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Emergence of Ofloxacin Resistance in *Mycobacterium tuberculosis* Clinical Isolates from China as Determined by *gyrA* Mutation Analysis Using Denaturing High-Pressure Liquid Chromatography and DNA Sequencing▽

Ruiru Shi,[1,2] Jianyuan Zhang,[3] Chuanyou Li,[3] Yuko Kazumi,[1] and Isamu Sugawara[1]*

Mycobacterial Reference Center, The Research Institute of Tuberculosis, Tokyo, Japan[1]; Henan Provincial Research Institute of Tuberculosis, Zhengzhou, China[2]; and Beijing Tuberculosis and Lung Tumor Research Institute, Beijing, China[3]

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A high rate of double point mutations in *gyrA* (56% of 87 ofloxacin-resistant *Mycobacterium tuberculosis* clinical isolates) indicates the emergence of fluoroquinolone resistance. This is the first report to describe denaturing high-pressure liquid chromatography analysis of mutations in *gyrA* of *M. tuberculosis* in a large number of clinical isolates.

Up to the present, fluoroquinolones have been studied as a first-line treatment for tuberculosis (9). However, fluoroquinolone resistance among *Mycobacterium tuberculosis* strains is emerging, with important implications for treatment (4, 6). Fluoroquinolones have been widely used for tuberculosis treatment in China for more than 10 years and have been given routinely as monotherapy for the empirical treatment of numerous outpatient infections. Thus, China may be one of the countries with the highest rate of fluoroquinolone abuse and resistance in the world. The goal of this work was to identify quinolone resistance-determining regions (QRDRs) of *gyrA* in ofloxacin-resistant isolates from China by denaturing high-pressure liquid chromatography (DHPLC) and DNA sequencing methods.

(Most of this study was presented at the 41st US-Japan Cooperative Medical Science Program Tuberculosis and Leprosy Research Conference at Kagoshima, Japan, in July 2006.)

The 109 clinical isolates (87 shown to be ofloxacin resistant and 22 shown to be susceptible by a routine proportional method) were collected from patients with pulmonary tuberculosis (65 males and 44 females, aged 17 to 73 years, with 2 to 6 months of fluoroquinolone treatment) over a period of 2 years (2002 to 2003) at the Beijing Tuberculosis and Lung Tumor Research Institute, Tongzhou, China. MICs of ofloxacin were detected by an absolute concentration method in Lowenstein-Jensen medium, and the concentrations were 0.125, 0.25, 1, 2, 4, 8, 10, 16, 20, and 32 μg/ml. For DHPLC analysis, *M. tuberculosis* H37Rv (ATCC 25618) and *M. tuberculosis* Erdman (ATCC 35801) were used as reference strains. DHPLC was performed with a WAVE DNA fragment analysis system (Transgenomic Inc.). The melting temperature for *gyrA* analysis was 67.7°C. The conditions for DNA hybridization and DHPLC analysis have been described in detail elsewhere (10). For DNA sequencing, a 227-bp DNA fragment corresponding

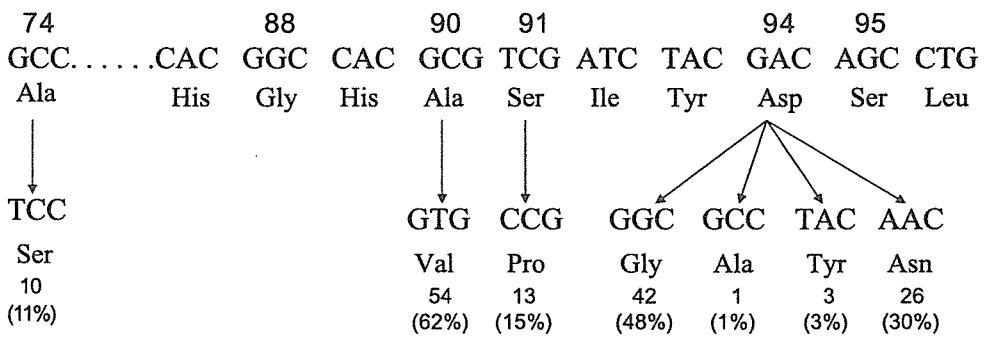

FIG. 1. Nucleotide sequence and missense mutations within the QRDRs of *gyrA*. All the isolates contain a naturally occurring polymorphism, codon 95 AGC→ACC. Seventy-three (84%) of the 87 ofloxacin-resistant clinical isolates were found to carry a codon 94 mutation, and 49 (56%) were found to harbor a double point mutation.

* Corresponding author. Mailing address: Mycobacterial Reference Center, The Research Institute of Tuberculosis, 3-1-24 Matsuyama, Kiyose, Tokyo 204-0022, Japan. Phone: 81 42 493 5075. Fax: 81 42 492 4600. E-mail: sugawara@jata.or.jp.

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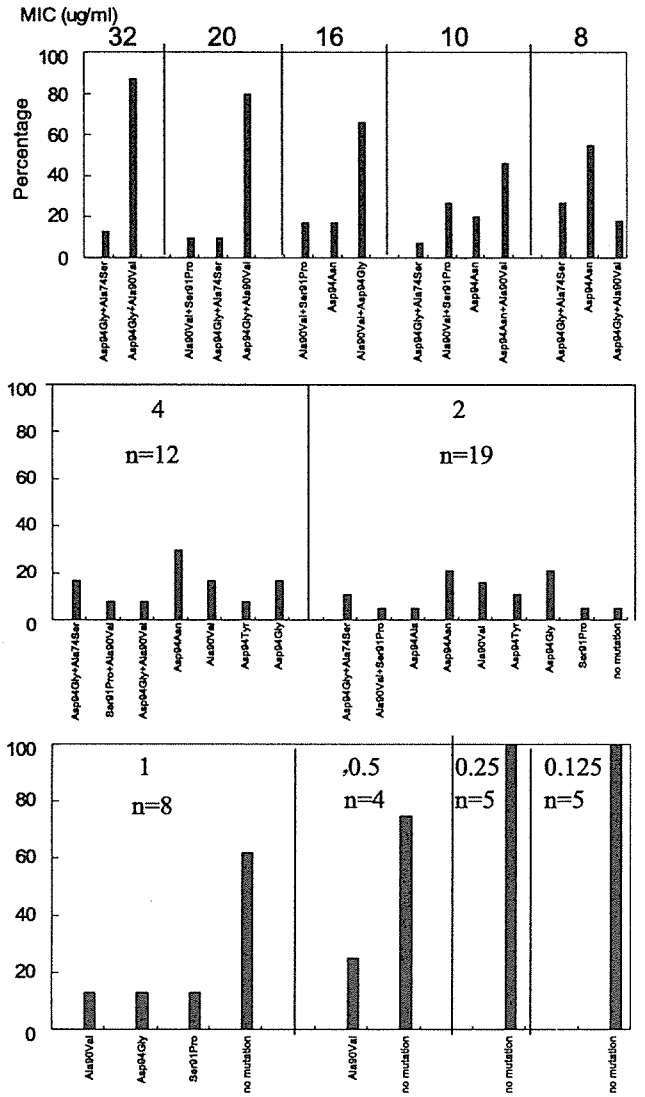

FIG. 2. Ofloxacin MIC relative to the *gyrA* QRDR allele spectrum. Ofloxacin MICs are given above each panel. n, number in each MIC group. Bars indicate the percentage represented by each allele.

to the QRDR was generated by PCR with the following primer set: forward, 5′-GACCGCAGCCACGCCAAG-3′, and reverse, 5′-AGCATCACCATCGCCAACG-3′. After purification, the PCR product (5 ng) was used as a template for TaqCycle sequencing using ABI Prism BigDye Terminator sequencing kits (Applied Biosystems). Cycle sequencing products were subsequently analyzed on an ABI PRISM 310 genetic analyzer (Applied Biosystems).

gyrA mutations were found to occur predominantly at codons 90, 91, and 94 and in four types of codon 94 mutation (94Asp→Gly, Ala, Tyr, and Asn) (Fig. 1), largely confirming the findings of other researchers (1, 2, 7, 11, 12). The previously reported mutation involving codon 88 was not found (5). All of the 109 clinical isolates had a codon 95 ACC natural polymorphism, which paralleled the results for 138 other isolates from China (2). However, two new findings were unexpected. One was that 49 of the 87 ofloxacin-resistant isolates (56%) carried double point mutations, and the other was that among these double-mutated isolates, 20% (10/49) harbored an $Ala^{74}Ser$ mutation (Fig. 2), which has not been reported previously for *M. tuberculosis*. Double point mutation of *gyrA* is relatively rare (2, 5, 12) and is generally thought to be uncommon in clinical isolates. The $Ala^{74}Ser$ mutation has been reported only for other bacteria (8, 12). This indicates that fluoroquinolone resistance is already emerging in China.

DHPLC analysis. First, *M. tuberculosis* H37Rv was routinely used as a reference strain, and this revealed that all 109 isolates carried mutations (aberrant peak patterns in Fig. 3). DNA sequencing showed that all the strains possessed a natural codon 95 AGC→ACC (Ser→Thr) polymorphism, which did not have a significant impact on fluoroquinolone susceptibility (4). To improve the DHPLC detection capacity, the other reference strains were selected from H37Ra, *M. tuberculosis* Kuruno, *M. tuberculosis* Erdman, and *Mycobacterium bovis* BCG Pasteur (data not shown). We found that *M. tuberculosis* Erdman (fluoroquinolone susceptible, with codon 95 ACC in *gyrA* QRDRs) was the best as the second reference strain in this study. Those isolates with only the codon 95 AGC→ACC polymorphism showed a normal peak (Fig. 3). Thus, the influence of this natural polymorphism was successfully avoided. When *M. tuberculosis* H37Rv and *M. tuberculosis* Erdman ref-

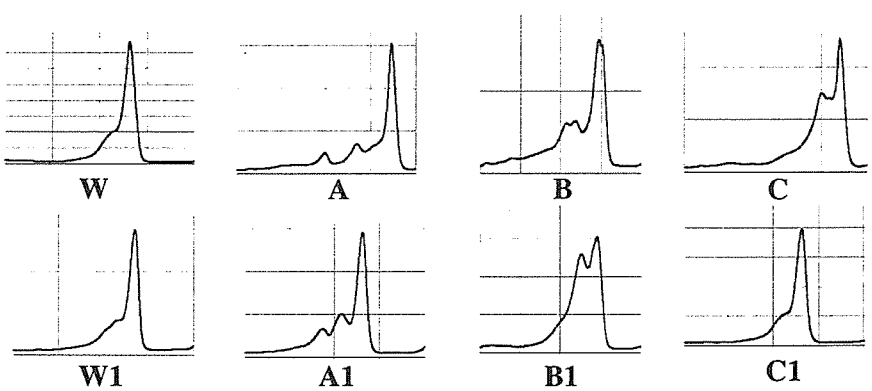

FIG. 3. DHPLC patterns of *gyrA* genes of the 109 clinical isolates when *M. tuberculosis* H37Rv was used as a reference strain. Patterns A, B, and C are shown as examples (details are shown in Fig. 4). W, H37Rv wild type. When *M. tuberculosis* Erdman was used as a reference strain, A, B, and C were changed to A1, B1, and C1, respectively. W1 indicates the *M. tuberculosis* Erdman wild type. Isolates (MIC less than 2 μg/ml) that harbored only the codon 95 ACC natural polymorphism with no other mutation in *gyrA* QRDRs showed the wild-type pattern C1.

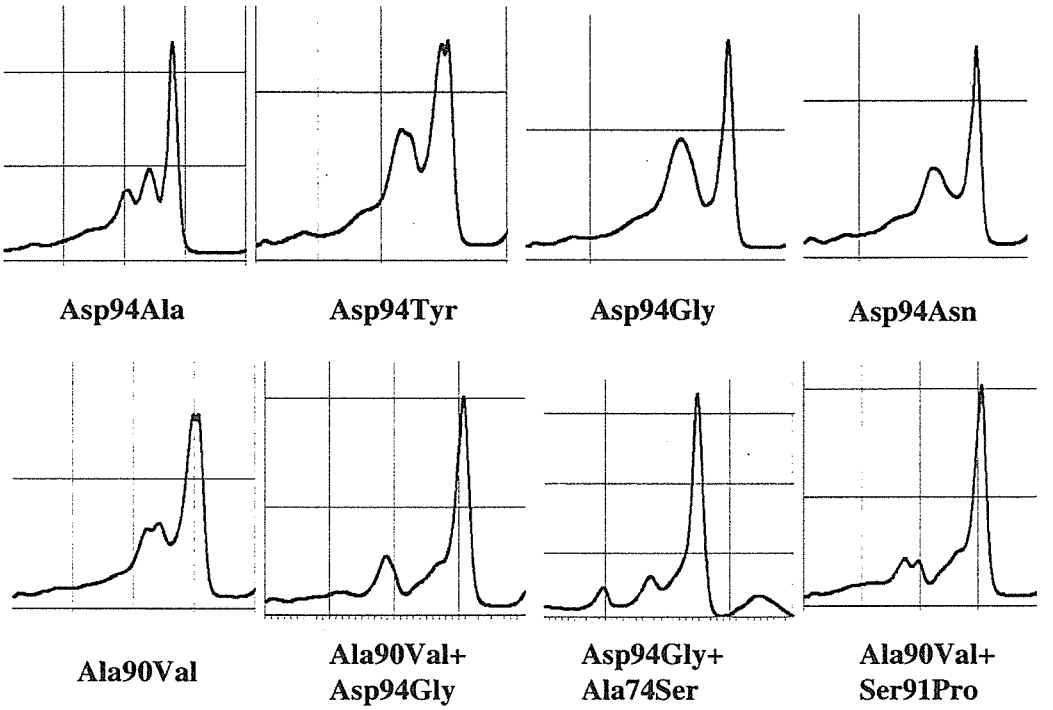

FIG. 4. Specific DHPLC pattern of each *gyrA* QRDR mutation type.

erence strains were used, a wild-type peak pattern appeared, indicating no point mutation in *gyrA* QRDRs. Of course, if an isolate carries any point mutation at a codon except codon 95, an aberrant peak pattern will appear. One interesting thing is that most of the isolates with the same mutation showed the same DHPLC patterns. The peak profiles of each mutant are shown in Fig. 4. Asp^{94}Gly and Asp^{94}Asn changes revealed similar patterns that were difficult to distinguish from each other. Other mutations had their own peak patterns. Therefore, it is thought that, to some extent, specific DHPLC patterns may predict the types of resistance.

DHPLC is a relatively new technique utilizing heteroduplex formation between wild-type and mutated DNA strands to identify mutations and was predicted to be a potentially useful genotypic screening method for gene mutations conferring drug resistance in *M. tuberculosis* (3, 8, 10). In this study, by introducing *M. tuberculosis* H37Rv and *M. tuberculosis* Erdman as two reference strains, the interference from the codon 95 AGC→ACC natural polymorphism was successfully avoided. Since no other polymorphism has been found in *gyrA* QRDRs except for that in codon 95, and all the point mutations in codons 74, 88, 90, and 91 correlate with fluoroquinolone resistance, the DHPLC method devised in this study can be regarded as a useful and powerful tool for analysis of *gyrA* mutation in tuberculosis.

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Short Communication

Imaging of Pulmonary Granulomas Using a Photon Imager

Isamu Sugawara*, Satoru Mizuno, Taiga Tatsumi[1] and Tadayoshi Taniyama[2]

Mycobacterial Reference Center, The Research Institute of Tuberculosis;
[1]Sowa Trading Co., Inc., Tokyo 204-0022; and [2]National Institute of Infectious Diseases, Tokyo 162-8640, Japan

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SUMMARY: To clarify the location of pulmonary granulomas in vivo, we prepared a *Mycobacterium tuberculosis* H37Rv mutant in which the gene for a green fluorescent protein (GFP) (GFP-H37Rv) was introduced. Five weeks after aerosol infection with GFP-H37Rv, the infected lungs from guinea pigs and mice were subjected to imaging using a photon imager. Pulmonary granulomas more than 1 mm in diameter were localized clearly by the photon imager. Therefore, if a method for binding a dye (GFP, fluorescein isothiocyanate [FITC], etc.) specifically to *M. tuberculosis* can be developed, it will be possible to visualize granulomas using a photon imager.

When tubercle bacilli enter lung alveoli, they eventually induce granulomas unless they are killed. Such granulomas are similar to carcinomas in that they are solid and spread to other organs hematogenously. Therefore, for the diagnosis of granulomas, it is useful if their locations within organs can be pinpointed. As a first step toward this goal, we prepared H37Rv in which the gene for a green fluorescent protein (GFP) was introduced (GFP-H37Rv). Briefly, a BCG hsp60 promoter-GFP mut 3.1 was prepared by using the pCR 2.1-BCG hsp60 promoter and pGFP mut 3.1 (Clontech Labs,. Inc., Pal Alto, Calif., USA). Then, a pHSP 60 promoter-GFP mut 3.1 was constructed by utilizing pHSP 60 promoter-GFP mut 3.1 B and pGFM-12 (kindly supplied by Dr. C. Locht) (1). *Mycobacterium tuberculosis* H37Rv strain (ATCC25618) was then transformed with the pHSP 60 promoter-GFP mut 3.1 M to obtain a stable GFP-H37Rv mutant. This mutant was shown to remain stable for a year after subcutaneous administration to C57BL/6 mice. There was no statistically significant difference in in vitro growth between H37Rv and the GFP-H37Rv mutant (Fig. 1).

Next, the GFP-H37Rv mutant was smeared on glass slides and fixed with 4% paraformaldehyde for 30 min. The slides were then observed using a confocal laser microscope (Digital Eclipse C1; Nikon Optical Co., Tokyo, Japan). As shown in Fig. 2, clustered GFP-H37Rv tubercle bacilli emitted intense green fluorescence. We then used the RAW264.7 mouse macrophage cell line to determine whether the GFP-H37Rv

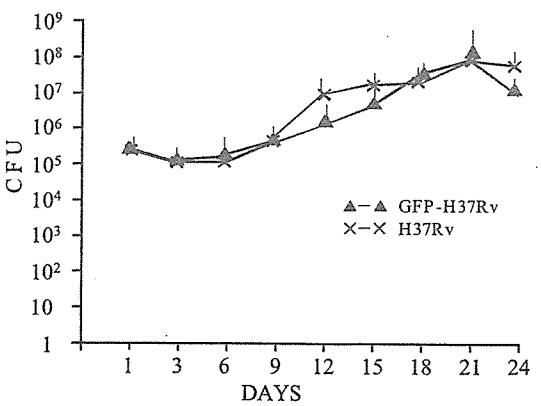

Fig. 1. Growth curve of GFP-H37Rv and H37Rv (parental strain).

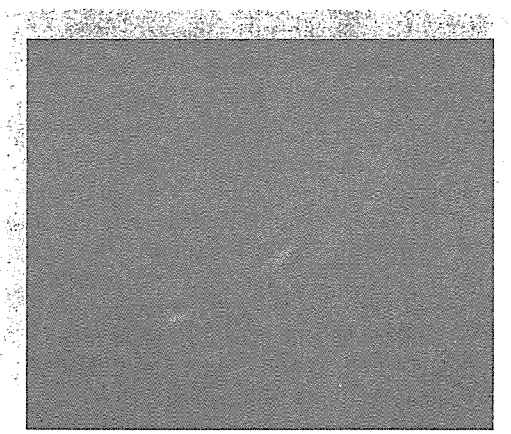

Fig. 2. GFP-H37Rv mutant emitting green fluorescence. After the GFP-H37Rv mutant had been smeared on glass slides and fixed with 4% paraformaldehyde for 30 min, it was observed by confocal laser microscopy. × 600.

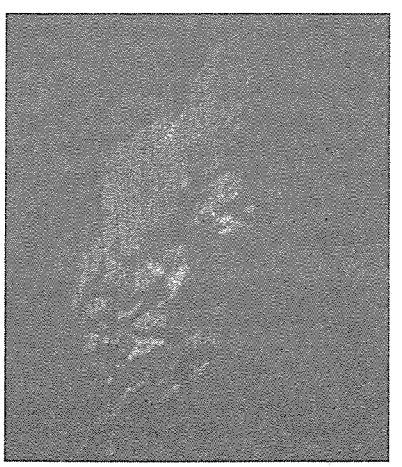

Fig. 3. GFP-H37Rv mutant in phagosomes of the RAW264.7 murine macrophage cell line. After the GFP-H37Rv mutant had been added to RAW cells (multiplicity of infection, 50:1) they were fixed with 4% paraformaldehyde for 60 min, and observed by confocal laser microscopy. × 600.

*Corresponding author: Mailing address: Mycobacterial Reference Center, The Research Institute of Tuberculosis, 3-1-24 Matsuyama, Kiyose, Tokyo 204-0022, Japan. Tel: +81-424-93-5075, Fax: +81-424-92-4600, E-mail: sugawara@jata.or.jp

mutant maintained its green fluorescence intracellularly. The GFP-H37Rv mutant was added to a culture of RAW264.7 cells (multiplicity of infection, 50:1) and cultured in RPMI 1640 with 10% heat-inactivated fetal bovine serum overnight. After fixation with 4% paraformaldehyde for 60 min, the cells were observed using a confocal laser microscope. As shown in Fig. 3, the GFP-H37Rv mutant in macrophages still emitted intense green fluorescence.

We next attempted to determine the fate of the GFP-H37Rv mutant in vivo. Permission for animal experimentation was given by the Animal Experiment Committee of The Tuberculosis Research Institute. Female guinea pigs and BALB/c female mice were infected with the GFP-H37Rv mutant (1×10^6 CFU) by an airborne infection apparatus (Model 099CA424; Glas-Col, Inc., Terre Haute, Ind., USA). The concentration was calculated to result in the uptake of around 200 viable bacilli by guinea pig lungs and around 70 viable bacilli by mouse lungs after inhalation exposure for 90 min under the experimental conditions employed (2). Five weeks later, the lungs were removed and fixed with 4% paraformaldehyde for 2 days. The guinea pig and mouse lungs were scanned for green fluorescence using a Φ imager (photon imager; Biospace Mesures, Paris, France). This imager is based on a 3rd generation GaAs intensified charge-coupled device (ICCD) camera that allows real-time photon counting over a wide spectral range (400-900 nm). This imager amplifies every photon up to 10^6 light spots in a detector using an ICCD chip. The detection conditions of this imager were as follows: spatial resolution, equivalent to $1,080 \times 1,440$ pixels CCD; dynamics, 2,000 counts/pixel/min; excitation, 485 nm; emission, 535 nm. As shown in Fig. 4, signals with green fluorescence were recognized to varying degrees in both guinea pig and mouse lungs. No signal was detected in exudative inflammation. Fig. 5 shows that the signals corresponded to granulomas (proliferative inflammation) of various sizes. The granulomas contained significant numbers of tubercle bacilli (GFP-H37Rv mutant) as evaluated by Ziehl-Neelsen staining for acid-fast bacilli (data not shown).

Fig. 4. Pulmonary granulomas visualized by a photon imager. Guinea pigs and BALB/c mice were infected with *M. tuberculosis* Kurono strain by aerosol infection. Five weeks after infection, lung tissues from guinea pigs (large) and mice (small) were visualized by a photon imager. Granulomas of various sizes were localized in the lungs. × 10.

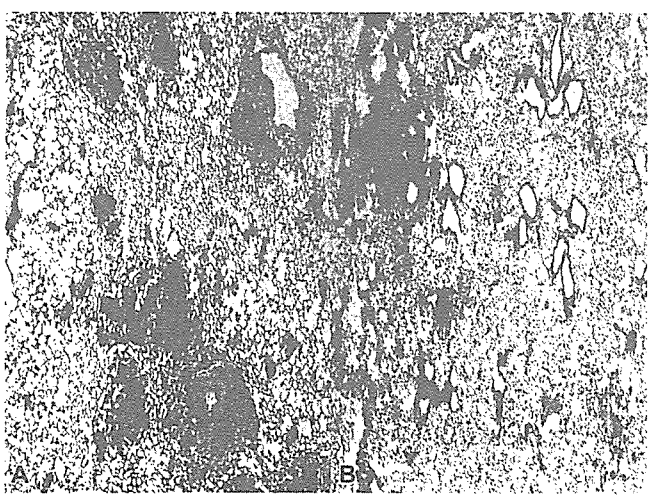

Fig. 5. Histopathology of infected lung tissues of guinea pigs (A) and mice (B). Pulmonary granulomas of various sizes are shown in this picture. Hematoxylin & eosin stain. × 20.

The spatial visualization technique (tumor imaging) is commonly utilized in the diagnosis of lung cancer (3-8). So far, however, there has been no research report on pulmonary granuloma imaging. The present study showed that it is possible to detect pulmonary granulomas by green fluorescence emission. This system detects signals from cyanin, fluorescein isothiocyanate (FITC), and rhodamine as well as GFP. If a technique for binding a dye specifically to tubercle bacilli could be developed, it would be possible to visualize granulomas (proliferative inflammation) in other organs as well as the lungs.

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Tuberculosis

Protective efficacy of recombinant (Ag85A) BCG Tokyo with Ag85A peptide boosting against *Mycobacterium tuberculosis*-infected guinea pigs in comparison with that of DNA vaccine encoding Ag85A

I. Sugawara[a,*], T. Udagawa[a], T. Taniyama[b]

[a]*Mycobacterial Reference Center, The Research Institute of Tuberculosis, 3-1-24 Matsuyama, Kiyose, Tokyo 204-0022, Japan*
[b]*National Institute of Infectious Diseases, Tokyo 162-8640, Japan*

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Peptide booster;
IFN-γ

Summary
A recombinant form of BCG Tokyo with an Ag85A gene insert was administered once subcutaneously to guinea pigs and its protective efficacy was compared with that of a DNA vaccine encoding Ag85A from *Mycobacterium tuberculosis* administered twice to guinea pigs by epidermal gene gun bombardment. Vaccination with either the recombinant BCG Tokyo or Ag85A DNA significantly reduced the severity of pulmonary pathology and the number of pulmonary and splenic colony-forming units (cfu) ($p<0.001$). The recombinant BCG Tokyo was better than Ag85A DNA in terms of protective efficacy against *M. tuberculosis*. When immunogenic synthetic Ag85A peptide was further used as a booster together with recombinant BCG Tokyo (Ag85A) or Ag85A DNA, lung pathology was improved significantly and the number of pulmonary CFU was reduced significantly. Neither recombinant BCG Tokyo, Ag85A DNA, nor the parental BCG Tokyo protected the guinea pigs from hematogenous spread of tubercle bacilli to the spleen because splenic granulomas without central necrosis were recognized. The spleen tissues from guinea pigs vaccinated with recombinant BCG Tokyo or Ag85A DNA expressed IFN-γ and IL-2 mRNA at significantly high levels ($p<0.001$) as evaluated by reverse transcription polymerase chain reaction. It is concluded that peptide boosting is important for the induction of higher protective efficacy by recombinant BCG Tokyo or a tuberculosis DNA vaccine and both recombinant BCG Tokyo (Ag85A) and Ag85A DNA vaccine induce Th2 cytokine mRNA expression significantly.

*Corresponding author. Tel.: +81 424 93 5075; fax: +81 424 92 4600.
E-mail address: sugawara@jata.or.jp (I. Sugawara).

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Introduction

Tuberculosis (TB) still remains a major health problem affecting millions of people worldwide. The only TB vaccine currently available is *Mycobacterium bovis* BCG. However, the efficacy of BCG still remains controversial, especially against pulmonary TB in young adults, and development of a better vaccine is urgently required to counteract the global threat of TB.[1–3]

Vaccination with naked plasmid DNA encoding Ag85A and Ag85B can stimulate strong humoral and cell-mediated immune responses.[4,5] It has been reported that intramuscular needle injection, but not epidermal gene gun bombardment, is capable of inducing a protective immune response with an Ag85A DNA vaccine.[6] In mouse models, Ag85A DNA vaccine is effective during the first weeks after *Mycobacterium tuberculosis* challenge, but its protection decreases over time.[7] However, Ag85A from *M. tuberculosis* induces significant humoral and cell-mediated immune responses. Recent reports of successful DNA vaccination against *M. tuberculosis* in small-animal models suggest that DNA vaccines act by reducing lung pathology without sensitizing animals to tuberculin testing. On the other hand, vaccination of guinea pigs with DNA encoding the mycobacterial antigen MPB83 influences pulmonary pathology.[8] We have previously shown that vaccination with Ag85A DNA twice by gene gun bombardment significantly reduced the severity of pulmonary pathology and the number of colony-forming units (cfu) in guinea pigs.[9] However, there are some drawbacks to the use of a TB DNA vaccine in the tissues. It induces low immunogenicity and transfection efficacy is low. It is very difficult to measure protein derived from a TB DNA vaccine, even though its protective efficacy has been demonstrated. Furthermore, anti-DNA antibody produced by a TB DNA vaccine may damage the host.

On the other hand, a recombinant BCG, in which a specific gene has been introduced, is interesting to study because BCG is used routinely throughout the world and the biology of BCG is well known. There are several publications reporting recombinant BCG vaccines that over-express Ag85.[10,11] We selected Ag85A among Ag85 families because the immunological property of Ag85A is relatively well-known.[4–7] Therefore, we generated a recombinant form of BCG Tokyo in which the Ag85A gene had been introduced, and compared its protective efficacy with that of Ag85A DNA vaccine. We found that the recombinant BCG Tokyo was better than Ag85A DNA in terms of protective efficacy against *M. tuberculosis*. When immunogenic synthetic Ag85A peptide was further used as a booster, lung pathology was improved significantly and the number of pulmonary CFU was reduced significantly.

Materials and methods

Construction of recombinant BCG Tokyo

The Ag85A gene was amplified by PCR and subcloned into the pCR4 vector. The presence of the Ag85A gene was then confirmed by DNA sequencing. The gene was inserted into the pBBN vector (Ag85A-HA) possessing a hemagglutinin (HA) tag at its 5′ end. At this stage, the Ag85A-HA was expressed in *E. coli*, and then the Ag85A-HA gene was introduced into the downstream region of the pHPS integration vector. The vector was then electroporated into BCG Tokyo. The resulting transformants were cultured individually and the content of the extracted lysate that contains Ag85 protein was confirmed by western blotting.

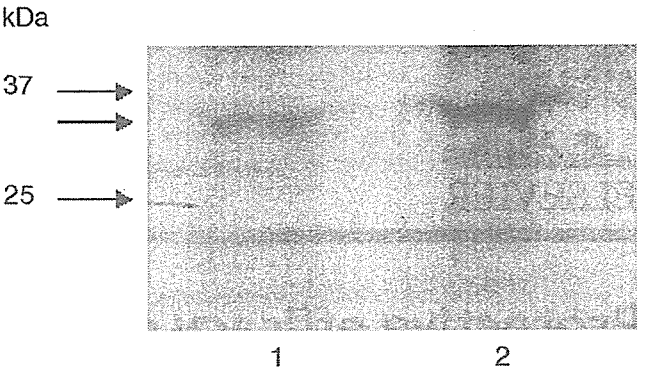

Figure 1 Western blot of BCG Tokyo (lane 1) and recombinant BCG Tokyo (Ag85A)(lane 2). → Shows size markers. For the results by Western blotting, a densitometric analysis was carried out using the NIH image software version 1.62.

Anti-Ag85 monoclonal antibody (TD 32.15) was generated in Dr. K. Huygen's laboratory.[12] The Ag85 protein content of the recombinant BCG Tokyo was 15–20% higher than that of the parental BCG Tokyo, as evaluated by Western blotting (Fig. 1). There was no statistically significant difference in in vitro growth between BCG Tokyo and recombinant BCG Tokyo (Ag85A).

Bacterial strain

M. tuberculosis Kurono strain (ATCC35812) was passed through mice and grown once in 7H9 liquid medium before titration and storage in aliquots at −85 °C. The culture strain was filtered through a membrane filter (4 μm pore size; Millipore, Bedford, MA, USA) before use to ensure even dispersal.[13]

Inoculation of guinea pigs

Hartley female guinea pigs (group 2) were vaccinated with BCG Tokyo (Group 1), or recombinant BCG Tokyo (5×10^6 CFU/guinea pig) (Group 2) subcutaneously once. The 20-mer Ag85A peptide (141–160) was synthesized on a peptide synthesizer (Applied Bio-systems) and subsequently purified by HPLC.[9] Three weeks after the inoculation, 500 μg of 20-mer Ag85A peptide (141–160) was administered subcutaneously once (Group 3). The guinea pigs were also vaccinated on the shaved dorsal skin using the Helios Gene Gun System (Bio-Rad) at a helium discharge pressure of 500 lb/in^2 as described previously.[9] Two gene gun immunizations were carried out 3 weeks apart, consisting of 50 non-overlapping shots of 1-μm gold beads coated with 50 μg of plasmid DNA (Ag85A DNA (v1J.ns) or control DNA) (Group 4 and Group 6). Several guinea pigs without any treatment were infected with *M. tuberculosis* (Group 7). After the second immunization with Ag85A DNA, 500 μg of Ag85A synthetic peptide emulsified in Freund's incomplete

adjuvant was administered to the guinea pigs subcutaneously (Group 5). For BCG vaccination, guinea pigs were injected subcutaneously with 5×10^6 cfu BCG Tokyo (Group 1). Preliminary experiments and mouse experiments with the gene gun showed that 50 μg of TB DNA vaccine was sufficient to induce protective efficacy to guinea pigs.[9]

Airborne infection of guinea pigs

One week after the last challenge, the guinea pigs were infected by the airborne route by placing them in the exposure chamber of an airborne infection apparatus (Model 099CA4241; Glas-Col, Inc., Terre Haute, IN, USA). The nebulizer compartment was filled with 5 ml of a suspension containing 2.5×10^5 cfu of the Kurono strain (ATCC35812). The concentration was calculated to result in uptake of approximately 150 viable bacilli by the lungs after inhalation exposure for 90 min under the experimental conditions used in this study. Just after the inhalation exposure experiments, the lung tissues were removed from the guinea pigs and homogenized using a rotating blade macerator system. The lung homogenates were plated on 7H10 Middlebrook agar. Viable counts were determined using serial dilutions of the homogenates. Four weeks later, the colonies were counted and approximately 150 viable tubercle bacilli were obtained. Seven weeks after aerial infection, the guinea pigs were sacrificed for histopathology and enumeration of mycobacteria.

Histopathology

Prior to detailed examination of fixed lungs and spleens from the guinea pigs, the number of visible lesions on the surfaces of all lobes was recorded on a diagram of the lungs.[9] Tissue sections from paraffin blocks containing lung, spleen and liver were stained with hematoxylin and eosin (H & E), or the Ziehl–Neelsen method for acid-fast bacilli.[14] The degree of pulmonary lesions was judged blindly by two workers (I.S. and T.U.).

Bacterial enumeration

Part of lung and spleen tissues (five pieces each) were homogenized carefully in 5 ml of sterile distilled water using small ceramic jars. The lung or spleen homogenates were plated on 7H10 Middlebrook agar. Viable counts were determined using serial dilutions of the homogenate, and preparations were examined for growth of mycobacteria after 4 weeks of incubation at 37 °C.[14,15] The lung and spleen tissues were weighed and the results were expressed as cfu ± SE/whole organ.

Reverse transcription polymerase chain reaction (RT-PCR)

PCR was performed using a mixture of equivalent amounts of cDNA of the samples 1 week after the last immunization (1 μl), 0.05 μl Takara EX *Taq* polymerase, 1.0 μl 10 × EX buffer, 0.8 μl dNTPs (TAKARA BIO INC., Otsu Shiga, Japan), 0.5 μl each of gene-specific primer sets for GAPDH (internal control), IFN-γ, and IL-2, and finally 6.15 μl of distilled water (total volume: 10 μl in a 200 μl microcentrifuge tube). The sequences of the primer sets and annealing temperatures have been described previously.[14] The mRNAs purified with an Oligotex™-dT30 〈Super〉 mRNA purification kit and not subjected to RT were used as negative controls in the PCR reaction to confirm that there was no genomic DNA contamination. All PCRs were performed as follows: denaturation at 94 °C for 5 s, annealing at 60 or 65 °C for 5 s, and extension at 72 °C for 10 s, with a GeneAmp PCR System 9700 (Applied BioSystems, CA, USA). The expected sizes of the amplified products of GAPDH, IFN-γ and IL-2 mRNA were 343, 171, and 353 bp, respectively. PCR products were visualized by ethidium bromide staining of 4% SeaKemGTG agarose and NuSieve GTG (1:3) gels after electrophoresis.

Statistical analysis

We performed analysis of variance (ANOVA) for repeated measurements using the baseline results at screening as a covariate on log-transformed data to compare between groups.

Results

Gross pulmonary lesions in vaccinated guinea pigs

Guinea pigs were vaccinated with recombinant BCG Tokyo, recombinant BCG Tokyo plus synthetic Ag85A peptide, plasmid encoding Ag85A, plasmid encoding Ag85A plus synthetic Ag85A peptide, control vector plasmids or no vaccination. One additional group of guinea pigs was vaccinated with the parental BCG Tokyo. All animals survived until 7 weeks after challenge and were then killed humanely and used to evaluate vaccine efficacy. Figure 2 shows the number of visible lesions on the surfaces of the fixed lungs in seven different groups. Vaccination with recombinant BCG Tokyo, recombinant BCG Tokyo plus Ag85A synthetic peptide, Ag85A DNA, Ag85A DNA plus Ag85A synthetic peptide, and parental BCG Tokyo reduced the mean number of lesions compared with control plasmid DNA or no vaccination significantly ($p < 0.001$). Vaccination with recombinant BCG Tokyo plus synthetic Ag85A peptide reduced the number of gross lesions most markedly, but there was no significant difference in the number of gross lesions between recombinant BCG (Ag85A) group and recombinant BCG Tokyo (Ag85A) plus Ag85A peptide group. There was a significant difference in the number of gross lesions between the TB DNA plus peptide group and the control plasmid group and between the recombinant BCG Tokyo plus peptide group and no vaccination group ($p < 0.001$).

Histopathology of vaccinated guinea pigs

All test treatments reduced the extent of granulomatous inflammation compared with control plasmid DNA or no vaccination. Representative examples of the histopathology observed in vaccinated guinea pigs and animals treated with

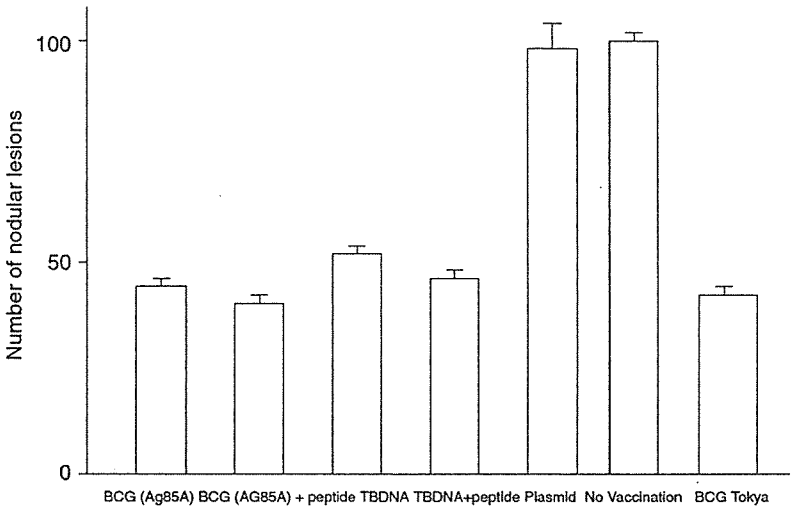

Figure 2 Number of gross lesions on the surfaces of fixed lungs 7 weeks after aerial infection. Each bar represents the mean number of gross lesions from three guinea pigs. Error bars indicate standard errors of the means.

parental BCG Tokyo are shown in Fig. 3. Although vaccination with recombinant BCG Tokyo plus synthetic Ag85A peptide reduced the number of pulmonary lesions significantly, hematogenous spread of tubercle bacilli to the spleen was recognized (data not shown). Granulomas with central necrosis were found in guinea pigs treated with recombinant BCG Tokyo, recombinant BCG Tokyo plus Ag85A synthetic peptide, Ag85A DNA, Ag85A DNA plus Ag85A synthetic peptide, parental BCG Tokyo and control plasmid DNA. After follow-up of five guinea pigs treated with parental BCG Tokyo or recombinant BCG Tokyo, recombinant BCG Tokyo plus synthetic Ag85A peptide, Ag85A DNA or Ag85A DNA plus Ag85A synthetic peptide for 6 months, the number of gross lesions on the surfaces of the fixed lungs was reduced (38, 35, 32, 36 and 35, respectively, as mean values). Furthermore, the granulomatous lesions were fibrotic and no tubercle bacilli were evident by Ziehl–Neelsen staining.

Replication of tubercle bacilli in the lung and spleen tissues of vaccinated guinea pigs

Table 1 shows bacterial replication in lung and spleen tissues from Hartley guinea pigs vaccinated with recombinant BCG Tokyo, recombinant BCG Tokyo plus synthetic Ag85A peptide, Ag85A DNA, Ag85A DNA plus Ag85A peptide, parental BCG Tokyo, control plasmid DNA, or no vaccination and then challenged with *M. tuberculosis* aerially. Both recombinant BCG Tokyo and Ag85A DNA treatment reduced the number of pulmonary and splenic cfu significantly ($p<0.001$). There were significant differences in the number of pulmonary cfu among the recombinant BCG Tokyo plus synthetic Ag85A peptide group, the TB DNA plus peptide group, and the no vaccination group ($p<0.001$). The extent of the reduction in the groups treated with recombinant BCG Tokyo plus peptide was higher than that with TB DNA plus peptide and that in the group treated with parental BCG Tokyo. When Ag85A synthetic peptide was given further as a booster to the guinea pigs treated with recombinant BCG Tokyo, this combination reduced the number of pulmonary cfu more significantly than recombinant BCG Tokyo (Ag85A) ($p<0.001$). Control DNA treatment did not reduce the number of pulmonary and splenic cfu significantly.

Splenic IFN-γ and IL-2 mRNA expression in vaccinated guinea pigs

The spleen tissues were removed one week after the last immunization, and RT-PCR was carried out with gene-specific primer sets for IFN-γ (171 bp) and IL-2 (353 bp). We conducted a densitometric analysis of the electrophoretic RT-PCR results using the NIH Image software package version 1.62. GAPDH (343 bp) was used as an internal control. The background expression levels of IFN-γ and IL-2 mRNAs in spleen tissues of unvaccinated guinea pigs were very low. The expression levels of IFN-γ and IL-2 mRNAs were increased in spleen tissues from guinea pigs that had been vaccinated with parental BCG Tokyo, recombinant BCG Tokyo and Ag85A DNA compared with those from unvaccinated guinea pigs (Fig. 4). Among them, the expression levels of IFN-γ and IL-2 mRNAs were the highest in recombinant BCG Tokyo. There was a significant difference in mRNA expression between the vaccinated and the unvaccinated guinea pigs ($p<0.001$).

Discussion

Recombinant BCG Tokyo plus Ag85A peptide as a booster reduced the severity of pulmonary pathology and the number of pulmonary cfu significantly and had better protective efficacy than recombinant BCG Tokyo, Ag85A DNA vaccine or parental BCG Tokyo. Ag85 complex proteins are major secretory products of *M. tuberculosis* and induce strong cellular and humoral immune responses in infected experimental animals and humans.[4,5] Ag85A is highly conserved amongst all mycobacterial species and is present in all strains of BCG.[16] This is why Ag85A was selected in this study. Our recombinant BCG Tokyo contained 15–20% more

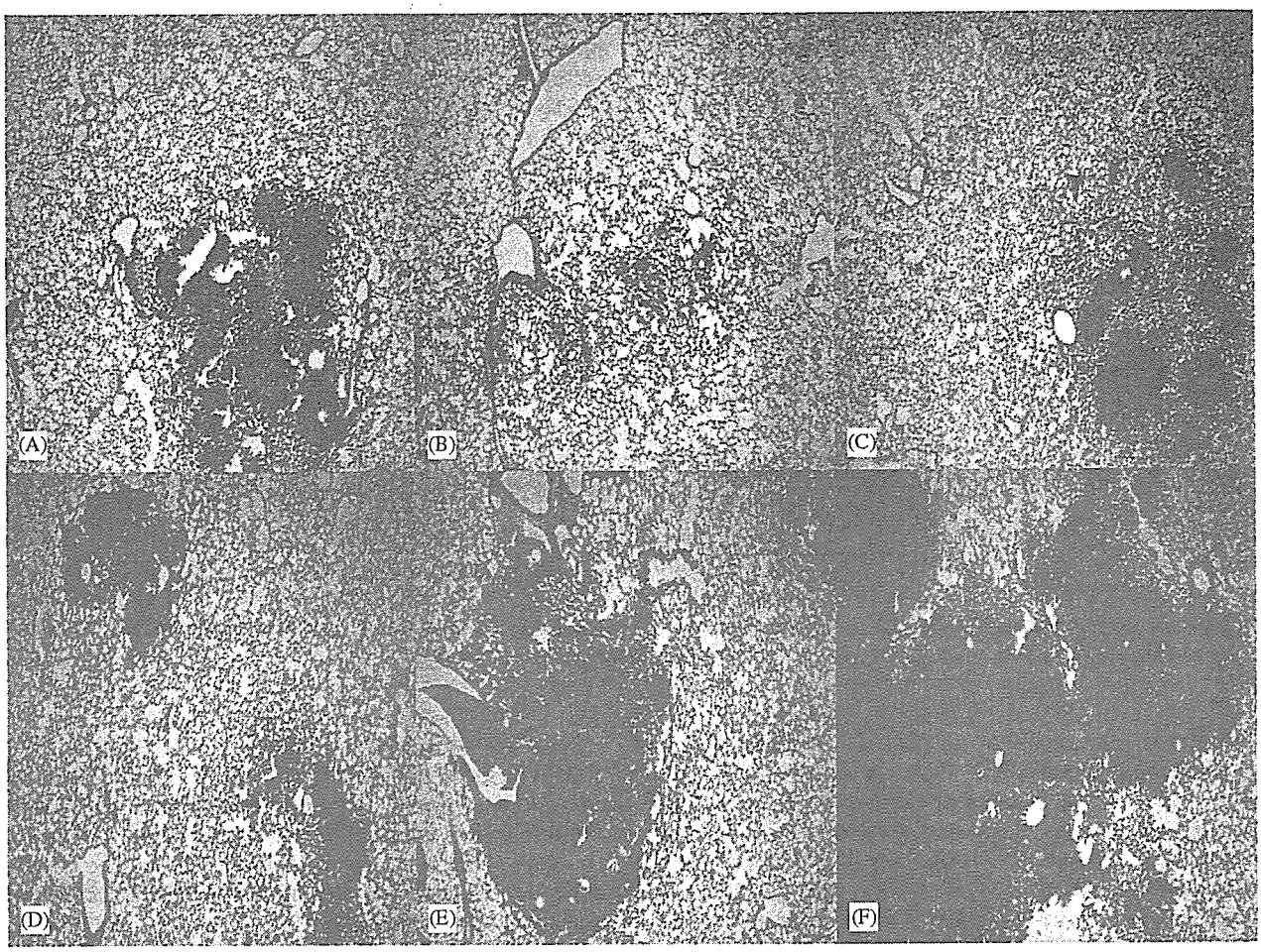

Figure 3 Lung histopathology of vaccinated guinea pigs (hematoxylin & eosin stain). × 40: (A) lung pathology of a guinea pig vaccinated with recombinant BCG Tokyo and infected with airborne *M. tuberculosis*; (B) lung pathology of a guinea pig vaccinated with recombinant BCG Tokyo plus Ag85A peptide and infected with *M. tuberculosis* aerially; (C) lung pathology of a guinea pig vaccinated with TB Ag85A DNA and infected with *M. tuberculosis* aerially; (D) lung pathology of a guinea pig vaccinated with TB Ag85A DNA plus Ag85A peptide and infected with *M. tuberculosis* aerially; (E) lung pathology of a guinea pig vaccinated with parental BCG Tokyo and infected with *M. tuberculosis* aerially; (F) lung pathology of a guinea pig treated with plasmid DNA (negative control) and infected with *M. tuberculosis* aerially. The sizes of the pulmonary granulomas (A–E) are reduced, except for F. Lung histopathology without any vaccination is similar to that with plasmid DNA (F).

Table 1 Bacterial replication in lungs and spleens from guinea pigs vaccinated with recombinant BCG (Ag85A), Ag85A TB vaccine, boosted with Ag85A peptide, and challenged with *M. tuberculosis* Kurono strain.

Vaccine used	No. of cfu/lung*	No. of cfu/spleen*
Recombinant BCG (Ag85A)	5.20±0.19	3.43±0.11
Recombinant BCG (Ag85A)+Ag85A peptide	4.70±0.22	2.84±0.10
Ag85A TB DNA	5.81±0.30	4.01±0.21
Ag85A TB DNA+Ag85A peptide	5.35±0.30	3.90±0.32
Control plasmid	6.96±0.51	5.18±0.20
BCG Tokyo	5.36±0.22	3.85±0.30
No vaccination	7.33±0.40	5.59±0.42

*Mean number of cfu±SD (log_{10} values) as determined by plating on Middlebrook 7H10 agar.

Ag85 protein than the parental strain in Western blotting. Furthermore, vaccination with recombinant BCG Tokyo-induced IFN-γ and IL-2 mRNA expression significantly in the spleen. RT-PCR was utilized because anti-guinea pig IFN-γ and IL-2 antibodies were not available for enzyme-linked immunosorbent assay (ELISA).[17]

Tanghe et al.[18] reported that protective efficacy of a TB DNA vaccine encoding Ag85A was improved by Ag85A protein

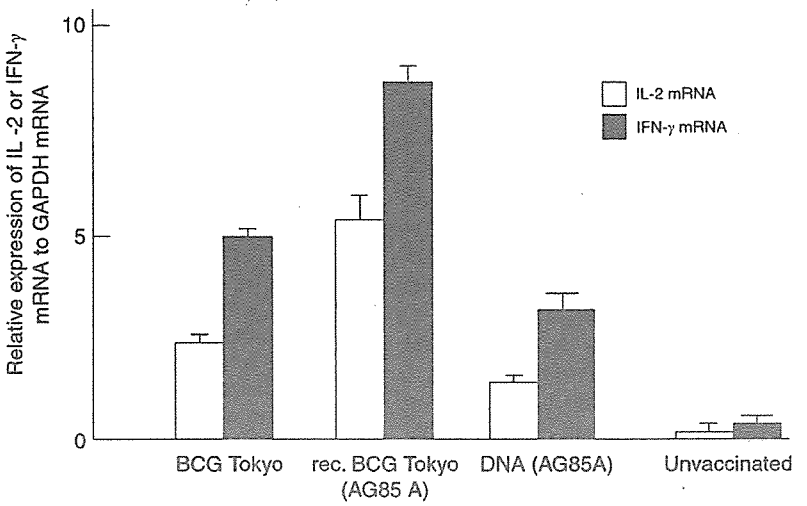

Figure 4 Relative expression of IL-2 or IFN-γ mRNA in guinea pig spleens in comparison with that of GAPDH mRNA. RT-PCR was utilized to examine the expression levels of IL-2 and IFN-γ mRNAs in the spleen tissues of the vaccinated guinea pigs. Thereafter, we conducted densitometric analysis of the electrophoretic RT-PCR results using NIH Image software version 1.62. Relative densitometric ratios were determined using GAPDH mRNA as an internal control.

boosting in TB mouse models. It was also shown that a 20-mer Ag85A synthetic peptide (141–160) was immunogenic in terms of interferon-γ induction.[18] Therefore, in the present study, 500 μg of Ag85A synthetic peptide (141–160) was used as a booster. We used Ag85A synthetic peptide in this study because it was easy to prepare purified Ag85A synthetic peptide than purified secretory Ag85A protein and anti-Ag85A peptide in sera was measured by ELISA.[19] A combination of recombinant BCG Tokyo plus Ag85A peptide reduced the number of pulmonary cfu significantly in a guinea pig TB model ($p<0.001$) and it tends to ameliorate the severity of pulmonary pathology. It is suggested that recombinant BCG Tokyo followed by exogenous protein boosting is an effective way of increasing the protective efficacy of an experimental TB DNA vaccine encoding Ag85A.

The ability to prevent hematogenous spread is a recognized feature of BCG vaccination.[20–22] In this study, none of the vaccines were able to prevent the dissemination of tubercle bacilli to the spleen because granulomatous lesions without central necrosis were recognized in guinea pigs vaccinated with recombinant BCG Tokyo, recombinant BCG Tokyo plus Ag85A peptide, Ag85A DNA, Ag85A DNA plus Ag85A peptide, or parental BCG Tokyo. When these vaccinated guinea pigs were followed up for a further 6 months, splenic granulomas without central necrosis disappeared completely in those vaccinated with parental BCG Tokyo, recombinant BCG Tokyo plus Ag85A peptide or Ag85A DNA plus Ag85A peptide emulsified in Freund's incomplete adjuvant. Moreover, the pulmonary granulomas were surrounded by collagen fibers and no tubercle bacilli were recognized inside them. Therefore, it is necessary to follow up vaccinated animals for a long period in order to evaluate the protective efficacy of TB vaccines accurately. When we followed up the guinea pigs vaccinated with parental BCG Tokyo, recombinant BCG Tokyo plus Ag85A peptide or Ag85A DNA plus Ag85A peptide for 6 months, protection was still maintained. We administered 10×10^6 cfu recombinant BCG/guinea pig subcutaneously and observed no adverse effects (body weight loss, liver damage and so on) during one year of follow-up.

It is thought that DNA TB vaccines, based on selective expression of mycobacterial antigens, have several advantages over live vaccines, but there are also disadvantages for TB DNA vaccines. Anti-DNA antibodies may be generated in vaccinated hosts and the anti-DNA antibody is detrimental to hosts immunized with DNA vaccines. Very recently, recombinant modified vaccinia virus Ankara expressing Ag85A was administered to BCG-vaccinated humans and there were no serious adverse reactions.[23] However, hsp60 and Ag85A DNA vaccination against TB has been reported to produce pulmonary necrosis.[24]This previously unanticipated safety problem indicates that DNA vaccines should be used carefully in individuals who may already have been exposed to TB. Our Ag85A DNA-vaccinated guinea pigs did not exhibit such necrosis. In this respect, the immunization protocol (one administration to non-immunized hosts) seems to be important. It is also difficult to measure antigens expressed in host tissues when DNA vaccines are used for immunization. Thus, details of how much antigen should be used for immunization and how much is expressed have remained unknown. In this study we were unable to measure Ag85A antigen in sera of the guinea pigs after intradermal immunization with Ag85A DNA vaccine. Therefore, it is essential to determine the optimal conditions including the concentration of DNA vaccine, the concentration of the antigen expressed in the cells after vaccination, the immunization method and the route of immunization.[9,22,25] On the contrary, we were able to measure Ag85A antigen in sera of the guinea pigs by ELISA after subcutaneous immunization with recombinant BCG Tokyo (Ag85A) (data not shown).

Several TB vaccines are currently being tested. These include recombinant BCG vaccine expressing Ag85B, recombinant modified vaccinia virus Ankara expressing Ag85A, TB polyprotein vaccine, Mtb72F, ESAT-6 subunit vaccine, auxotrophic vaccines for TB, and recombinant

BCG overexpressing major extracellular proteins (rBCG30).[8,10,17,26–29] The phase 1 study of modified vaccinia virus Ankara (Ag85A), involving intradermal administration to individuals vaccinated previously with BCG, has now been completed, and the results suggest that it is safe and highly immunogenic.[17] We think the combination of these TB vaccines is important for achieving maximal protective efficacy against adult TB. Some researchers have used a combination of DNA vaccine followed by a booster protein, or BCG followed by a polyprotein vaccine.[30] On the basis of our present findings, we favor the combined use of vaccines (recombinant BCG followed by subunit vaccine). We selected BCG Tokyo as the parental strain because it is less virulent than BCG Pasteur and other BCGs.

In summary, the results of this study indicate clearly that recombinant BCG Tokyo containing plasmids encoding a single mycobacterial gene with the peptide boosting is an effective way of inducing protective immunity in guinea pig models of TB, and that long-term follow-up of vaccinated animals infected with *M. tuberculosis* is required to better evaluate the protective efficacy of recombinant BCG Tokyo and TB DNA vaccines. The lack of a sufficient safety record for DNA vaccines makes delivery of the recombinant subunit using an adjuvant approach more desirable. We consider that our approach using recombinant BCG Tokyo followed by Mtb72F protein as a booster is practical, as long as data in the literature related to TB vaccines is sufficiently evaluated.

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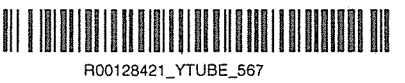

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Method for efficient storage and transportation of sputum specimens for molecular testing of tuberculosis

H. Guio,* H. Okayama,† Y. Ashino,* H. Saitoh,* P. Xiao,* M. Miki,† N. Yoshihara,‡ S. Nakanowatari,† T. Hattori *

* Department of Infectious and Respiratory Diseases, Graduate School of Medicine, Tohoku University, Sendai, † Japanese Red Cross Sendai Hospital, Sendai, ‡ Japanese Research Center of Tuberculosis, Tokyo, Japan

SUMMARY

SETTING: The polymerase chain reaction (PCR) is a highly sensitive method for the detection of *Mycobacterium tuberculosis* and is available in most countries, though to a lesser extent in rural areas.

OBJECTIVE: To amplify *M. tuberculosis* DNA sequences of sputum spotted on FTA® cards and compare them with the results of microscopic examination among culture-positive samples.

DESIGN: A total of 102 sputum specimens of TB patients in treatment were spotted on FTA cards and stored at room temperature until DNA analysis. We assessed the IS*6110* region of *M. tuberculosis*. The efficacy of the PCR assay for the direct detection of *M. tuberculosis* was evaluated and compared with the results of cultures (Middlebrook 7H9 broth) and smears of fresh sputum specimens.

RESULTS: We were able to detect 10 fg/μl of mycobacterial DNA even after 6 months in storage. The PCR sensitivity and specificity using the FTA card system were 82% and 96%, while microscopic examination showed 41% and 95%, respectively.

CONCLUSION: The FTA® card system for the storage of bacterial DNA from sputum samples should be considered for the molecular diagnosis of tuberculosis. Samples can easily be obtained from geographically isolated populations and shipped by mail for accurate molecular diagnosis.

KEY WORDS: tuberculosis; sputum; PCR; FTA® card

CURRENT GLOBAL TUBERCULOSIS (TB) control efforts are based on the diagnosis of cases followed by adequate treatment. Difficulties involved in the collection, transport and processing of samples in clinical practice have also been a major issue. An initial error made by microscopic diagnosis will not be known until weeks later, when the clinical signs are more evident (in false negatives). Microscopic examination of sputum acid-fast bacilli (AFB) smear is still the most widely available diagnostic tool for TB. Unfortunately, smear microscopy is neither specific for *Mycobacterium tuberculosis*, nor is it very sensitive. Depending on the number of specimens examined, smear microscopy detects 30–60% of culture-positive TB suspects.[1] In most situations in which TB is diagnosed by AFB microscopy, it should be assumed to be *M. tuberculosis* until proven otherwise.[2] Detection of AFB smear-negative patients, who make up a significant proportion of all TB patients, is even more problematic.[3,4] As *M. tuberculosis* grows very slowly, diagnosis by culture is a long process, requiring 3–8 weeks in solid media and 1–4 weeks in liquid media.[5]

The development of rapid and accurate procedures for the diagnosis of TB has been a long-standing goal for two main reasons—to improve case finding and case management, and to improve disease surveillance.[5] Molecular amplification assays such as polymerase chain reaction (PCR), which can specifically amplify large quantities of DNA from small starting quantities (10–100 mycobacteria/ml), have been shown to be a promising alternative even for developing countries.[6,7] Microscopic examination requires $>10^3$ to 10^4 mycobacteria/ml, and it is necessary for the diagnostic laboratory to either process the specimen shortly after collection or store it at 4°C to inhibit the growth of contaminating micro-organisms. The latter procedure entails additional labour costs for the processing and conservation of specimens, and reductions in sensitivity.[8]

The FTA® card system (Whatman International Ltd, Abingdon, Cambridge, UK) was originally developed for storing blood samples for DNA testing; its matrix binds and lyses cells, resulting in amplifiable DNA being immobilised on the paper sections, which can

Correspondence to: Dr Toshio Hattori, Division of Respiratory and Infectious Diseases, Postgraduate Division, Tohoku University, 1-1 Seiryo-machi, Aoba-ku, Sendai 980-8574, Japan. Tel: (+81) 22 717-8220. Fax: (+81) 22 717-8221. e-mail: hattori.t@rid.med.tohoku.ac.jp

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be punched out directly for use. The card's size allows storage of several hundred samples at room temperature. The filter is impregnated with a chaotropic agent that denatures infectious agents, and thus, as the samples no longer represent a biohazard, their storage and transport can be managed without burdensome precautions.

In this study, we set out to investigate the sensitivity and specificity of a PCR system for the detection of *M. tuberculosis* in sputum samples spotted on FTA® cards and to evaluate the potential of using this method to overcome the difficulties of transporting and storing sputum samples during the TB diagnosis process.

INDIVIDUALS, MATERIALS AND METHODS

Patient and clinical specimens

A total of 102 sputum specimens from 35 TB patients at the Japanese Red Cross Sendai Hospital were collected prospectively. The clinical diagnosis of TB was established by patient histories and clinical and radiological findings, as recommended by the American Thoracic Society.[9] All the patients provided written informed consent. The study was approved by the Tohoku University Committee on Clinical Investigation and by the Ethics Committee of the Red Cross Hospital.

Evaluation and application of the sputum onto the FTA® cards

Spontaneously produced sputum was the specimen of choice. No patient was assisted by respiratory therapy technicians nor stimulated with hypertonic saline aerosol to produce acceptable sputum. The gross appearance of the sputum was evaluated according to Miller & Jones' classification: M1 (pure mucus), M2 (little purulent content), P1 (purulent sputum less than one third of the volume), P2 (purulent sputum between one third and two thirds of the volume) and P3 (purulent sputum more than two thirds of the volume).[10] Standard precautions, such as the use of gloves and a mask, were taken when manipulating sputum specimens.

To improve the chance of detecting *M. tuberculosis*, we chose the thicker (purulent) particles of the sputum and applied them directly onto a FTA® card using a foam-tipped applicator (Whatman®, Tokyo, Japan) that was squeezed over an area of 2.5 cm in diameter. The card was then allowed to dry for 1 h at room temperature. Heat was not used during the drying period. The cards were then put into storage desiccant packets (Whatman®, Japan) and stored at room temperature until DNA analysis.

Culture and hybridisation

Equal volumes of N-acetyl-L-cysteine/NaOH (4%) were mixed with the specimens for digestion and decontamination. The resulting mixtures were allowed to stand for 15 min at room temperature, then centrifuged at 3000 × g for 15 min. The sediment was re-suspended in 2 ml of phosphate buffered saline (PBS) and 0.5 ml was inoculated into Middlebrook 7H9 broth (Middlebrook, Becton Dickinson, Cockeysville, MD, USA). DNA was extracted in all the culture-positive samples to identify and confirm *M. tuberculosis* using a DNA-DNA calorimetric microdilution plate hybridisation kit (DDH Mycobacteria; Kyokuto Pharmaceuticals, Tokyo, Japan).

Preparation of isolated DNA from FTA® cards

Four discs of 1.2 mm from the spotted area of the filters were cut out using a sterile hole puncher (Harris Micro punch 1.2 mm, Whatman®, Japan) and placed in 1.5 ml PCR tubes. The punch was cleaned by placing the end of the punch in the flame of a Bunsen burner and by sterilisation with 70% ethanol between cuts from different samples. The discs were washed three times for 5 min with 800 µl FTA® purification reagent (Whatman®, Japan), which removes PCR inhibitors and other potential contaminants to ensure the quality of the DNA for downstream analysis. The discs were also rinsed twice with 800 µl TE buffer (10 mM Tris HCL, 0.1 mM EDTA, pH 8.0) (Promega, Tokyo, Japan) for 5 min each time. The discs were then dried at room temperature for 1 h and used directly as templates in the PCR reaction mix in the same tube.

PCR procedure

The primers were synthesised using an Automated Multiplex Oligonucleotide Synthesizer (Roche Diagnostics, Tokyo, Japan) (Table 1). Two different PCR assays were performed. First, as an internal control for monitoring successful DNA extraction, β-globin was amplified by using primers GH21 and PCO3 to yield a 250-bp product.[11] The total reaction volume was 50 µl and the reaction mixture contained each primer (10 pmol each), 2U taq DNA polymerase (Invitrogen Cat N°10342-020), 5 µl 10x PCR buffer (200 mM Tris pH 8.4, 500 mM KCl), 1 µl 10 mM dNTP mixture (Invitrogen Cat N°18427-013), 1.5 µl 50 mM $MgCl_2$ and 38.1 µl distilled water. PCR was performed in an MJ Research PTC-200 DNA Engine Thermal Cycler (MJ Research, Waltham, MA, USA) using the following amplification conditions: 95°C for 5 min, 35 cycles at 95°C for 30 s, 55°C for 45 s, 72°C for 30 s and one cycle at 72°C for 10 min. Finally, a 123-bp sequence of insertion element IS*6110* was amplified using T4-T5 primers.[12] This insertion sequence is present in different numbers and locations in the genomes of most clinical isolates of *M. tuber-*

Table 1 PCR primers used in this study

Target	Sequencing	bp	Ref.
β-globin GH21-PCO3	5'-GGA-AAA-TAG-ACC-AAT-AGG-CAG-3' 5'-ACA-CAA-CTG-TGT-TCA-CTA-GC-3'	250	10
M. tuberculosis IS*6110* T4-T5	5'-CCT-GCG-AGC-GTA-GGC-GTC-GG-3' 5'-CTC-GTC-CAG-CGC-CGC-TTC-GG-3'	123	11

culosis, which makes it a useful probe for diagnostic and epidemiological purposes. The final composition of the PCR mix was the same as in the previous case. The PCR conditions were preheating at 94°C for 5 min, then 40 cycles at 94°C for 2 min, 68°C for 2 min, 72°C for 2 min, and then 72°C for 10 min.

Determination of sensitivity

To determine the sensitivity of *M. tuberculosis* detection, 10-fold serial dilutions (1 ng to 10 fg) of H37Rv DNA (kindly provided by Dr I Sugawara, Research Institute of Tuberculosis, Tokyo, Japan) were performed using T4-T5 primers. The final composition and PCR conditions were the same as for amplifying IS*6110*.

All amplification products were detected on 1.5% agarose gel in 1 × TAE buffer stained with ethidium bromide and visualised by ultraviolet transillumination.

Control procedures

A positive control tube containing 0.1 ng H37Rv DNA and a negative control tube containing no DNA were included with each set of reactions. To evaluate cross-contamination during sampling, we performed control punches using unspotted cards.

Statistical methods

The sensitivity and specificity of each pair of primers for the detection of *M. tuberculosis* were calculated on the basis of the study reference standards, with the liquid culture method taken as a gold standard. In addition, the results of individual PCR were employed for the analysis of smear-positive and -negative samples according to the culture results.

RESULTS

Patient characteristics

We evaluated by PCR 102 sputum samples from 35 TB patients (23 male, 12 female), all of whom were receiving anti-tuberculosis treatment for periods ranging from 2 weeks to 1 year. The average age was 51.4 years.

Gold standard

Twenty-two positive culture samples were taken as gold standard. In all of these samples, *M. tuberculosis* was identified by hybridisation assay.

Appearance of the specimens

Eighty-five per cent of smear positives, 68% of culture positives and 81% of PCR positives contained >30% of purulent sputum (P2 or P3 in Miller & Jones' classification[10]).

Sensitivity detection

Four small discs of the FTA® card system were used as templates for the PCR processing. The criterion for using four discs was based on the assumption that the mycobacteria were scanty and heterogeneously distributed during the absorption process; in addition, in a pilot study we determined that using four disks increased the degree of amplification (data not shown). The detection limit of the PCR assay for the amplification of IS*6110* was 10 fg/μl of purified *M. tuberculosis* H37Rv (Figure 1A). The amplification of the 123 bp fragments by PCR using the FTA® card system is depicted in Figure 1C.

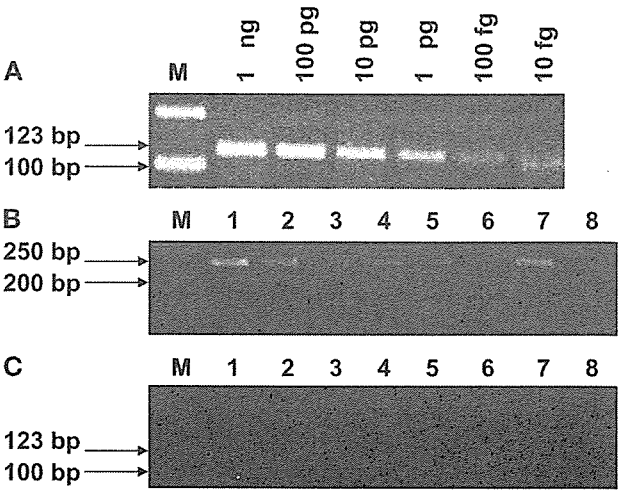

Figure 1 PCR results. A. Sensitivity of detection of *M. tuberculosis*: ten-fold serial dilution of H37Rv DNA was amplified. B. PCR amplicons from β-globin (250 bp): M: marker; lanes 1–7: results using FTA® cards from TB patients; lane 8: negative control. C. PCR amplicons from *M. tuberculosis* (123 bp) stored on FTA® cards: M: marker; lane 1: positive control (H37Rv); lanes 2, 4 and 6: cards from TB patients; lanes 3, 5 and 7: no spotted cards (control punch); lane 8: negative control. In each case, 10 μl samples were electrophoresed through a 1.5% agarose gel and photographed under UV illumination. PCR = polymerase chain reaction; bp = base pairs; TB = tuberculosis; UV = ultraviolet.

Effect of PCR inhibitors

The ability to detect *M. tuberculosis* by PCR can be impaired by the presence of substances inhibitory to Taq DNA polymerase. The β-globin PCR assay generated the expected 250-bp band (Figure 1B) in 90 (89%) of the samples. All the culture-positive samples were also positive in the amplification of β-globin. This finding may suggest that the PCR-negative, culture-positive samples contained low concentrations of TB bacteria rather than PCR inhibitors, which would tend to rule out inhibition as a cause.

PCR and smear results compared with culture

Among the 22 culture-positive samples, 18 (82%) were PCR-positive and 9 (41%) smear-positive (Table 2). The remaining four culture-positive samples were both PCR- and smear-negative. All 9 smear- and culture-positive samples were also positive by PCR. The sensitivity and specificity of PCR were 82% and 96%, compared to 41% and 95%, respectively, for smear examination. Differences were observed on comparing the sensitivity of smear microscopy with that of

Table 2 Comparison of PCR with smear and culture for detection of *M. tuberculosis*

Culture	Positive *n* (%)	Negative *n*
PCR*		
Positive	18 (82)†	3
Negative	4	77
Smear		
Positive	9 (41)†	4
Negative	13	76

* PCR using the FTA® card system.
† (%) sensitivity.
PCR = polymerase chain reaction.

PCR ($P < 0.05$), but there was no observed difference in specificity (Figure 2).

We repeated the PCR procedure for all culture- and smear-positive samples. The second experiment showed the same results as the first.

Sensitivity of PCR by smear result

The sensitivity of PCR for smear-positive, culture-positive samples was 9/9 (100%), whereas that for smear-negative, culture-positive samples was 9/13 (69%). These results show that, even in the paucibacillary form of TB resulting from treatment, this PCR system could provide rapid and sensitive detection of *M. tuberculosis* DNA impregnated on the FTA® card.

Stability and control procedures

All of the 102 samples were analysed by PCR at two time points—at the time of sample collection and after 6 months of storage—obtaining positive amplifications in both cases, clearly showing that storage for 6 months did not affect the amplification. PCR products were not detected in negative controls or control punch cards, confirming the absence of contamination during the procedure (Figure 1C). The total assay time was 9 h.

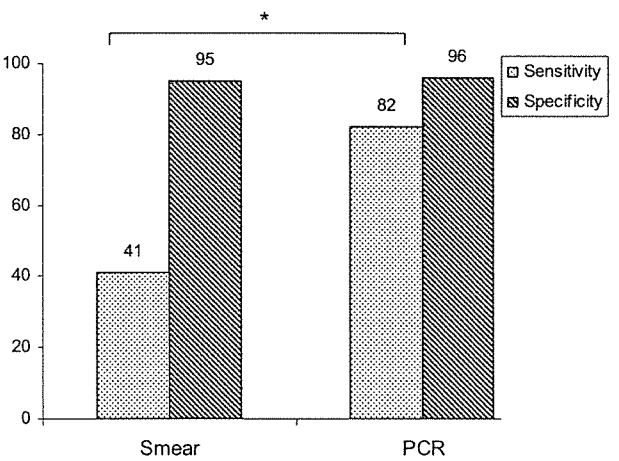

Figure 2 Accuracy of the methods. Sputum smear examination (smear) and PCR assay using the FTA® card system (PCR) among the 22 positive culture samples. * $P < 0.05$. PCR = polymerase chain reaction.

DISCUSSION

The present study demonstrated that the DNA of *M. tuberculosis* can be amplified using sputum spotted on an FTA® card. We found that the *M. tuberculosis* DNA stability with this card at room temperature was up to 6 months. However, care must be taken to avoid cross-contamination between specimens during sampling and handling. The present findings are relevant to patient care and clinical trials and suggest that sputum stored on FTA® cards could provide a simple, economical method for the collection, storage and transport of suspected TB specimens for later testing.

In addition, samples can easily be obtained from geographically isolated populations where access to and/or availability of TB diagnostic testing may be limited. Samples collected may be shipped by mail to a central laboratory for molecular diagnosis without the triple packing system otherwise required for transport.[13]

Depending on the gold standard and other methodological factors, studies have shown PCR sensitivities ranging from 77% to >95% and PCR specificities of >95% in TB patients before treatment.[4,5] Regarding patients under treatment, Kennedy et al. found 76% agreement between culture and PCR.[14] In our study, three culture-negative samples (one smear-positive and two smear-negative) were detected by our method, which may be explained by the limited quantity of TB DNA.[15] While the sensitivity of smear is dependent on the type and quality of the specimen, our method could be employed with accuracy even 6 months after obtaining the sample. However, as we recommend taking the purulent part of the sputum, it will be necessary to help patients understand that secretions from deep in the lung are required.

More sensitive methods exist, including the use of automated culture systems, but the best tests are not always available for the people who most need them.[16]

In summary, the present system appears to be a promising method for transporting and storing sputum samples. Other advantages are the simplicity of the sample preparation and the use of the small disc as a template during the PCR process, using specific targets, without the need for extensive nucleic acid purification.

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RÉSUMÉ

CADRE : La réaction polymérase en chaîne (PCR) est sensible pour la détection de *Mycobacterium tuberculosis*, et est disponible dans la plupart des pays, mais dans une moindre mesure dans les zones rurales.
OBJECTIFS : Amplifier les séquences d'ADN de *M. tuberculosis* des crachats repérées sur les cartes FTA et les comparer avec les résultats des examens microscopiques dans les échantillons de cultures positives.
MÉTHODES : Au total, 102 échantillons de crachats de patients tuberculeux ont été marqués sur les cartes FTA et stockés à l'air ambiant. La spécificité et la sensibilité de deux amorces de PCR qui amplifient la région IS*6110* de *M. tuberculosis* ont été évaluées et comparées à celles de cultures (milieu liquide 7H9), et de l'examen microscopique d'échantillons frais de crachats.
RÉSULTATS : Il a été possible de détecter 10 fg/µl de DNA mycobactérienne même après 6 mois de stockage. L'analyse PCR des deux paires d'amorces révèle une sensibilité et une spécificité respectivement de 82% et 96%, alors que celles de l'examen microscopique sont respectivement de 41% et de 95%.
CONCLUSION : Le système de carte FTA permettant le stockage de l'ADN bactérien issu d'un échantillon de crachats devrait être envisagé pour le diagnostic moléculaire de la tuberculose. Les échantillons de crachats peuvent être facilement obtenus dans des populations géographiquement isolées, stockés et adressés par courrier afin d'établir à distance un diagnostic moléculaire précis.

RESUMEN

CONTEXTO : La reacción en cadena de la polimerasa (PCR) es un método sensible para la detección de *Mycobacterium tuberculosis* y se encuentra al alcance en la mayoría de los países, aunque en menor medida en zonas rurales.
OBJETIVO : Amplificar secuencias del ADN de *M. tuberculosis* a partir de manchas de esputo en papel de filtro (*FTA cards*®) y comparar estos resultados con los resultados de la baciloscopia, en muestras con cultivo positivo para micobacterias.
MÉTODOS : Se recogió un total de 102 muestras de esputo de pacientes con tuberculosis en curso de tratamiento, las cuales se almacenaron como manchas en papel de filtro a temperatura ambiente hasta el momento del análisis. Con la PCR se amplificó un fragmento de 123 pares de bases de la secuencia de inserción IS*6110* de *M. tuberculosis*. Se evaluó la eficacia de la PCR en la detección de *M. tuberculosis* y los resultados se compararon con los resultados de los cultivos en medio líquido 7H9 (método de referencia) y de la baciloscopia, de muestras frescas de esputo.
RESULTADOS : El método permitió detectar hasta 10 fg/µl de ADN micobacteriano en muestras almacenadas durante más de 6 meses. La PCR a partir de las manchas de esputo en tarjetas FTA® mostró una sensibilidad del 82% y una especificidad del 96%, comparada con una sensibilidad del 41% y una especificidad del 95% de la baciloscopia.
CONCLUSIÓN : El sistema con tarjetas FTA® debería tenerse en cuenta como método de conservación del ADN micobacteriano presente en las muestras de esputo, para el diagnóstico molecular de la tuberculosis. Así, en poblaciones geográficamente aisladas, de manera sencilla podrían obtenerse las muestras de esputo, almacenarlas y expedirlas por correo con el fin de establecer un diagnóstico molecular exacto.

第1章 概念・定義と疫学

結核：世界と日本の現状

緒言

結核は世界最大の感染症であり，多くの国の大きな保健問題であります．WHO は塗抹陽性患者の 70％ を見つけ，発見患者の 85％ を治癒させることを目標と定め，DOTS 戦略を進めています．我が国の結核は高年齢層と都市部に偏在する傾向が強まっています．大都市にはホームレス者などの対応困難な人々が多く，対策の課題となっています．また，多数の既感染者が存在し，患者が発生し続けている現状にあることから，日常診療の場で忘れてはならない感染症であります．

世界の結核の現状

結核は世界最大の感染症であり，世界の総人口の約 3 分の 1 の人々が結核に感染していると推定され，いまだに多くの国の重要な保健問題となっています．新発生患者数は 892 万人，喀痰塗抹陽性者 394 万人，死亡数 169 万人の状況にあります（2004 年 WHO 推定数）[1]．HIV 感染者の増加や社会変動などにより，結核の蔓延が加速している国もあります（表 1）．患者数の多い上位 22 ヵ国で世界の結核患者の 80％ を占めています．これらの国々は結核高負担国と呼ばれています．インド，中国，インドネシア，ナイジェリア，バングラディッシュ，パキスタンは，人口が多く，しかも人口の移動，人口の都市集中も激しく，かつ結核患者が集中している国であります．地域別にはアジア地域を始めとする開発途上国に患者発生が集中している現状にあります（表 2）．サハラ以南のアフリカ諸国のジンバブエ，南アフリカ，モザンビークなどでは HIV 感染の影響で罹患率が高くなっています．これらの国では，貧困化，都市化，HIV の流行があり，しかも保健医療体制も十分でない中で結核が猛威をふるい続けています[2]．十分な結核対策が講じられないとさらに深刻になる可能性があ

◉キーワード
結核
DOTS
HIV
都市化

表1 世界の推定新発生患者数の8割を占める22の結核高負担国（2004年WHO推定）

国	人口（100万人）	全結核（千人）	罹患率	HIV有病率（%）	塗抹陽性（千人）	全結核死亡数（千人）
インド	1,087	1,824	168	5.2	815	329
中国	1,308	1,325	101	0.9	595	217
インドネシア	220	539	245	0.9	242	101
ナイジェリア	129	374	290	26.6	161	106
南アフリカ	47	339	718	60.2	138	64
バングラディッシュ	139	319	229	0.1	144	70
パキスタン	155	281	181	0.6	126	63
エチオピア	76	267	353	21.0	116	60
フィリピン	82	239	293	0.1	108	39
ケニア	33	207	619	28.5	89	45
コンゴ	56	204	366	20.8	89	44
ロシア	144	166	115	6.8	74	30
ベトナム	83	147	176	3.0	66	19
タンザニア	38	131	347	36.0	55	29
ウガンダ	28	112	402	19.1	49	26
ブラジル	184	110	60	16.9	48	14
アフガニスタン	29	95	333	0.0	43	26
タイ	64	91	142	8.5	40	12
モザンビーク	19	89	460	48.4	37	25
ジンバブエ	13	87	674	68.0	35	20
ミャンマー	50	85	171	7.1	38	10
カンボジア	14	70	510	13.0	31	13

Published in Global TB Control 2006 (www.who.int/tb/publications/global_report). Visit www.who.int/tb/country/tb_burden for 2000 estimates published in Corbett et al. 2003. Arch Intern Med 163: 1009–1021.

ります．このような深刻さが増しているのに社会的な危機感が乏しいことから，WHOは1993年4月に全世界に対して結核緊急事態宣言を発令し，結核対策の強化を訴えました．また，世界のすべての国の結核患者の治療をリファンピシン（RFP）を入れた短期化学療法に切り替えた方が費用対効果が大きいことを示して，世界各国からの資金供与を得て，世界中の患者に標準治療を実施することを目指しました．これが，発見した患者に対し服薬によって完全に治療を終了できるように支援するWHOの“Directly Observed Treatment, Short course (DOTS)”戦略であります．WHOは，新発生塗抹陽性患者の70％を見つけ出し，発見した患者の85％を治癒させることを目標と定め