) that allows the amplification of the IS*6110* flanking regions. The method to obtain the IS*6110* flanking regions by SL-PCR was shown schematically in Fig. 1. Genomic DNA (50ng/μl) was cleaved using a restriction enzyme *Sau*3A I followed by ligation. The resulting self ligation products were then used as templates for enzymatic amplification by PCR using oligonucleotide primers homologous to IS*6110* 150 bp *Sau*3A I restriction fragment (Fig. 2) to amplify the DNA restriction fragments flanking this DNA fragment.

For uniform amplification of multiple DNA fragments, the size distribution of target DNA is one of the crucial factors. Theoretically, small fragments can be efficiently amplified by PCR than larger fragments. Thus we chose the restric-

tion endonuclease *Sau*3A I that had a 4-bp recognition sequence and produces a 4-bp 5′ overhang (GATC) that allowed the generation of the short restriction fragment and efficient ligation of the restriction fragments, respectively. The restriction endonuclease *Sau*3A I was found to cut IS*6110* into 16 fragments with two fragments flank the neibouring DNA of IS *6110*. One of them, 151 bp fragment was chosen for the target of our SL-PCR. The distribution of the size of TB DNA generated by *Sau*3A I range from 50 to 600 bp with the average length of 250 bp. This size range allowed efficient amplification of up to 20 restriction fragments carrying IS*6110* sequences. Otal et al. demonstrated the possibility of the utilization of inverse-PCR for the molecular epidemiology of tuberculosis[9]. The basic concept of this method is similar to the SL-PCR described in this study. However, they got much smaller number of amplified fragments than that of the RFLP analysis. In contrast, the numbers of amplified bands are slightly smaller than that of the RFLP analysis but much larger than that of the inverse PCR. The difference of the numbers of bands between Otal et al. and us may be explained by the choice of restriction endonuclease for the digestion of *M. tuberculosis* genomic DNA. They used *Pst* I, a 6 base cutter, for their experiment, where *Sau*3A I was used in our study. The size of restriction fragment generated by *Pst* I range from 2000 to 6000 bp with the average size of 4000 bp. This is 15 times longer than the restriction fragments by *Sau*3A I. The larger size of restriction fragment prevented the uniform amplification of IS*6110* flanking sequences and resulted in the smaller number of PCR products. And further more, the discriminatory capacity of SL-PCR was much higher than that of inverse-PCR described by Otal et al.

In this study we have genotyped 34 isolates of tuberculosis by using SL-PCR and compared with the fingerprint patterns obtained by ML-PCR and RFLP analysis (Fig. 3 and 4). The SL-PCR produced up to 15 bands, whereas the RFLP and the ML-PCR patterns contained up to 20 and 30 bands, respectively. In SL-PCR, the molecular size of the amplified DNA fragments was between 50 and 400 bp, where it was between 100 and 1400 in ML-PCR.

To evaluate the ability of the SL-PCR to differentiate clinical isolate, analysis of 21 clinically independent isolates by SL-PCR, ML-PCR and RFLP were performed. We obtained different patterns in all isolates in three methods, though the number of bands with difference were smaller in SL-PCR. This result indicated that SL-PCR carries the compatibility with the fingerprint pattern obtained by ML-PCR and RFLP analysis. Thirteen outbreak isolates in five groups were analyzed by three methods. Four out of five cases exhibited good correlation between these three methods. However, only the good compatibility was found between SL-PCR and RFLP analysis (Fig. 4). This result indicated the superiority of SL-PCR over ML-PCR.

The main advantage of SL-PCR and ML-PCR over the traditional RFLP analysis is its independence from subculture of tuberculosis bacilli. The DNA fingerprinting requires a 3- to 4-weeks subculture of the isolate or heavy growth on the original slant for analysis, whereas by SL-PCR and ML-PCR, fingerprint pattern can be obtained from much less amount of bacilli. In addition, between SL-PCR and ML-PCR, SL-PCR is more favorable because of its simplicity. To assay one complete analysis in ML-PCR takes many technical steps and also time consuming[7]. But in SL-PCR needs few technical steps and not time consuming (8 hours). So, SL-PCR is very simple and easier than ML-PCR.

The results in this paper demonstrate that the potential of the SL-PCR for identifying and monitoring outbreaks. For strains with distinct RFLP patterns, most of the SL-PCR patterns were unique. Some strains could not be reliably differentiated by SL-PCR, as their patterns differed by only one or two bands. However, numerous studies based on IS*6110* RFLP analysis have shown epidemiologically related strains may present RFLP patterns differing by one or two bands[15)~18)]. It should be noted that

M. tuberculosis strains carry multiple IS*6110* copies, isolates which contain only one or a few copies have been observed[19)~22)]. There has been no study about the stability of IS*6110*. But some studies postulated that genotype instability in small numbers of *M. tuberculosis*, and the causes of IS*6110* genotypic instability are unclear[23)].

In conclusion, the SL-PCR reported in this paper is technically simple and reproducible and requires much less DNA than the standard RFLP typing method. This method is useful for first choice in transmitting *Mycobacterium tuberculosis*, and after that isolates should be confirmed with IS*6110* RFLP. High and middle prevalence countries of *Mycobacterium tuberculosis* having similar patterns of one or two additional bands. Of course, outbreaks and a familial infection also having one additional band about 29%[23)]. These characteristics may be of great value for molecular epidemiology of tuberculosis. Due to its speed, this technique may be useful as a screening method for contact tracing investigation or identification of tuberculosis epidemic.

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