Table 1. The conditions for DNA hybridization and DHPLC analysis have been described in detail elsewhere [8].

2.4. DNA sequencing

rpsL and *rrs* gene PCR products of 115 streptomycin-resistant, and 100 streptomycin-susceptible clinical isolates, and the streptomycin-dependent strain 18b, were sequenced. The sequencing primer sets were the same as those used for PCR. After purification, the PCR product (5 ng) was used as a template for *Taq*Cycle Sequencing using ABI Prism Big Dye Terminator sequencing kits (Applied Biosystems). Cycle sequencing products were subsequently analyzed on an ABI PRISM 310 Genetic Analyzer (Perkin Elmer Applied Biosystems) [9].

3. Results

3.1. rpsL mutations in clinical isolates

The results of DNA sequencing showed that all of the 100 streptomycin-susceptible isolates had wild-type *rpsL*. Of the 115 streptomycin-resistant isolates, 88 (76.5%) were found to have *rpsL* mutations, the majority at codon 43 (Fig. 1). Seven (6.1%) isolates rpsL88 mutation (AAG → AGG, Lys → Arg) and 81 (70.4%) had an rpsL43 (AAG → AGG, Lys → Arg). Seven of the isolates with an rpsL43 mutation harbored a second mutation in *rrs* and *rpsL* genes: one isolate had rpsL39 (ACC → ACT, no amino acid change), one had rpsL71 (GGC → AGC, Gly → Ser), one had rrs462 (T → G, 645 A deletion), one had rrs513 (A → C), one had rrs1400 (A → G) and two had rrs1401 (C → T) mutations. No compound mutation of codon 88 was found.

Codon 88 was the other mutation point. Seven (6.1%) isolates carried codon 88 mutation (AAG → AGG, Lys → Arg). No compound mutation of codon 88 was found.

3.2. rrs mutations in clinical isolates

No mutation was found among 100 streptomycin-susceptible isolates as evaluated by DNA sequencing. Of the 115 streptomycin-resistant clinical isolates, a total of 11 (9.5%) isolates had *rrs*A mutation (Fig. 2), among which two had compound mutation with *rpsL* codon 43 mutation as described above, and nine possessed mono-mutation. Among the mutation types, two isolates had 513 A → C and 645 deletion, five had 513 A → C, one had 516 C → T, and one had 464 A → C.

*rrs*B: Only one (0.9%) isolate showed *rrs*B 906 A → C mutation.

*rrs*C: Three (2.6%) isolates were found to have *rrs*C mutation (one with 1400 A → G, two with 1401 C → T), all of them compound with *rpsL* codon 43 mutation, as described above.

3.3. Streptomycin resistance gene mutation and MIC analysis

Fig. 3 shows the relationship between mutation types and MIC levels of 98 streptomycin-resistant isolates in which *rpsL* or *rrs* mutation was detected. Seventy-four isolates with *rpsL*43 AAG → AGG mutation were found at different MICs ranging from 20 to 800 μg/ml. The MICs of nine isolates with *rrs*A mono-mutation ranged from 50 to 800 μg/ml. There was no significant difference in MIC among the mutation types. These results revealed no close correlation between mutation type and streptomycin resistance level.

3.4. Mutations in streptomycin-dependent strain 18b

DNA sequencing results revealed one insertion of an additional cytosine residue between positions 512 and 513 in the 530 loop and one deletion of adenosine at position 645 in the *rrs*A gene. No mutation was found in *rpsL*, *rrs*B or *rrs*C.

3.5. DHPLC analysis

The results of DHPLC analysis were completely consistent with those of DNA sequencing. With *M. tuberculosis* H37Rv as a reference strain, DHPLC analysis revealed that all the streptomycin-susceptible isolates, and those isolates that were streptomycin-resistant but with no *rpsL* or *rrs* mutation, had a normal peak pattern, which was the same as that of H37Rv. All the 88 isolates that were found to carry *rpsL* mutation showed an aberrant DHPLC pattern. Four types of mutation in the *rpsL* gene were found by DNA sequencing, and four corresponding peak patterns were shown by DHPLC analysis (Fig. 4). Seventy-six codon 43 AAG → AGG mono-mutated isolates, seven codon 88 AAG → AGG mono-mutated isolates,

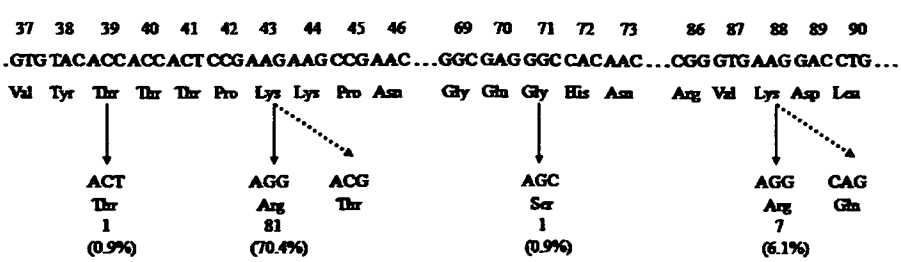

Fig. 1. Nucleotide sequence and missense mutations in the *rpsL* gene. ——▶ shows the mutations found in this work. - - - ▶ shows mutations from other reports.

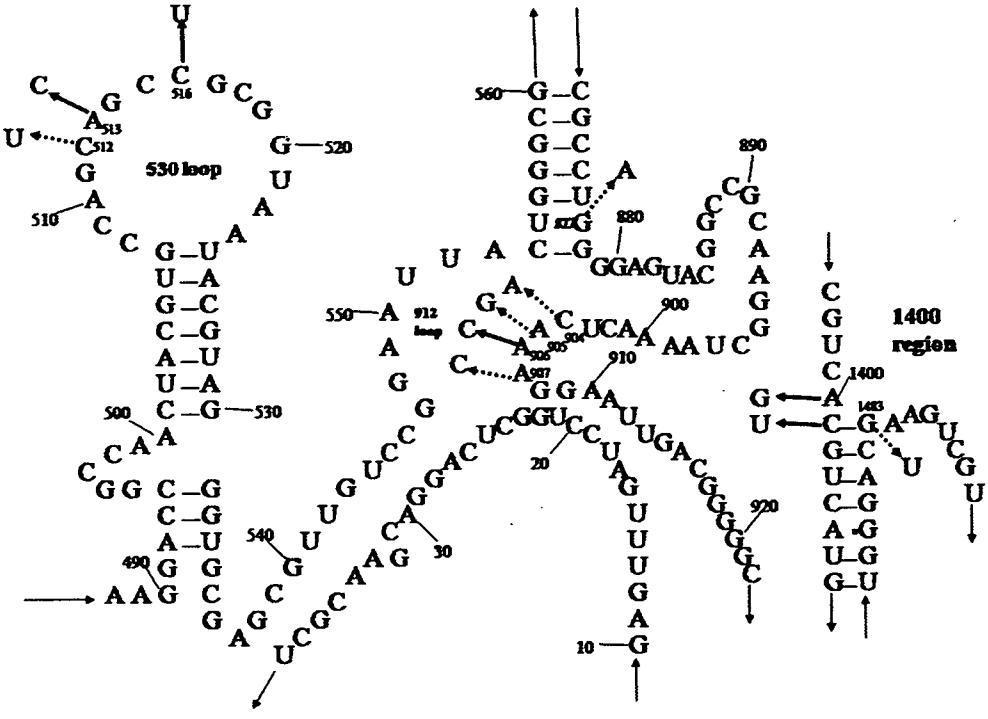

Fig. 2. Mutations located in the 530 loop, the 912 loop and the 1400 region in 16S rRNA associated with streptomycin resistance. The figure is based on a model structure of *M. tuberculosis* 16S rRNA [15]. ——▶ shows the mutations found in this work. - - - ▶ shows mutations from other reports.

one codon 43 AAG → AGG compound with codon 39 ACC → ACT mutated isolate, and one codon 43 AAG → AGG compound 71 GGC → AGG mutated isolate showed their own specific and aberrant DHPLC patterns, respectively.

Four types of *rrs*A gene mutation, one type of *rrs*B gene mutation and two types of *rrs*C gene mutation were found in this study. Their corresponding DHPLC patterns are shown in Fig. 4 and all of them differs from the susceptible reference strain, H37Rv. With regard to the streptomycin-dependent strain 18b, the results of *rpsL* and *rrs* gene DHPLC analysis are also shown in Fig. 4.

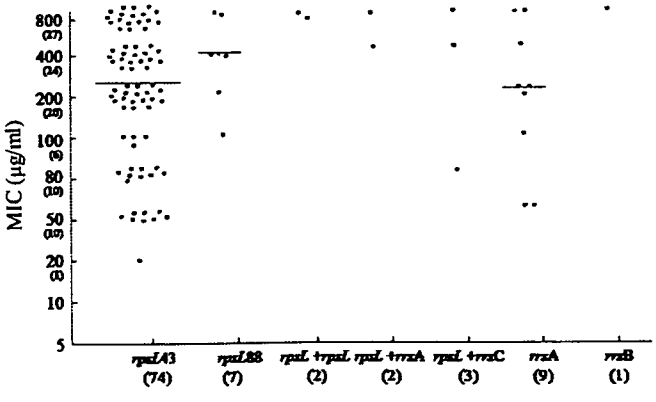

Fig. 3. Relationship between mutation types and MICs of 98 streptomycin-resistant isolates. Numbers in bracket indicate total number of clinical isolates harboring one type of mutation or one level of MIC. ——— indicates average MIC level in a group. No significant difference was found among *rpsL*43AAG ——▶ AGG, *rpsL*88AAG ——▶ AGG, *rrs*A mutation, *rrs*B mutation and three kinds of double mutations.

4. Discussion

One practical implication of the present findings is that that the DHPLC method has wide clinical application for *rpsL* and *rrs*A mutation analysis. Currently, control of MDR-TB and XDR-TB is a major issue throughout the world. It is useful to detect *rpsL* and *rrs* mutations in kanamycin- and amikacin-resistant *M. tuberculosis* strains. Kanamycin and amikacin are commonly used in second-line therapy of TB. Detection of drug-resistant phenotypes of *M. tuberculosis* using routine methods takes several weeks. The establishment of a rapid, simple and reliable method for detection of drug-resistant phenotypes of *M. tuberculosis* is one of the most urgent requirements for effective treatment of tuberculosis patients. Compared with DNA sequencing and drug susceptibility testing, DHPLC has been confirmed to be a simple, reliable and cost-effective method with high sensitivity and specificity, and has already been applied for detection of *rpoB* and *gyrA* gene mutation in order to predict rifampicin and fluoroquinolone resistance in *M. tuberculosis* [7–9]. This is the first report of the use of DHPLC for streptomycin resistance gene mutation analysis using a large series of clinical samples. The results of DHPLC are completely consistent with those of DNA sequencing: In all of the susceptible clinical isolates, no mutation was found; 84.3% of streptomycin-resistant clinical isolates revealed *rpsL* or *rrs*A mutation; one type of peak patterns corresponded to one specific mutation type. The DHPLC method devised in this study can be regarded as a useful and powerful tool for analysis of *rpsL* and *rrs* mutation in *M. tuberculosis*. It should be mentioned that the frequency of streptomycin resistance is very high in China. One obvious

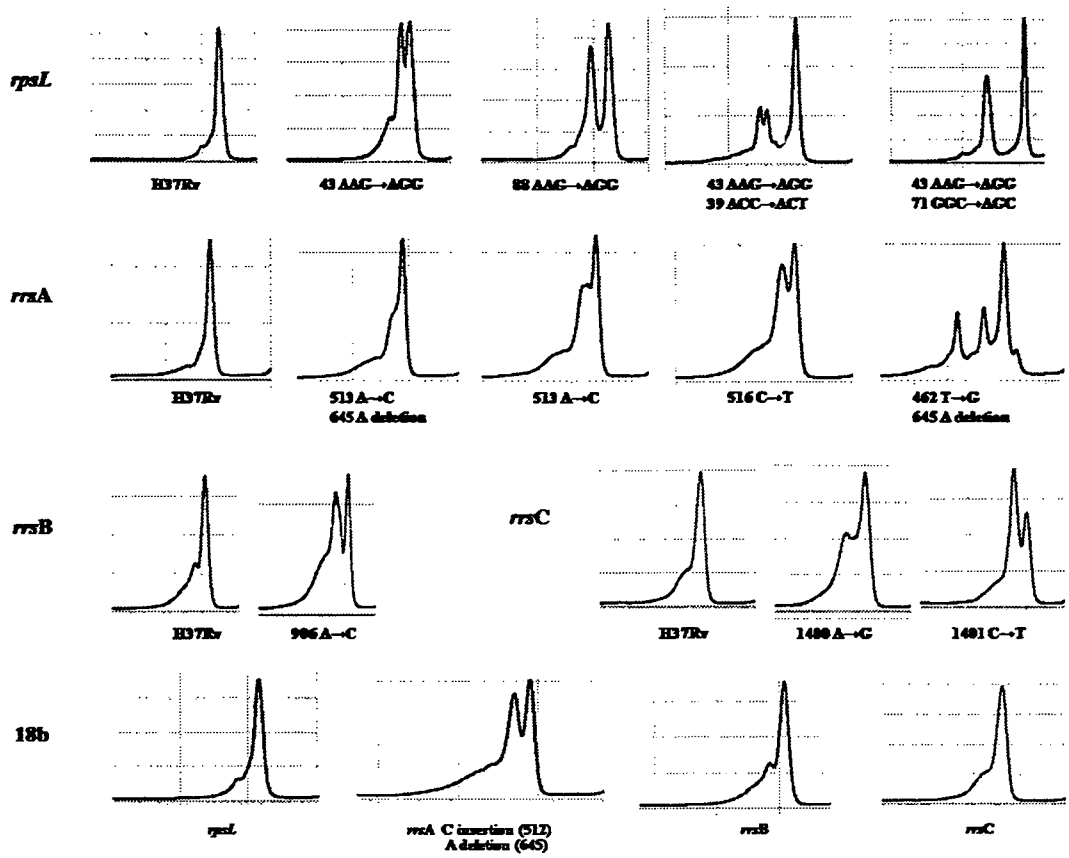

Fig. 4. DHPLC peak patterns of the *rpsL* and *rrs* genes of streptomycin-resistant clinical isolates and streptomycin-dependent strain b18.

reason is that streptomycin has been used for many years in China as an anti-TB drug. In China, Japan and other countries with a high frequency of *rpsL* and *rrs* mutation in streptomycin-resistant clinical isolates, it is anticipated that this DHPLC method will have a high predictive value.

In this study, DNA sequencing and the DHPLC method were applied to investigate the molecular mechanism of streptomycin resistance in *M. tuberculosis* clinical isolates from China. The results revealed that 85.2% of streptomycin-resistant isolates had mutation in the *rpsL* or *rrs* gene. This rate is much higher than those of 56–68% reported in America [10,11], 48% in Germany [12], 60% in France [13], 52% in Poland [14], 24% in Mexico [15], and 77.8% in Japan [16], and indicates the different geographic distribution of *rpsL* and *rrs* mutations among streptomycin-resistant *M. tuberculosis* isolates, those from China showing the highest frequency. There is a possibility that a high transmission frequency could be the cause for the high frequency of *rrs* and *rpsL* mutations in our isolates compared to those found in previous reports [10–16]. It was found in this study that *rpsL* played a more important role (76.5%, 88/115) than the *rrs* gene (*rrs*A: 9.6%, 11/115) in the mechanism of streptomycin resistance, and no *rpsL* or *rrs* mutation was found among 100 streptomycin-susceptible clinical isolates, strongly confirming and extending the findings of other investigators [11–16]. The relative proportions of *rpsL* versus *rrs* mutations in the streptomycin-resistant isolates are similar compared with other studies [11–16]. No relationship between mutation type and resistance level was found in this study, thus differing from the reports of Cooksey [5] and Bottger [17]. The reason may be related to the difference in areas from which the strains were derived, similarly to the differences in the geographic distribution of mutation types discussed above. It is also possible that there are other reasons for no relationship between MIC and mutation type. Unknown mechanisms may exist between different strain families of *M. tuberculosis*.

In this study, *rpsL* mutations were found to occur predominantly at codon 43 (91%, 81/88 isolates). Codon 88 mutation played a minor role (9%, 7/88 isolates). These results largely confirm the findings of other researchers [21]. Two new point mutations found in this study, at codon 39 (ACC → ACT) with no amino acid substitution, and at codon 71 (GGC → AGC, Gly → Ser), both compounded with codon 43 AAG → AGG, seemed to be of only minor importance. Previously reported mutations involving codon 43 AAG → ACG (Lys → Thr) and codon 88 AAG → CAG (Lys → Gln) were not found [21]. This is because they have been proved to be restrictive mutations leading to fitness cost and show attenuated virulence. Only non-restrictive mutations such as codon 43 AAG → AGG (Lys → Arg), which has unaltered virulence properties, can be widely transmitted and finally dominate in clinical isolates [22]. In this study, a total of 15 isolates

(13%) were found to have *rrs* mutation. In contrast to most bacteria that have multiple copies of the *rrs* gene, *M. tuberculosis* and other slow-growing mycobacteria have only one copy [4,21]. Mutations in the *rrs* gene, which encodes the loops of 16S rRNA, the highly conserved 530 loop, the 912 loop and the 1400 region that interact with the S12 ribosomal protein, constitute an easily selected resistance site. In *rrs*A, 513 A → C, 516 C → T, 464 A → C mutations were demonstrated in these isolates from China, while 512 C → T mutation and 491 C → T polymorphism [15,23] were not found. Since only one isolate carried *rrs*B mutation and three *rrs*C-mutated isolates were all compounded with *rpsL* codon 43 AAG → AGG mutation concurrently, it can be concluded that *rrs*B and *rrs*C mutations are not common, while the majority of *rrs* point mutations producing streptomycin resistance in *M. tuberculosis* occur in *rrs*A, which encodes the 530 loop of 16S rRNA. Two isolates had identical *rpsL* codon 43 mutation plus *rrs*C codon mutation. There is a possibility of transmission for the two isolates each with two identical mutations, although no data are available. It is reported that *rrs*C 1400 A → G mutation is the main mechanism involved in resistance to other aminoglycosides (amikacin and kanamycin) in *M. tuberculosis* [24]. It is interesting that the double mutations result in a higher MIC, although the numbers are small. Further study will be required to clarify the mechanism to undergo double mutations. In this study, 14.8% (17/115) of streptomycin-resistant isolates revealed no mutation in the *rpsL* or *rrs* gene. This observation implies that there is at least one additional mechanism conferring streptomycin resistance, and that future molecular genetic studies should be aimed at identifying the gene(s) involved. Recently, Okamoto *et al.* [25] reported that mutations within the *gidB* gene, which encodes a conserved 7-methylguanosine methyltransferase specific for the 16S rRNA, played a role in the mechanism of streptomycin resistance. In fact, two of the 17 clinical isolates in this study that were streptomycin-resistant but with no *rpsL* or *rrs* mutation were found to harbor *gidB* gene mutation (DHPLC results not shown), and the results were also confirmed by DNA sequencing and therefore further study is needed. Apart from this, a growth inhibition experiment [17] has revealed that membrane-active agents such as Tween 80 are capable of reducing the level of resistance significantly by approximately 10-fold, supporting a hypothesis that membrane permeability may play a role in streptomycin resistance. It seems entirely possible that a combination of different resistance mechanisms may operate in a drug-resistant clinical isolate. Therefore, for example, a permeability barrier would be expected to increase the level of resistance of isolates with an altered *rpsL* or *rrs* gene. Other factors, for example a membrane efflux pump [26,27], may have some relationship to streptomycin resistance. In order to further explore the molecular mechanism of streptomycin, we have also sequenced the streptomycin-dependent strain 18b. As is the case for *E. coli*, *M. tuberculosis* also has three streptomycin phenotypes: sensitive, resistant and dependent. Streptomycin-dependent strain 18b was isolated in Japan in 1955 by Hashimoto [28], and in 1995 Cole [29] found that it possessed a novel mutation in the 530 loop of the 16s rRNA: insertion of an additional cytosine between 512 C and 513 A. In the present study, we found a second mutation in its 16s rRNA: a 645 A deletion. How streptomycin has helped to stabilize the conformational structure of 16s rRNA and S12 ribosomal protein and eventually become a necessary component for the process of translation is not clear. The mechanism responsible for the streptomycin dependence of 18b still remains to be discovered.

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Recombinant BCG Tokyo (Ag85A) protects cynomolgus monkeys (*Macaca fascicularis*) infected with H37Rv *Mycobacterium tuberculosis*

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KEYWORDS
Mycobacterium tuberculosis;
H37Rv;
Ag85A;
Recombinant BCG;
Cynomolgus monkey

Summary
One tuberculosis vaccine candidate that has shown a promising degree of protective efficacy in guinea pigs is recombinant BCG Tokyo (Ag85A)(rBCG-Ag85A[Tokyo]). As a next step, cynomolgus monkeys were utilized because they are susceptible to *Mycobacterium tuberculosis* and develop a continuous course of infection that resembles that in humans both clinically and pathologically. The recombinant BCG vaccine was administered once intradermally in the back skin to three groups of cynomolgus monkeys, and its protective efficacy was compared for 4 months with that of its parental BCG Tokyo strain. Vaccination of the monkeys with the rBCG-Ag85A[Tokyo] resulted in a reduction of tubercle bacilli CFU ($p<0.01$) and lung pathology in animals challenged intratracheally with 3000 CFU H37Rv *M. tuberculosis*. Vaccination prevented an increase in the old tuberculin test after challenge with *M. tuberculosis* and reaction of *M. tuberculosis*-derived antigen. Thus, it was shown in monkeys that rBCG-Ag85A[Tokyo] induced higher protective efficacy than BCG Tokyo. This warrants further clinical evaluation.
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Introduction

Tuberculosis (TB) still remains a major health threat affecting millions of people worldwide. The only TB vaccine currently available is *Mycobacterium bovis* BCG. However, the efficacy of BCG against adult pulmonary TB still remains controversial.[1–4] Thus, development of a better TB vaccine is urgently required to counteract the global threat of TB.

We have previously reported the protective efficacy of a TB DNA vaccine (Ag85A) and a recombinant strain BCG Tokyo (Ag85A) in small-animal models challenged with *M. tuberculosis* Kurono strain.[5,6] We found that recombinant BCG

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Tokyo was better than Ag85A DNA in terms of protective efficacy against *M. tuberculosis*.[2] The spleen tissues from guinea pigs vaccinated with rBCG-Ag85A[Tokyo] or Ag85A DNA expressed IFN-γ and IL-2 mRNA at significantly high levels.[6] This finding prompted us to explore further the efficacy of rBCG-Ag85A[Tokyo] in cynomolgus monkeys. We chose cynomolgus monkeys because this animal is reportedly protected more efficiently than rhesus monkeys by BCG vaccination.[7] Previous studies have shown that whereas the rhesus macaque is highly susceptible to *M. tuberculosis*, the closely related cynomolgus macaque is more resistant.[8–10] Cynomolgus monkeys are more efficiently protected by BCG vaccination than rhesus monkeys and therefore afford a good experimental model for the evaluation of new TB vaccine candidates.

Several TB vaccines are currently being tested using various models[11,16–19] and several recent reviews on TB vaccines have been published.[12–15] 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). However, there have been few reports on the efficacy of TB vaccine candidates in cynomolgus monkey models due to lower availability of monkey P3 facilities. Vaccination of cynomolgus monkeys with Ag85B-ESAT-6 reportedly induces protective immune responses.[20] DNA vaccine (HSP65+IL-12/HVJ) as well as 72f recombinant BCG provide better protective efficacy in cynomolgus monkeys.[21] In order to find a better TB vaccine, it is progression to the primate model after positive results in the small animal models. In the present investigation, we examined the efficacy of rBCG-Ag85A[Tokyo] in cynomolgus monkeys, and found that it induced higher protective efficacy than BCG Tokyo.

Materials and methods

Construction of recombinant BCG Tokyo (rBCG-Ag85A[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 *Escherichia 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 (rBCG-Ag85A[Tokyo]) were cultured individually and the content of the extracted lysate that contains Ag85 protein was confirmed by western blotting.[6]

Bacterial strain

M. tuberculosis H37Rv (ATCC 25618) 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.

Monkeys

A total of 18 cynomolgus male monkeys (*Macaca fascicularis*) (5–7 kg, 6–8 years old) were used. All animals were housed at the animal biosafety level (ABSL) 3 facility of Wuhan University, Wuhan, China. The animals were studied in groups of six. Before the start of the studies, all animals were examined clinically and radiologically, and tuberculin skin-tested. For intratracheal challenge, animals were anesthetized with ketamine. Prior to commencement, experiments were reviewed and approved by the Wuhan University ethics committee.

Inoculation of monkeys

The monkeys were randomly assigned to three groups. Group 1 (6 monkeys) received one intradermal injection of 2×10^6 CFU/ml rBCG-Ag85A[Tokyo]. Group 2 received one intradermal injection of 2×10^6 CFU/ml BCG Tokyo. Group 3 comprised 6 unvaccinated monkeys that received physiological saline as a control.

Intratracheal infection of monkeys

Eight weeks after vaccination, the animals were challenged by intratracheal instillation of 1 ml (3000 CFU) of H37Rv *M. tuberculosis*. All animals were challenged on the same day with the same preparation, and were then observed for 4 months after infection. As PPD did not give better positive results to the monkeys, old tuberculin was used. The old tuberculin test (Chemo-Sero-Therapeutic Research Institute, Kumamoto, Japan) was carried out 1, 2 and 3 months after infection. Briefly, 0.1 ml of old tuberculin solution was injected intradermally into the left palpebral skin and 0.1 ml saline was injected intradermally into the right palpebral skin. Two days later, swelling and redness on both sides were compared. When the diameter of redness is more than 10 mm, it was judged as +, and when more than 11 mm, it was judged as 2+. All animals were housed in animal biosafety level (ABSL) 3 facilities.

Animal care

After infection, animals were observed daily by the animal caretakers for changes in behavior, eating and coughing. Weight, erythrocyte sedimentation rate (ESR) and temperature were recorded at times of blood sampling. Body temperature was measured rectally.

Immunological examination

Blood from the femoral vein was used to obtain serum. TB Dot assay (Shanghai Upper Biotech and Pharma Co., Shanghai, China) was carried out according to the instruction sheet provided by the manufacturer. Briefly, two drops of blocking buffer were spotted on a TB blot membrane previously coated with 38-kDa *M. tuberculosis*-derived

antigen.[22] Then, 40 μl serum was added and semi-dried. Thereafter, six drops of washing buffer were added and semi-dried. Then, two drops of gold-labeled anti-human antibody solution were added and semi-dried. Finally, six drops of washing buffer were added and allowed to dry completely. When a reddish spot appeared, it was judged as positive and when no reddish spot appeared, it was judged as negative.

Bacterial enumeration

Just after death of the unvaccinated monkeys, 10 pieces from the upper and lower lobes of lungs, and also spleen tissue, about 0.5 cm^3 in size were taken randomly. For the vaccinated monkeys, similar samples were taken randomly at necropsy. After being weighed, the samples were combined, homogenized and diluted with physiological saline. For the vaccinated groups, pyrazinamide (200 μg/20 μl) was added to determine BCG Tokyo-derived colonies (background count). Pyrazinamide kills *M. tuberculosis*, but does not kill BCG Tokyo. Triplicate 10-fold dilutions were incubated for 4 weeks in 1% Ogawa solid slant agar and the number of colonies was counted. To examine *M. tuberculosis*-derived colonies, the background count was subtracted from the number of colonies. The lung and spleen tissues were weighed and the results were expressed as CFU ± SD/whole organ.

Histopathology

Necropsies were undertaken on unvaccinated monkeys just after death and on vaccinated monkeys after euthanasia. The removed organs were fixed with 15% formalin for 10 days. Tissue sections from paraffin blocks containing lung, spleen, hilar lymph nodes and liver were stained with hematoxylin and eosin or the Ziehl–Neelsen method for acid-fast bacilli. The severity of pulmonary lesions was judged independently by two experts (I.S. and T.U.).

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

Clinical course

The monkeys vaccinated with rBCG-Ag85A[Tokyo] or parental BCG Tokyo and their non-vaccinated controls were infected intratracheally with H37Rv *M. tuberculosis*. No coughing was observed in the animals after challenge. None of the vaccinated animals gained weight during the infection period. Their ESR was within the normal range (1–2 mm/h). None of the non-vaccinated animals showed an appreciable increase in body weight. Two of the monkeys (17 and 18) showed a gradual decrease in weight (50 g). The other four non-vaccinated control animals showed a severe decrease in weight of 550–800 g, and their ESR was higher than the reference value (55, 10, 8 and 10 mm/h)(Table 1).

On radiographs of the chest, the non-vaccinated animals exhibited early development of multilobar pneumonia in the right lung and rapid progression to bilateral pneumonia. Lobar consolidation and atelectasis in the involved lungs and hilar lymphadenopathy were observed frequently in the non-vaccinated groups (Table 1).

In the vaccinated groups, pneumonia was slight to mild (Figure 1).

Immunological responses

Two immunological methods (the old tuberculin test and serum TB diagnosis) were utilized to clarify the severity of *M. tuberculosis* infection. In the vaccinated monkeys 2 months after infection, the old tuberculin test gave a positive result (+). In the non-vaccinated animals, the reaction was strongly positive (2+) and marked palpebral reddish skin swelling was observed.

The TB dot assay gave a negative result in all vaccinated and non-vaccinated animals 1 month after infection. However, 2 months after infection, the result was positive in the vaccinated monkeys, and strongly positive (2+) in the non-vaccinated monkeys (Table 1).

Gross pathology and histopathology of the vaccinated and non-vaccinated monkeys

At necropsy, all unvaccinated animals showed extensive bilateral lung pathology characterized by the presence of multiple granulomas. These granulomas showed conglomeration to larger caseous areas, especially in the hilar region. Granulomas were also present in the liver and spleen. In the vaccinated animals, a few small granulomas were evident, but these showed no caseous changes. Small liver granulomas were noted in two of the BCG Tokyo-vaccinated monkeys, but such granulomas were not observed in the recombinant BCG-vaccinated monkeys. Four of the non-vaccinated monkeys died of advanced TB 50, 67, 70 and 84 days after infection (Figure 2). These were necropsied just after death for further examination.

On microscopic examination, the non-vaccinated animals showed multifocal, coalescing granulomas with central necrosis and pronounced cellular infiltrates in the periphery (Figure 3). The vaccinated animals showed markedly less severe histopathology. In particular, the peripheral inflammatory cell infiltration was notably more pronounced in the unvaccinated than in the vaccinated animals. Histological examination of the animals that had received the recombinant BCG (Ag85A) showed almost normal lung tissue without granulomas in five of them. The remaining vaccinated animal showed a solitary small granuloma without central necrosis. Two BCG Tokyo-vaccinated animals showed a single small granuloma (Table 1).

Replication of tubercle bacilli in the lung and spleen tissues of vaccinated and non-vaccinated monkeys

At autopsy, 10 different pieces of lung and spleen tissue were taken for determination of CFU. Background culture

Table 1 Summary of the monkey experiments.

Monkey no.	Vaccination	Gross pathology	Lung histopathology	ESR (mm/h, 2 months)	Old tuberculin (2 months)	Serum diagnosis (1 month, 2 months)	Chest X-ray before death or necropsy
1	rec BCG	Lung, spleen, LN	Solitary granuloma	2	+	−, +	Slight pneumonia
2	rec BCG	Lung, spleen, LN	Pneumonia	1	+	−, +	Slight pneumonia
3	rec BCG	Lung, spleen, LN	Pneumonia	1	+	−, +	Slight pneumonia
4	rec BCG	Lung, spleen, LN	Pneumonia	1	+	−, +	Slight pneumonia
5	rec BCG	Lung, spleen, LN	Pneumonia	1	+	−, +	Slight pneumonia
6	rec BCG	Lung, spleen, LN	Pneumonia	1	+	−, +	Slight pneumonia
7	BCG Tokyo	Lung, spleen, LN	Pneumonia	1	+	−, +	Slight pneumonia
8	BCG Tokyo	Lung, spleen, LN	Pneumonia	1	+	−, +	Slight pneumonia
9	BCG Tokyo	Lung, spleen, LN	Pneumonia	1	+	−, +	Slight pneumonia
10	BCG Tokyo	Lung, spleen, LN, liver	Solitary granuloma	3	+	−, +	Slight pneumonia
11	BCG Tokyo	Lung, spleen, LN	Pneumonia	1	+	−, +	Slight pneumonia
12	BCG Tokyo	Lung, spleen, LN, liver	Solitary granuloma	2	+	−, +	Slight pneumonia
13	Saline	Lung, spleen, LN, liver	Miliary tuberculosis	55	2+	−, 2+	Severe pneumonia, consolidation
14	Saline	Lung, spleen, LN, liver	Miliary tuberculosis	10	2+	−, 2+	Severe pneumonia, consolidation
15	Saline	Lung, spleen, LN, liver	Miliary tuberculosis	8	2+	−, 2+	Severe pneumonia, consolidation
16	Saline	Lung, spleen, LN, liver	Miliary tuberculosis	10	2+	−, 2+	Severe pneumonia, consolidation
17	Saline	Lung, spleen, LN, liver	Miliary tuberculosis	5	2+	−, 2+	Severe pneumonia, consolidation
18	Saline	Lung, spleen, LN, liver	Miliary tuberculosis	6	2+	−, 2+	Severe pneumonia, consolidation

Criteria of old tuberculin test and serum diagnosis: +, positive; and 2+, strongly positive. rec BCG; recombinant BCG.

CFU of BCG Tokyo or rBCG-Ag85A[Tokyo] after pyrazinamide was added to the tissue homogenates was one or two. The lung tissue of animals vaccinated with recombinant BCG showed a significant 1000-fold decrease in the number of bacteria compared to the non-vaccinated animals ($p<0.01$). The number of CFU in BCG Tokyo-vaccinated animals after 16 weeks of infection was reduced 100-fold relative to that in the non-vaccinated animals ($p<0.01$). There was a statistically significant difference in the number of pulmonary CFU between recombinant BCG-vaccinated and BCG Tokyo-vaccinated animals ($p<0.01$) (Figure 4).

A similar tendency was also observed in the number of splenic CFU. The spleen tissues of animals vaccinated with recombinant BCG or BCG Tokyo showed a significant 1000-fold decrease in the number of bacteria compared to the non-vaccinated animals ($p<0.01$). However, there was no significant difference in the number of splenic CFU between recombinant BCG-vaccinated and BCG-vaccinated animals.

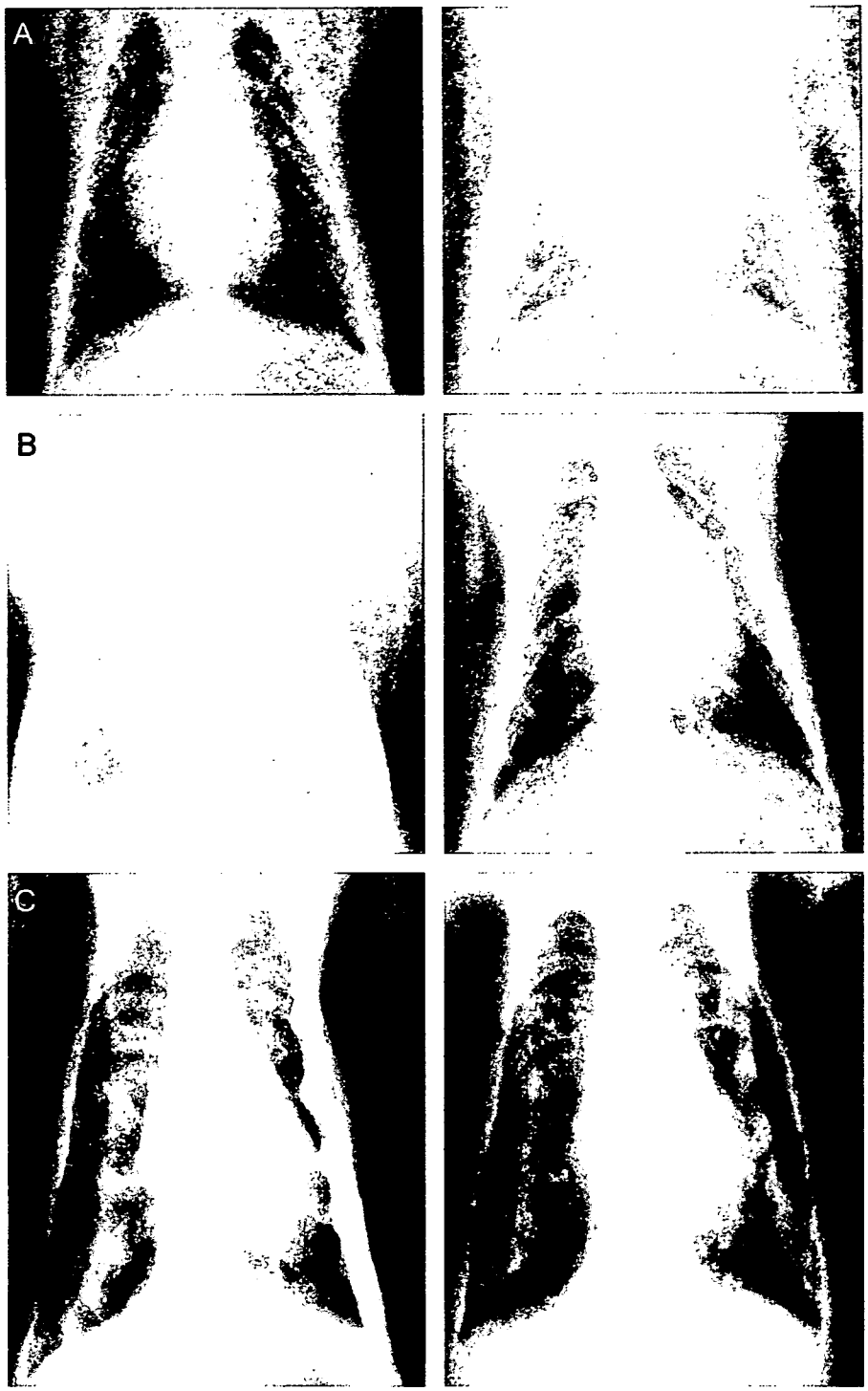

Figure 1 Chest radiologic examinations after challenge with H37Rv *M. tuberculosis*. (A) The monkey (No. 2) vaccinated with rBCG-Ag86A[Tokyo]. The chest X-ray picture was taken 1 day before necropsy. (B) The monkey (No. 9) vaccinated with parental BCG Tokyo. The chest X-ray picture was taken 1 day before necropsy. (C) The non-vaccinated monkey (No. 13) 2 days before death. After H37Rv challenge, the non-vaccinated monkeys rapidly developed extensive bronchopneumonia. Many nodular shadows (→) were recognized, but the vaccinated monkeys had negative chest X-ray findings 3 months after H37Rv challenge.

Discussion

In the present study, we have demonstrated that vaccination of cynomolgus monkeys with recombinant BCG (Ag85A)(rBCG-Ag85A[Tokyo]) induces protection against infection with H37Rv *M. tuberculosis*. In addition to measurement of protection in terms of reduction in bacterial number and/or lung pathology, we have also shown that recombinant BCG vaccination prevented the development of a number of important clinical and

immunological changes during infection. These changes included an increase of the ESR and the development of strong immune responses to a wide spectrum of mycobacterial antigens (old tuberculin). When we inoculated monkeys once with 2×10^6 CFU rBCG-Ag85A[Tokyo], there was a significant reduction of CFU in lung and spleen tissues compared to that in BCG Tokyo-inoculated monkeys. We showed for the first time that the H37Rv strain could also be used for intratracheal infection instead of the Erdman strain. Many researchers use the Erdman strain (1000 CFU or more) for optimal intratracheal infection.[10,16,17] We chose 3000 CFU as the dose for the H37Rv strain because it is less virulent than Erdman strain, and we were recommended to use H37Rv instead of the Erdman strain at the Animal Biosafety Level 3 Facility of Wuhan University.

When parental BCG Tokyo was used for vaccination, we found several grayish tubercles in the liver in two of six monkeys, but no such tubercles were evident in monkeys vaccinated with recombinant BCG. Moreover, there was a significantly lower number of CFU in lung tissues of monkeys vaccinated with recombinant BCG than in monkeys vaccinated with BCG Tokyo ($p<0.01$). Taken together, the results suggest that the recombinant BCG bearing the introduced Ag85A gene gives better protective efficacy than BCG Tokyo. However, to evaluate the efficacy of the Ag85A antigen carefully, it will be necessary to lower the dose of recombinant BCG because 2×10^6 CFU BCG Tokyo alone is still sufficiently effective.

TB dot assay, which targets the 38-kDa antigen from *M. tuberculosis*, gave a negative result 1 month after infection, but a positive one 2 months after infection. Therefore, care is needed when diagnosing TB in the early phase. The old tuberculin test may be more useful for early-phase TB diagnosis.

We selected Ag85A as a promising immunogen because the protein from *M. tuberculosis* induces significant humoral

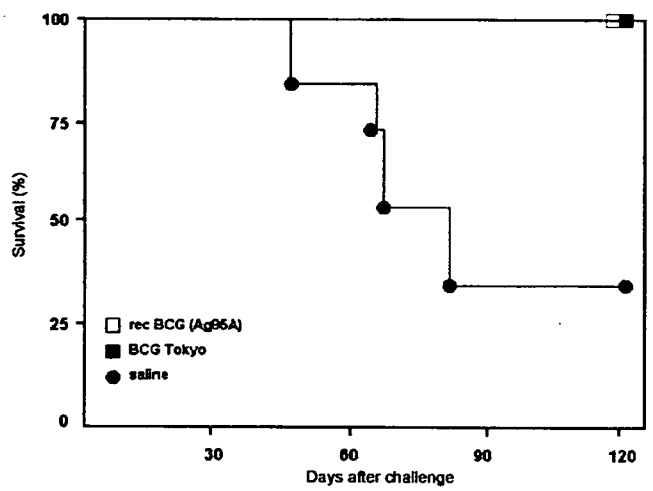

Figure 2 Mortality curve after challenge. Animals in groups of six were challenged by intratracheal inoculation with 3000 CFU of H37Rv. Non-vaccinated animals (six per group) were inoculated with saline.

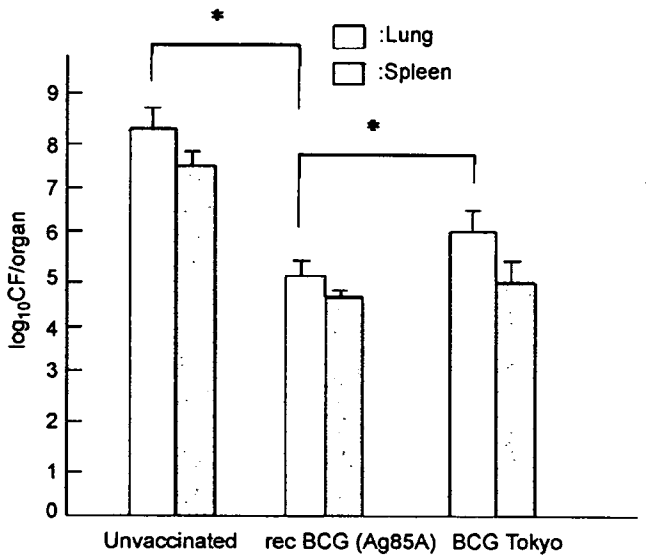

Figure 4 CFU counts in lung and spleen tissues of H37Rv *M. tuberculosis*-infected cynomolgus monkeys vaccinated with recombinant BCG Tokyo (Ag85A), and in non-vaccinated controls. *Statistical difference at $p<0.01$.

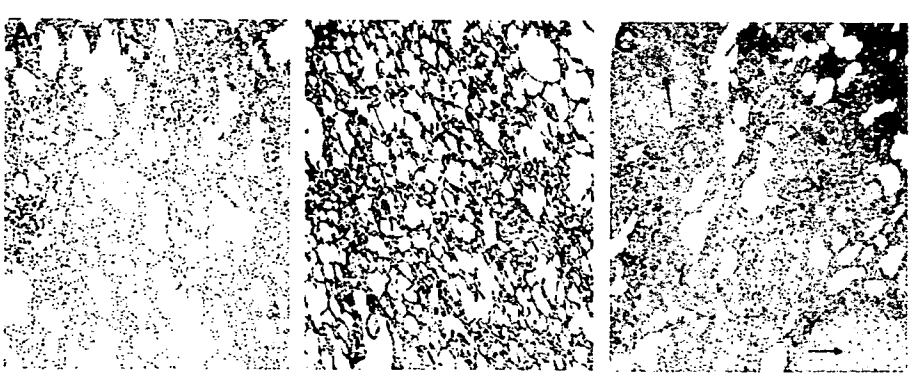

Figure 3 Histopathology of lung tissues from *M. tuberculosis*-infected monkeys vaccinated with rBCG-Ag85A[Tokyo] (A), BCG Tokyo (B) or non-vaccinated controls (C) at necropsy. The miliary granulomas with caseating necrosis are surrounded peripherally by a dense infiltrate of epithelioid cells and lymphocytes (C), but in the vaccinated monkeys interstitial pneumonia is evident (A and B) × 100. Hematoxylin and eosin stain.

and cell-mediated immune responses.[23,24] 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, rBCG-Ag85A[Tokyo], and Ag85A DNA vaccine. Among them, the expression levels of IFN-γ and IL-2 mRNAs were the highest in rBCG-Ag85A[Tokyo].[6] Furthermore, the sera from the rBCG-Ag85A[Tokyo]-vaccinated guinea pigs reacted with Ag85A peptide we used in our previous study significantly (data not shown). We have shown previously that vaccination with Ag85A DNA twice by gene gun bombardment or with rBCG-Ag85A[Tokyo] once significantly reduced the severity of pulmonary pathology and the number of CFU in guinea pigs.[5,6] When the immunogenic synthetic Ag85A peptide was further used as a booster together with recombinant BCG (Ag85A), lung pathology was improved significantly, together with a significant reduction in the number of pulmonary CFU.[6] Although a single intradermal inoculation of 2×10^6 CFU BCG (Ag85A) was enough to induce protective efficacy in the present study, it would be desirable to use Ag85A peptide as a booster, Ag85B-ESAT-6 fusion protein and 72f fusion protein in combination with recombinant BCG Tokyo (Ag85A) to achieve much better protective efficacy.[6,18]

In summary, we have shown that vaccination of primates with rBCG-Ag85A[Tokyo] induces good protective immune responses. Using the macaque challenge model, further optimization of the dose and timing, and use of a booster, may well lead to levels of protection that are better than those achieved with BCG.

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Anti-tuberculosis drug susceptibility testing of *Mycobacterium bovis* BCG Tokyo strain

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SUMMARY

SETTING: The *Mycobacterium bovis* bacille Calmette-Guérin (BCG) vaccine is the only vaccine against tuberculosis (TB), owing to its valuable protective effects and low virulence. However, it can occasionally cause systemic infection in immunocompromised hosts. Isoniazid (INH), rifampicin (RMP), streptomycin (SM) and ethambutol (EMB) are known to be effective anti-tuberculosis drugs and are used for the treatment of BCG infections. Unfortunately, there are few studies of the susceptibility of BCG vaccine strains to these drugs.

OBJECTIVE: To measure the minimum inhibitory concentrations (MICs) of BCG Tokyo vaccine products for anti-tuberculosis drugs and assess vaccine safety in terms of drug susceptibility.

DESIGN: We measured the MIC for one seed and five product lots of BCG Tokyo strain for INH, RMP, SM and EMB using Middlebrook 7H11 agar plates.

RESULTS: The MIC results for INH were 0.06 and 0.125 μg/ml for the product and seed lots, respectively. The MIC results for RMP, SM and EMB were 0.25–0.5, 0.25 and 2–4 μg/ml, respectively.

CONCLUSION: Our results indicate that the BCG Tokyo strain was susceptible to the major anti-tuberculosis drugs and treatable even in cases of severe adverse events, including systemic infection.

KEY WORDS: BCG; minimum inhibitory concentration; drug susceptibility

TUBERCULOSIS (TB) is an infectious disease of international importance that remains a major life-threatening disease worldwide. It is estimated that approximately one third of the world's population is infected with *Mycobacterium tuberculosis*. Every year, approximately 9 million people develop active disease and 1.7 million die of TB.[1]

Bacille Calmette-Guérin (BCG) vaccines are safe, attenuated live bacteria and have been shown to have valuable protective effects against TB. The BCG Tokyo strain is recognised as a low virulence strain among all BCGs,[2] and is widely used in several countries as a vaccine strain. If used properly, it protects against the development of TB and the dissemination of TB bacilli. Few severe complications have been reported.[3] However, systemic BCG infection may occur frequently when it is administered to immunocompromised hosts with congenital or acquired immunodeficiency such as human immunodeficiency virus (HIV) infection.[4,5] BCG is contraindicated in symptomatic HIV diseases. When general BCG infection occurs, patients are treated empirically using anti-tuberculosis drugs because there is limited information about the drug susceptibility of BCG strains. It is therefore very important to evaluate the drug susceptibility of BCG Tokyo strain to ensure the safety of the vaccine.

Isoniazid (INH), rifampicin (RMP), streptomycin (SM) and ethambutol (EMB) are the first-line anti-tuberculosis drugs most commonly used in standard TB treatment regimens. These drugs are currently available even in developing countries. The present study aimed at measuring the minimum inhibitory concentrations (MICs) of these drugs against the BCG Tokyo strain to estimate the effect of clinical treatment in case of infection by the BCG Tokyo strain.

MATERIALS AND METHODS

BCG Tokyo strain

Five lots of vaccine product (number 1003 as 'Lot A', 1960 as 'Lot B', 1036 as 'Lot C', 1061 as 'Lot D', 1998 as 'Lot E') and one seed lot were provided by the Japan BCG Laboratory (Tokyo, Japan) and used in this study. These vaccines were produced by Japan BCG Laboratory for vaccination from the seed lot in 2004. The experiment was carried out in a type II-B

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biological safety cabinet at the Research Institute of Tuberculosis, Tokyo.

Minimum inhibitory concentrations

The MICs were measured modifying the proportion method described in M24-A of the Clinical and Laboratory Standards Institute (CLSI, former National Committee for Clinical Laboratory Standards) and in previous reports.[6,7] The following procedure was used: lyophilised BCG Tokyo products were suspended in 1 ml of distilled water and were cultured on Middlebrook 7H10 agar (DIFCO, Becton Dickinson Microbiology Systems, Cockeysville, MD, USA) supplemented with oleic acid, albumin, dextrose and catalase (OADC: BBL Prepared Culture Media, Becton Dickinson) at 37°C until sufficient growth was observed. After harvesting colonies from culture media, each lot strain of BCG Tokyo was dispersed by vortex mixing with glass beads (disperser tube: Nichibi, BCG Laboratory, Tokyo, Japan) and two drops of 10% Tween 80 (LC-MS, Santa Fe, CA, USA). After vortex mixing for 30 s, 1 ml of distilled water was added to each sample and they were vortexed again for 10 s. The supernatant of each bacterial suspension was transferred to 10 ml of Middlebrook 7H9 broth supplemented with albumin, dextrose and catalase (BBL Prepared Culture Media, Becton Dickinson), and the suspension density was adjusted to an optical density (OD) of 0.05 at 530 nm. These culture tubes were incubated at 37°C with daily mixing and OD checking. When the OD reached 0.2, they were used as the original bacterial suspension.

To prepare 10^{-2} dilutions, a 100 µl aliquot was transferred into 10 ml of distilled water. In a similar way, 100 µl of the 10^{-2} dilution was added to 10 ml of distilled water for 10^{-4} dilutions. One hundred microlitres of the 10^{-2} dilution were inoculated onto Middlebrook 7H11 agar plates with anti-tuberculosis drugs at the designated concentrations. Final INH concentrations were 0.03, 0.06, 0.125, 0.5, 1.0 and 2.0 µg/ml. RMP (0.03, 0.06, 0.125, 0.25, 0.5, 1.0, 2.0, 4.0 µg/ml), SM (0.25, 0.5, 1.0, 2.0, 4.0, 8.0, 16, 32 µg/ml) and EMB concentrations (0.25, 0.5, 1.0, 2.0, 4.0, 8.0, 16, 32 µg/ml) were adjusted accordingly. The 10^{-2} and 10^{-4} suspensions were inoculated onto Middlebrook 7H11 medium containing no drugs for growth control and 1% proportion measurements. These plates were incubated at 37°C. When the 10^{-2} dilution control showed sufficient growth (>100 visible colonies), the MICs were measured as the lowest concentration of drug that inhibited more than 99% of the bacterial population compared with the number of colonies on drug-containing media and the 10^{-4} growth control. Each test was performed in triplicate.

RESULTS

The MICs of one seed and five product lots were measured in triplicate. The MICs of the anti-tuberculosis drugs varied slightly with the lots tested, but were identical among the triplicate tests. The MICs for all tested drugs are shown in the Table. The MICs of INH were 0.06 µg/ml and the seed lot MIC was 0.125 µg/ml. The MIC in test 3 of lot A was not determined due to contamination. For RMP, the MICs for lots A, B and C were 0.25 µg/ml; those for lots D and E were 0.5 µg/ml. It was considered that the MICs of RMP were between 0.25 and 0.5 µg/ml. For SM, the MICs were determined to be 0.25 µg/ml in all tests. For EMB, the MICs were 4 µg/ml for lots A, B and C, while the MICs for lots D and E were 2 µg/ml. The MIC of EMB was 2–4 µg/ml.

DISCUSSION

The BCG vaccine was developed by Calmette and Guérin in 1921. All BCG vaccines consist of live attenuated *Mycobacterium bovis* bacteria. BCG vaccination is commonly performed on neonates and infants once or twice in middle to high tuberculosis prevalence countries, and more than 100 million children have received BCG in recent years.[8] Its safety is therefore a priority issue.

BCG vaccination may sometimes cause complications as a pathogen. Local adverse effects of BCG vaccination have at times been observed and usually improve spontaneously, although severe complications in immunocompromised patients have been reported. McKenzie et al. reported systemic haematological dis-

Table MIC values of four first-line drugs for the BCG Tokyo strain

	MIC (µg/ml)											
	INH			RMP			SM			EMB		
Samples	Test 1	Test 2	Test 3	Test 1	Test 2	Test 3	Test 1	Test 2	Test 3	Test 1	Test 2	Test 3
Lot A	0.06	0.06	cont	0.25	0.25	0.25	0.25	0.25	0.25	4.0	4.0	4.0
Lot B	0.06	0.06	0.06	0.25	0.25	0.25	0.25	0.25	0.25	4.0	4.0	4.0
Lot C	0.06	0.06	0.06	0.25	0.25	0.25	0.25	0.25	0.25	4.0	4.0	4.0
Lot D	0.06	0.06	0.06	0.5	0.5	0.5	0.25	0.25	0.25	2.0	2.0	2.0
Lot E	0.06	0.06	0.06	0.5	0.5	0.5	0.25	0.25	0.25	2.0	2.0	2.0
Seed lot	0.125	0.125	0.125	ND	ND	ND	ND	ND	ND	ND	ND	ND

MIC = minimum inhibitory concentration; BCG = bacille Calmette-Guérin; INH = isoniazid; RMP = rifampicin; SM = streptomycin; EMB = ethambutol; cont = contaminated; ND = not done.

semination of BCG in a child with X-linked severe combined immunodeficiency.[9] Puthanakit et al. reported four cases of BCG infection in HIV-positive children receiving BCG vaccinations at birth; the strain was not indicated.[10]

BCG strains have also been utilised for immunotherapy in addition to TB prevention. BCG is injected into the urinary bladder for intravesical instillation therapy in the early stages of bladder carcinoma.[11,12] The BCG Tokyo strain is popular for such adjuvant therapy in Japan,[13] whereas the Connaught strain is popular in other parts of the world. In a recent study, Mugiya et al. described good, complete response rates of 84% with BCG Tokyo (40 mg administered every 6 weeks) against bladder carcinoma in situ.[14] However, adverse reactions can also occur after instillation therapy. Eichel et al. reported INH-resistant BCG cystitis successfully treated with RMP and EMB.[15]

There is at present no recommended treatment regimen for BCG infection. Anti-tuberculosis drugs are the most potent agents for treating BCG infection. Drug susceptibility testing (DST) of BCG strains has been reported using different methods. Durek et al. evaluated the Connaught BCG strain using a BACTEC 460TB system (Becton Dickinson).[16,17] DST was performed for 31 drugs, including INH, RMP, SM, EMB and rifabutin. The BCG Connaught strain was susceptible to all of the anti-tuberculosis drugs except pyrazinamide (PZA) (BCG has natural/intrinsic resistance to PZA) and some other drugs used for general bacterial infections. The BACTEC 460 TB system employs critical drug concentrations of 0.1, 1.0, 2.0 and 2.5 for INH, RMP, SM and EMB, respectively. Rousseau and Dupuis reported the DST for a seed lot of the BCG Montreal strain by using solid Dubos medium.[18] They showed that this strain was sensitive to INH (0.2 μg/ml), RMP (1.0 μg/ml), SM (2.0 μg/ml) and EMB (5.0 μg/ml). These reports are not, however, comparable because of the differences in testing methods. There is no standard method for the DST of BCG; however, they may be equivalent to each other in the concept of detecting 1% resistance in the strain population. The proportion method with Middlebrook 7H11/OADC media, which is commonly used for the DST of *M. tuberculosis*, was used for this study.

The MICs indicated in the present study were lower than the critical concentrations employed in the previous studies, except for EMB with MIC close to the critical concentration of BACTEC. In the previous studies, the MICs of EMB to *M. tuberculosis* vary between 0.5 μg/ml and 2.0 μg/ml,[19,20] in 7H12 BACTEC broth MIC varies between 0.95 and 3.8 μg/ml and on 7H10 agar between 1.9 and 7.5 μg/ml.[21] Heifets proposed possible guidelines for the interpretation of MIC to *M. tuberculosis* determined in Middlebrook 7H12 broth (radiometric), and MIC 4.0 μg/ml of EMB as moderately susceptible.[22] It is possible that the MIC of BCG Tokyo strain for EMB was higher than wild type *M. tuberculosis*. However, these reports show the tendency of lower MIC in liquid media than solid media. The plasma concentration (C_{max}) of EMB reaches 2.0–5.0 μg/ml[23] and EMB generally works in a time-dependent manner. For this reason it is suggested that EMB could be effective. Although BCG and *M. tuberculosis* are different species, these MICs and pharmacokinetic data would support the potentials of EMB for the treatment of BCG infection. It was therefore considered that, like the BCG Montreal and Connaught strains, the BCG Tokyo strain is susceptible to the four major anti-tuberculosis drugs.

Hesseling et al. reported that BCG in an HIV co-infected infant who received a BCG Danish 1331 strain vaccination developed INH and RMP resistance following treatment with INH and RMP.[24] The MICs of the original strain were 0.15 and <0.4 μg/ml for INH and RMP, respectively. However, they had risen above 0.3 and 32 μg/ml after treatment. These results suggest that the strain was already clinically resistant to INH (MIC 0.15 μg/ml for INH), and monotherapy with RMP against BCG resulted in RMP resistance. Su et al. reported two general disseminated cases of the BCG Tokyo vaccine strains.[5] One of them was treated using anti-tuberculosis drugs (INH, RMP, SM and EMB) based on the susceptible DST results, and the patient recovered. Another case died following one month's treatment with INH, RMP and EMB. However, no DST data were shown in the mortality case and the infant seemed to have died from severe combined immunodeficiency. The MICs of the BCG Tokyo strain indicated in this study were considered less than or equivalent to those of the previous cases, so it was estimated that BCG Tokyo could be treated successfully even in severe adverse events such as systemic dissemination.

The reason why BCG strains have different phenotypic characteristics with respect to drug susceptibility is not clear. BCG has lost several regions of difference (RD) compared to *M. bovis* as the ancestral strain. In particular, the RD1 deletion made a significant contribution to the attenuation of BCG.[24–26] RD1 encodes a 6 kDa early secreted antigenic target protein (ESAT-6)[27] and a 10 kDa culture filtrate protein (CFP-10)[28] associated with virulence in *M. tuberculosis* complex. The BCG vaccine therefore has attenuated virulence compared to wild *M. bovis* strains. The loss of virulence apparently occurred through repeated passages.

The BCG strains were originally donated by the Pasteur Institute (Paris, France), and have been subcultured by several tuberculosis institutes around the world (Russia, Brazil, Sweden, Denmark, Japan, etc.) since 1924. The donated BCG strains differ from the original BCG strains due to differences in passage cultivation, culture medium and storage conditions. In 1972, Hesselberg found that a Swedish/Norwegian BCG strain became resistant to INH during the period 1953–1964, which was the reason why the serial sub-

culture system was discontinued and a seed lot system was adopted.[29] However, in 2003, low-grade INH-resistant (MIC >0.5 μg/ml) Danish 1331 strains were reported again to the World Health Organization (WHO). The WHO therefore recognises the necessity of a new quality assurance method for BCG vaccines.[30,31]

The BCG Tokyo strain was obtained from Calmette in the Pasteur Institute in 1924. Passage cultivation of BCG Tokyo strain has been performed strictly according to Calmette's original instructions, while some of the other BCG strain passages were tailored to each institute's needs. The BCG Tokyo 172 strain, which has undergone 172 passages since the Second World War II, has been used as the seed lot for lyophilised BCG Tokyo vaccines. In this study, the BCG Tokyo strain proved to be susceptible to the major anti-tuberculosis drugs; however, the results of this study do not apply to all BCG substrains. It will be necessary to ensure the safety of BCG vaccine by checking susceptibility to other antimicrobial agents.

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RÉSUMÉ

CONTEXTE : Le bacille de Calmette et Guérin (BCG) à base de *Mycobacterium bovis* est un vaccin unique contre la tuberculose (TB) en raison de ses effets protecteurs valables et de sa faible virulence. Toutefois, il peut causer occasionnellement une infection systémique chez les sujets en état d'immunodépression. L'isoniazide (INH), la rifampicine (RMP), la streptomycine (SM) et l'éthambutol (EMB) sont des médicaments antituberculeux reconnus comme efficaces et peuvent être utilisés dans le traitement des infections par le BCG. Il n'y a malheureusement que peu d'études concernant la sensibilité des souches de vaccin BCG à l'égard de ces médicaments.
OBJECTIF : Mesurer les concentrations minimales inhibitrices (CMI) du vaccin BCG Tokyo pour les médicaments antituberculeux et évaluer la sécurité du vaccin en ce qui concerne la sensibilité aux médicaments.
SCHÉMA : Nous avons mesuré les CMI sur plaques d'agar Middlebrook 7H11 pour la souche-mère et pour cinq lots de vaccin de la souche BCG Tokyo à la fois pour l'INH, la RMP, la SM et l'EMB.
RÉSULTATS : Les résultats des CMI pour l'INH ont été respectivement de 0,06 et de 0,125 μg/ml pour la souche-mère et pour les lots de vaccin. Les résultats des CMI pour la RMP, la SM et l'EMB ont été respectivement de 0,25–0,5, 0,25 et 2–4 μg/ml.
CONCLUSION : Nos résultats indiquent que la souche BCG Tokyo est sensible à l'égard des médicaments antituberculeux majeurs qui sont efficaces même en cas d'effets indésirables graves, y compris des infections systémiques.

RESUMEN

MARCO DE REFERENCIA : *Mycobacterium bovis*, el bacilo de Calmette y Guérin (BCG), es la única vacuna contra la tuberculosis (TB), debido a su valioso efecto de protección y a su baja virulencia. Sin embargo, esta vacuna puede causar en ocasiones infecciones generalizadas en individuos inmunodeprimidos. Isoniazida (INH), rifampicina (RMP), estreptomicina (SM) y etambutol (EMB) son medicamentos antituberculosos eficaces y se emplean en el tratamiento de las infecciones por BCG. Desafortunadamente, existen pocos estudios sobre la sensibilidad de la cepa de la vacuna antituberculosa a estos medicamentos.
OBJETIVO : Medir las concentraciones mínimas inhibitorias (CMI) de los medicamentos antituberculosos contra el BCG de Tokio contenido en las vacunas y evaluar su seguridad toxicológica en la concentración de sensibilidad al medicamento.
MÉTODOS : Se midieron las concentraciones inhibitorias mínimas de INH, RMP, SM y EMB para un lote de siembra y cinco lotes de vacuna de la cepa BCG de Tokio usando cultivos en placas de agar con Middlebrook 7H11.
RESULTADOS : La CMI para INH fue 0,06 con los lotes de siembra y 0,125 μg/ml con los lotes de vacuna. La CMI para los lotes de vacuna con RMP fue de 0,25 a 0,5 ; con SM fue 0,25 ; y con EMB fue de 2 a 4 μg/ml.
CONCLUSIÓN : Estos resultados indican que la cepa BCG de Tokio es sensible a los principales medicamentos antituberculosos y que es posible tratar los casos de reacciones adversas graves, incluida la infección generalizada.

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

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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

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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